# Heterozygous N-terminal deletion of IκBα results in functional nuclear factor κB haploinsufficiency, ectodermal dysplasia, and immune deficiency

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**Background: Nuclear factor κB (NF-κB) is a master transcriptional regulator critical for ectodermal development and normal innate and adaptive immune function. Mutations in the IκB kinase γ/NF-κB essential modifier have been described in male subjects with the syndrome of X-linked ectodermal dysplasia with immune deficiency that results from impaired activation of NF-κB.**

**Objectives: We sought to determine the genetic cause of ectodermal dysplasia with immune deficiency in a female patient.**

**Methods: Toll-like receptor–induced production of the NF-κB–dependent cytokines TNF-α and IFN-α was examined by means of ELISA, the patient's IκBα gene was sequenced, and NF-κB activation was evaluated by means of electrophoretic mobility shift assay and NF-κB–luciferase assays in transfectants.**

**Results: Toll-like receptor function was impaired in the patient. Sequencing of the patient's IκBα gene revealed a novel heterozygous mutation at amino acid 11 (W11X). The mutant IκBαW11X protein did not undergo ligand-induced phosphorylation or degradation and retained NF-κB in the cytoplasm. This led to roughly a 50% decrease in NF-κB DNA-binding activity, leading to functional haploinsufficiency of NF-κB activation. Unlike the only other reported IκBα mutant associated with ectodermal dysplasia associated with immune deficiency (ED-ID), S32I, IκBαW11X exerted no dominant-negative effect.**

**Conclusions: Functional NF-κB haploinsufficiency was associated with ED-ID, and this strongly suggests that normal ectodermal development and immune function are stringently dependent on NF-κB in that they might require more than half of normal NF-κB activity.**

**Clinical implications: Although ED-ID is well described in male subjects, female subjects can present with a similar syndrome of ectodermal dysplasia with immune deficiency resulting from mutations in autosomal genes within the NF-κB pathway.**

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***Key words:*** *Ectodermal dysplasia with immune deficiency, IκBα, nuclear factor κB, phosphorylation, degradation, Toll-like receptors, haploinsufficiency*

Nuclear factor κB (NF-κB) is a master transcription factor required for the normal development and function of the immune system. Effective host defense against invading pathogens requires an effective inflammatory response that is dependent on appropriate activation of NF-κB. Five NF-κB proteins have been described, including p65 (RelA), p105/p50, p100/p52, c-Rel, and RelB.[1,2] In resting cells NF-κB proteins are retained in the cytoplasm by the IκB (inhibitor of NF-κB) family of proteins, which includes IκBα, IκBβ, and IκBε.[3] Activation of a wide variety of cell-surface receptors results in NF-κB activation. Stimuli, including proinflammatory cytokines (TNF-α and IL-1) and Toll-like receptor (TLR) ligands, cause activation of the IκB kinase (IKK) complex, which phosphorylates IκBα on serines 32 and 36, leading to ubiquitination of lysines 21 and 22 and the subsequent degradation of IκBα.[4] Serines 32 and 36, as well as lysines 21 and 22, are contained within an N-terminal 73-amino-acid sequence designated the signal response domain because this region regulates the degradation of IκBα. Inflammation-induced degradation of IκBα releases NF-κB, primarily p50/p65 heterodimers, uncovering a nuclear localization signal that allows NF-κB to translocate to the nucleus, where it binds to consensus sequences in the promoters of a wide variety of genes and results in their transcription.[2] Importantly, transcription of IκBα is regulated by NF-κB.[5,6] In this manner NF-κB–induced transcription of IκBα leads to a feedback inhibition of NF-κB activity.

To date, mutations in 2 genes, IκB kinase γ/NF-κB essential modifier (IKKγ/NEMO) and IκBα, have been found to result in impaired activation of NF-κB in human subjects and in ectodermal dysplasia associated with immune deficiency (ED-ID).[7] This combination of clinical manifestations arises because normal ectodermal development (hair, teeth, and sweat glands), as well as effective innate and adaptive immune responses, depends

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*Abbreviations used*

ED-ID: Ectodermal dysplasia associated with immune deficiency
EMSA: Electrophoretic mobility shift assay
IκBα: Inhibitor of nuclear factor κB α
IKKγ/NEMO: IκB kinase γ/NF-κB essential modifier
p38 MAPK: p38 mitogen-activated protein kinase
TLR: Toll-like receptor
WT: Wild-type

on NF-κB activation.[8] IKKγ/NEMO is the scaffolding subunit of the IKK complex that links upstream receptor signaling components to the protein kinases IKKα and IKKβ.[3] Because IKKγ/NEMO is encoded on the X chromosome, IKKγ/NEMO deficiency affects only boys who have X-linked ED-ID. Numerous mutations in IKKγ/NEMO have been described.[9] A single mutation in one of the 2 IκBα alleles that substitutes Ser32 with isoleucine (S32I) has been identified as a cause of autosomal dominant ED-ID in 2 male patients.[10,11] IκBαS32I cannot be phosphorylated or degraded, resulting in impaired NF-κB activation. The IκBαS32I mutation was termed a hypermorphic mutation because IκBαS32I cannot be ''disinhibited,'' thereby exaggerating its function. In addition, the IκBαS32I mutation was shown to exert a dominant-negative effect because the IκBαS32I mutant was a significantly more potent inhibitor of NF-κB activity than wild-type (WT) IκBα in an NF-κB luciferase reporter assay.[10]

Impaired activation of NF-κB has deleterious effects on both innate and adaptive immune function.[12] TLRs and nucleotide-binding oligomerization domain proteins are pathogen recognition receptors within the innate immune system that detect invading pathogens, including bacteria, mycobacteria, fungi, and viruses.[13,14] Because TLRs and nucleotide-binding oligomerization domain proteins signal through NF-κB, defects in NF-κB activation can cause impaired inflammatory responses to invading pathogens, resulting in decreased production of proinflammatory cytokines and type I interferons.[7,15,16] Because the T-cell receptor and B-cell receptor signaling pathways also converge on NF-κB, impaired NF-κB function leads to deficits in antigen-specific immunity.[17] As a result, patients with X-linked ED-ID demonstrate increased susceptibility to a wide variety of bacterial, mycobacterial, fungal, and viral infections.[18] Analysis of immunoglobulins in these patients commonly reveals hypogammaglobulinemia with variably increased IgM or IgA levels. Specific antibody responses to protein and polysaccharide antigens are variably impaired. T-cell proliferation to mitogens (PHA, pokeweed mitogen, and concanavalin A) is generally intact; however, T-cell proliferation to antigens is variably diminished.[18]

In this report we describe a 10-year-old girl with ED-ID and a novel heterozygous nonsense mutation in IκBα (IκBαW11X) that deletes the N-terminus of the protein, resulting in a persistence-of-function mutant that cannot be degraded. Unlike IκBαS32I, the only previously described human IκBα mutant associated with ED-ID, IκBαW11X does not exert a dominant-negative effect and results in functional NF-κB haploinsufficiency. The association of ED-ID with the IκBαW11X mutant suggests a stringent requirement for NF-κB activation in ectodermal development and immune function.

## METHODS

### Production of cytokines in response to TLR ligands

Informed consent for blood and dermal biopsy samples was obtained from the patient and healthy control subjects in accord with the institutional review board at Children's Hospital, Boston. PBMCs were obtained by means of centrifugation through Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden). PBMCs (300,000 cells/200 μL) were stimulated in media and TLR ligands, as previously described.[19] Cell stimulations were 12 hours for TNF-α ELISA and 48 hours for IFN-α ELISA (Invitrogen, Carlsbad, Calif).

### Sequencing of IκBα

RNA and cDNA were generated from PBMCs, as previously described.[19] The IκBα transcript was amplified by using the forward primer 5′-CCAGCGAGGAAGCAGCG-3′ and the reverse primer 5′-CTAGGCAGTGTGCAGTGTGG-3′, with an annealing temperature of 61°C. The internal forward primer 5′-CATCCTGAAGGCTACCAACTAC-3′ and the reverse primer 5′-GAGGCTAAGTGTAGACACG-5′ were also used for sequencing. Genomic DNA was generated from the patient's fibroblasts by using the phenol/chloroform method. Exon 1 of IκBα was sequenced by using the forward primer 5′-GCAGAGGACGAAGCCAGTTC-3′ and the reverse primer 5′-CCACTTACGAGTCCCGTC-3′. Sequencing of IκBα was performed at the Molecular Biology Core Facility at Children's Hospital, Boston.

### Western blotting

Primary dermal fibroblast cultures were grown in RPMI media plus 10% FCS (Hyclone, Logan, Utah) plus L-glutamine, penicillin, and streptomycin (Invitrogen). Fibroblasts were stimulated with media or 25 ng/mL IL-1 (Invitrogen) for the indicated times. Western blotting was performed, as previously described,[19] by using anti-phospho-IκBα (Cell Signaling, Danvers, Mass), anti-full-length IκBα (Upstate, Charlottesville, Va), anti-N-terminal IκBα (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-phospho-p38 mitogen-activated protein kinase (p38 MAPK) and anti-p38 MAPK (Cell Signaling, Danvers, Mass), and anti-p65 and anti-p50 (Santa Cruz Biotechnology), according to the manufacturer's recommendations. Sheep anti-mouse horseradish peroxidase–conjugated and sheep anti-rabbit horseradish peroxidase–conjugated secondary antibodies were obtained from GE Healthcare (Piscataway, NJ).

### Immunoprecipitation

Fibroblasts or HEK293T cells were lysed in 1 mL of Triton buffer (20 mmol/L TRIS [pH 7.4] and 1% Triton X-100; Sigma, St Louis, Mo), 100 mmol/L NaCl, 10 mmol/L NaF, 2 mmol/L sodium orthovanadate, 1 mmol/L ethyleneglycol-bis-(β-aminoethylether)-N,N,N′N′-tetraacetic acid, and protease inhibitor cocktail (Sigma). Lysates were incubated at 4°C for 3 hours with 1 μg of anti-full-length IκBα, anti-Flag (Sigma), or nonspecific antibody and protein G–Sepharose (Calbiochem, La Jolla, Calif). Immunoprecipitates were washed 3 times in 1 mL of

Triton buffer, followed by boiling in sample buffer. Immunoprecipitates were resolved by means of SDS-PAGE, as above.

## Electrophoretic mobility shift assay

Equal numbers of primary fibroblasts were plated in 6-well tissue-culture plates. Fibroblasts were stimulated with 25 ng/mL IL-1 over a 1-hour period. Cytosolic and nuclear fractions were generated by using a nuclear extract kit (Active Motif, Carlsbad, Calif). The electrophoretic mobility shift assay (EMSA) for NF-κB was performed, as previously described, by using the phosphorous 32–labeled oligonucleotide 5′-TCGCTGGGGACTTTCCAGGGA-3′ and 2 μg of nuclear extract per condition.[20] Supershift of NF-κB complexes was performed with anti-p65 antibodies (Santa Cruz Biotechnology) included in one sample to demonstrate the specificity of the probe. In addition, Western blotting with anti–poly (ADP-ribose) polymerase, anti-p65, and anti-p50 (Santa Cruz Biotechnology) was performed as described above with 10 μg of nuclear extract per condition. Nuclear translocation of p65 and NF-κB binding to radiolabeled probe was normalized to the nucleus-specific protein PARP and quantitated with the ImageJ program from the National Institutes of Health.[21] Data are presented as the mean of 3 experiments ± SD. Statistical significance between the means was determined by using the 2-tailed Student *t* test.

## Constructs

Full-length WT human IκBα was cloned from cDNA generated from normal control blood cells by using the forward primer containing a *Bam*H1 overhang (5′-ATGGATCCGTCCGCGCCATGTTCC-3′) and the reverse primer containing a *Sal*1 overhang (5′-ATGTCGACTAACGTCAGACGCTGGCC-3′). The *Bam*H1-IκBα-*Sal*1 product was inserted into the expression vector pCMV-Tag4a, which contains a C-terminal Flag tag. The mutant human IκBα (IκBαW11X) construct was cloned from the cDNA derived from the mutant IκBα allele of the patient by using the forward primer containing a *Bam*H1 overhang (5′-ATGGATCCGTCCGCGCCATGTTCC-3′) and the reverse primer containing an *Eco*RV overhang (5′-ATGATATCTAACGTCAGACGCTGGCC-3′). The *Bam*H1-IκBαW11X-*Eco*RV product of IκBαW11X was initially inserted into the expression vector pcDNA6. The IκBαW11X mutant was then cut from pcDNA6 by using *Bam*H1 and *Sal*1 and then inserted into pCMV-Tag4a. The sequence of the IκBα constructs was verified by sequencing in the Molecular Biology Core Facility at Children's Hospital, Boston, Massachusetts.

## Luciferase assays

HEK293T cells were grown in Dulbecco's modified Eagle's medium plus 10% FCS (Hyclone) plus L-glutamine, penicillin, and streptomycin (Invitrogen). HEK293T cells were cotransfected with Fugene (Roche, Indianapolis, Ind) overnight with the indicated quantities of vector, WT IκBα, or IκBαW11X and 100 ng of NF-κB–luciferase reporter construct plus 10 ng of pRL TK–Renilla reporter construct. Transfected HEK293T cells were stimulated with media or 20 ng/mL human TNF-α (Invitrogen) for 6 hours. Luciferase activity was measured by using the Dual Luciferase Reporter Assay System (Promega, Madison, Wis), according to the manufacturer's recommendations. Luciferase activity was normalized with Renilla.

# RESULTS

## Case report

A 10-year-old girl presented with a history of 15 episodes of pneumonia since 2 months of age and evidence of bronchiectasis by means of computed tomographic scanning. To date, the patient has not had any documented episodes of bacteremia and no mycobacterial infections. She was born to unrelated parents, both of whom are healthy. Physical examination was significant for slightly thin hair, recessed hairline, pegged teeth (see Fig E1 in the Online Repository at www.jacionline.org), and coarse skin. She was noted to be heat intolerant and unable to sweat. She was subsequently given a diagnosis of ectodermal dysplasia.

Immunologic analysis was significant for a markedly increased serum IgA level and a low serum IgM level. Serum levels of IgG and IgG subclasses were normal, except for a modestly decreased IgG2 level. IgE level was normal (see Table E1 in the Online Repository at www.jacionline.org). The patient had protective titers to immunization with tetanus toxoid; however, she had no specific antibody response after immunization to any of the 14 polysaccharide antigens analyzed that were contained in the pneumococcal polysaccharide vaccine Pneumovax (Merck, Whitehouse Station, NJ) (data not shown). The patient had lymphocytosis, with normal percentages of T and B lymphocytes and natural killer cells (see Table E1). T-cell proliferation in response to PHA-P, anti-CD3, anti-CD3 plus anti-CD28, phorbol 12-myristate 13-acetate plus ionomycin, tetanus, and diphtheria was normal (see Fig E2 in the Online Repository at www.jacionline.org). Oxidative burst was normal, as determined by using the dihydrorhodamine reduction assay (data not shown).

## Impaired cytokine production in response to TLR ligands

The presence of ectodermal dysplasia and recurrent infections is consistent with defective activation of NF-κB.[7] To assess the patient's NF-κB function, we evaluated her ability to produce NF-κB–dependent cytokines in response to TLR ligands.[22] In 2 independent experiments stimulation of the patient's blood cells with Poly I:C (TLR3), LPS (TLR4), flagellin (TLR5), and ODN2216 (TLR9) demonstrated significant impairment in TNF-α production, whereas 3M-13 (TLR7) stimulation induced normal TNF-α production (Fig 1, *A*). Engagement of TLR3, TLR7, TLR8, and TLR9 stimulates production of type I interferons, which is also dependent on NF-κB activation. Stimulation of the patient's blood cells with TLR3 and TLR9 ligands demonstrated markedly impaired production of IFN-α, whereas IFN-α production in response to TLR7 ligand was less impaired (Fig 1, *B*). Thus the patient's blood cells demonstrate defects in the production of the NF-κB–dependent cytokines TNF-α and IFN-α.

## Heterozygous nonsense mutation in IκBα in the patient

To our knowledge, this is the first known female patient with ED-ID with clinical and laboratory findings similar to those found in male patients with X-linked ED-ID caused by mutations in IKKγ (NEMO). We therefore sequenced her IκBα gene, an autosomally encoded gene, from cDNA obtained from her blood cells. The results revealed a heterozygous nonsense mutation at codon 32 of IκBα

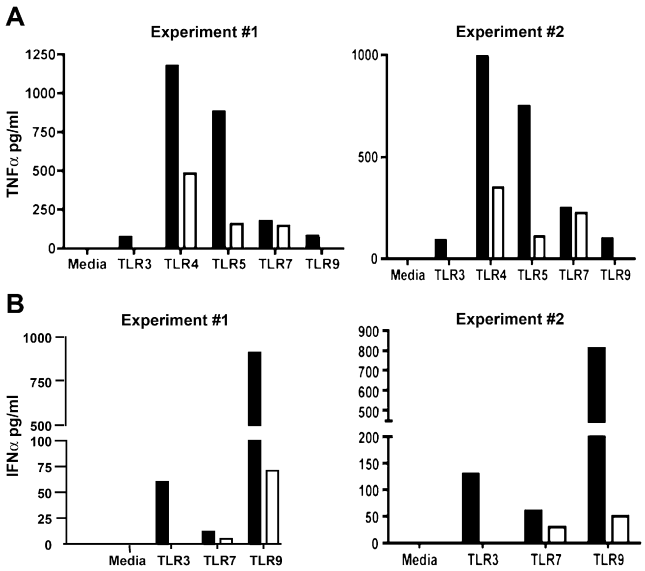


**FIG 1.** Impaired TLR-induced NF-κB–dependent cytokine production by patient PBMCs. PBMCs (control, *solid bars*; patient, *open bars*) were incubated with medium, Poly I:C (TLR3), LPS (TLR4), flagellin (TLR5), 3M-13 (TLR7), or ODN2216 (TLR9). TNF-α **(A)** and IFN-α **(B)** were quantitated 12 and 48 hours after stimulation, respectively. Two independent experiments are shown.

(G32A; see Fig E3, *A*, in the Online Repository at www.jacionline.org), which was confirmed by sequencing exon 1 of IκBα from the patient's genomic DNA obtained from fibroblasts. Sequencing of cDNA derived from the patient's mother's blood cells was normal (data not shown). Unfortunately, the patient's father is not available for analysis.

Examination of the coding sequence of IκBα reveals the presence of 2 ATG codons (Met13 and Met45 in the native IκBα) downstream from the nonsense mutation that are flanked by a consensus Kozak translation initiation sequence (see Fig E3, *B*, in the Online Repository at www.jacionline.org).[23] This might allow the in-frame translation of a truncated IκBα that lacks the N-terminal 12 and 44 amino acids, respectively.

## Patient fibroblasts express a mutant IκBαW11X protein that fails to be phosphorylated and degraded after IL-1 stimulation

To determine whether a protein product is translated from the mutant IκBα allele, cell lysates were prepared from unstimulated and IL-1–stimulated primary dermal fibroblasts of the patient and a healthy control subject and electrophoresed on 12% polyacrylamide gels to resolve proteins close in molecular weight. The gels were Western blotted for IκBα by using 2 different antibodies, one directed against full-length IκBα and another directed against an N-terminal peptide of IκBα (amino acids 1-30). Western blotting of unstimulated normal fibroblast lysates with both IκBα antibodies revealed a single band that corresponded to WT IκBα (Fig 2, lane 1, rows 1 and 2). In contrast, Western blotting of lysates from the patient with antibody to full-length IκBα revealed 2 species of IκBα, a band of normal size that corresponded to WT IκBα and a smaller-sized species (Fig 2, row 1, lane 6). The N-terminus antibody failed to recognize a smaller-sized IκBα band in the lysates from the patient's cells, which is consistent with N-terminal truncation of the mutant (Fig 2, lane 6, row 2). These results indicate that the heterozygous mutation in the patient results in the expression of an N-terminally truncated IκBαW11X protein.

Activation of NF-κB requires the phosphorylation of IκBα on serines 32 and 36, followed by ubiquitination of IκBα on lysines 21 and 22 and its degradation by the proteasome. This allows NF-κB to translocate to the nucleus and activate gene transcription.[4] Because the IκBαW11X mutant might contain serines 32 and 36, as well as lysines 21 and 22, we evaluated whether IκBαW11X undergoes normal phosphorylation and degradation. Control and patient fibroblasts were stimulated with IL-1, and cell lysates were Western blotted with anti-phospho-IκBα antibody and anti-IκBα antibody directed against the full-length protein. Western blotting with anti-phospho-IκBα antibody demonstrated that WT IκBα protein was phosphorylated within 5 minutes of stimulation in control fibroblasts (Fig 2, row 3, lane 2). This was accompanied by a shift in molecular weight in blots with anti-IκBα antibody (Fig 2, row 1, lane 2). Similarly, the product of the normal full-length WT IκBα allele was also phosphorylated and underwent a molecular weight shift at the 5-minute time point in the patient's fibroblasts (Fig 2, rows 3 and 1, lane 7), confirming the intact activation of the IKK complex in the patient's cells. In contrast, there was no evidence of phosphorylation of the IκBαW11X mutant, as indicated by the absence of a lower molecular weight band in the phospho-IκBα blot (Fig 2, row 3, lane 7). Lysates were

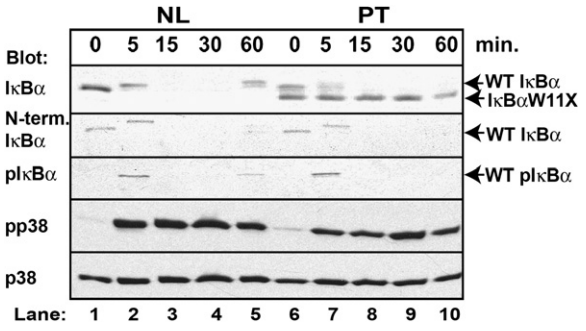


**FIG 2.** Patient fibroblasts express a mutant IκBαW11X protein that does not undergo IL-1–induced phosphorylation and degradation. Western blotting of lysates from control and patient fibroblasts stimulated for the indicated times with 25 ng/mL IL-1 is shown. The blot was first probed with anti-phospho-IκBα and then stripped and reprobed sequentially with anti-IκBα, anti-N-terminal IκBα, anti-phospho-p38 MAPK, and anti-p38 MAPK as a loading control. Data shown are representative of 3 independent experiments. *NL*, Healthy control subject; *PT*, patient.

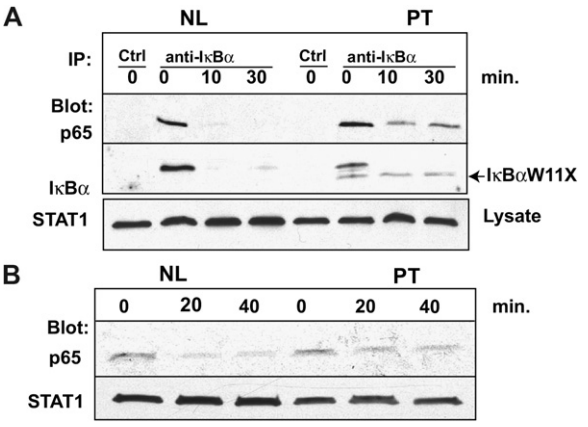


**FIG 3.** NF-κB p65 is persistently bound by mutant IκBαW11X in patient fibroblasts. **A**, Lysates of fibroblasts stimulated with media or 25 ng/mL IL-1 for the indicated times were immunoprecipitated with nonspecific rabbit IgG *(Ctrl)* or anti-IκBα and were Western blotted with mouse anti-p65 and anti-IκBα antibody. Fifteen milliliters of lysate was Western blotted with anti–signal transducer and activator of transcription 1 *(STAT1)* Western blotting to verify equal protein content. **B**, Western blotting of cytosolic fractions from unstimulated and stimulated fibroblasts with anti-p65 and anti-STAT1. Data are representative of 3 independent experiments. *NL*, Healthy control subject; *PT*, patient.

probed with anti-phospho-p38 MAPK antibodies to demonstrate that IL-1 stimulated control and patient fibroblasts. Stimulation of control and patient fibroblasts with IL-1 resulted in comparable activation of p38 MAPK (Fig 2, row 4).

Western blotting of IκBα demonstrated that WT IκBα was completely degraded within 15 minutes in both control and patient fibroblasts (Fig 2, row 1, lanes 3 and 8). In contrast, the IκBαW11X mutant was not degraded significantly over the course of 1 hour, as evidenced by the persistence of the lower molecular weight band recognized by the anti-IκBα antibody (Fig 2, row 1, lanes 6 through 10). Degradation of WT IκBα in IL-1–stimulated control fibroblasts was followed by its synthesis, as indicated by its reappearance and phosphorylation at 1 hour (Fig 2, row 1, lane 5). In contrast, 1 hour after IL-1 stimulation, reappearance of WT IκBα was not evident in the patient's fibroblasts (Fig 2, row 1, lane 10). Because the synthesis of IκBα is NF-κB dependent,[5] these results are consistent with impaired activation of NF-κB.

## The IκBαW11X mutant retains p65 in the cytoplasm after activation of the patient's cells

Because the IκBαW11X mutant does not undergo normal IL-1–induced phosphorylation and degradation, we examined whether it retains NF-κB after IL-1 activation. Control and patient fibroblasts were stimulated with IL-1, then IκBα was immunoprecipitated with an anti-IκBα antibody, and finally the IκBα immunoprecipitates were probed with antibodies to the p65 subunit of NF-κB to detect coprecipitating p65. In unstimulated fibroblasts from the healthy control subjects and the patient, p65 coprecipitated with IκBα. After stimulation of control fibroblasts with IL-1, p65 was no longer detected in IκBα precipitates, which is consistent with complete degradation of IκBα and release of p65. In contrast, a fraction of p65 remained associated with the IκBαW11X mutant after stimulation of the patient's fibroblasts with IL-1, which is consistent with the persistence of the IκBαW11X mutant (Fig 3, *A*). Because of the IκBα degradation, anti–signal transducer and activator of transcription 1 Western blotting was performed on equal-volume aliquots of fibroblast lysates to demonstrate equal protein content in the lysates used in the immunoprecipitation.

The persistent association of p65 with the IκBαW11X mutant after IL-1 activation suggests that a portion of p65 remains retained in the cytoplasm in the patient's cells. Cytosolic fractions of control and patient fibroblasts were prepared after IL-1 stimulation and probed for p65 to confirm this. As expected, Fig 3, *B*, shows that p65 virtually disappeared from the cytosolic fractions of control fibroblasts stimulated with IL-1. Twenty minutes after stimulation, only 18% ± 3% (n = 3) of total cellular p65 was retained in the cytoplasm. In contrast, 55% ± 10% (n = 3) of total cellular p65 remained in the cytosolic fractions of the patient's fibroblasts after IL-1 stimulation for 20 minutes ($P = .004$). These results suggest that IκBαW11X is a persistence-of-function mutant that sequesters NF-κB n the cytoplasm after receptor stimulation.

## Impaired nuclear translocation of NF-κB in the patient's cells

Because the IκBαW11X mutant retains NF-κB, we predicted that translocation of NF-κB to the nucleus and binding to DNA would be reduced in the patient's fibroblasts after activation with IL-1. Control and patient fibroblasts were stimulated with IL-1 over a 1-hour time course, and nuclear extracts were examined for the presence of p50 and p65. Fig 4, *A*, demonstrates that the nuclear translocation of p65 and p50 was reduced in

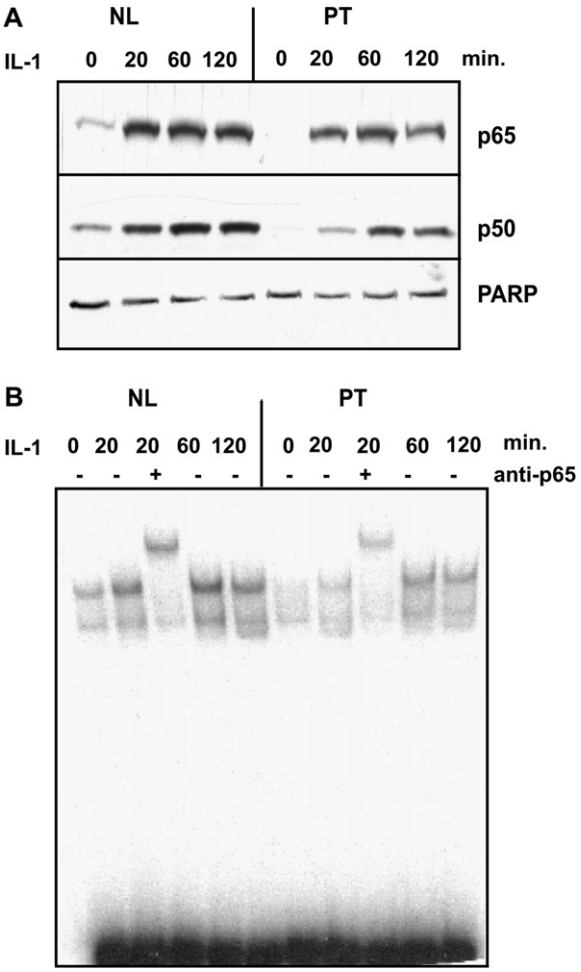


**FIG 4.** Reduced NF-κB nuclear translocation and DNA binding in nuclear extracts from the patient's fibroblasts. **A**, Nuclear extracts prepared from IL-1–stimulated fibroblasts for the indicated times were Western blotted sequentially with anti-p65 (p65), anti-p50 (p50), and anti-PARP as a loading control. **B**, EMSA with a radiolabeled NF-κB probe. Anti-p65 antibody was used in one reaction to verify the presence of p65 in the retarded bands. Data are representative of 3 independent experiments. *NL*, Healthy control subject; *PT*, patient.

the patient's cells. Densitometric analysis of the intensity of the p65 and p50 bands at the 20-minute time point in control and patient fibroblasts showed that nuclear accumulation of p65 and p50 in the patient's cells was reduced by 40% ± 5% and 50% ± 10%, respectively compared with that seen in control cells (n = 3). These results are consistent with impaired nuclear translocation of NF-κB in the patient's cells after stimulation.

The binding of NF-κB to DNA in nuclear extracts from control and patient fibroblasts was evaluated by means of EMSA. Fig 4, *B*, shows that stimulation with IL-1 resulted in an increase in the capacity of nuclear extracts from control fibroblasts to bind an NF-κB–specific oligonucleotide probe, as evidenced by an increase after stimulation in the intensity of the retarded bands present in unstimulated cells and by the appearance of a new retarded third band. Addition of anti-p65 antibody to the nuclear extracts supershifted the retarded complexes. Consistent with the impaired nuclear translocation of NF-κB in the patient's fibroblasts, binding of nuclear extracts from IL-1–stimulated patient's fibroblasts to the NF-κB–specific oligonucleotide probe was reduced by approximately 47% ± 6% (n = 3) compared with that seen in control cells, as determined by means of densitometric analysis of the supershifted bands (Fig 4, *B*).

### The IκBαW11X mutant does not exert a dominant-negative effect on NF-κB activation

HEK 293T cells were cotransfected with an NF-κB–luciferase expression construct and with increasing quantities of Flag-tagged WT IκBα or Flag-tagged IκBαW11X mutant to test whether the IκBαW11X mutant acts as a dominant-negative mutant, as has been reported for the IκBαS32I mutant. Consistent with the function of IκBα as an inhibitor of NF-κB activity, transfection with increasing amounts of WT IκBα led to a dose-dependent inhibition of NF-κB–luciferase activity. Transfection of the IκBαW11X mutant resulted in a comparable dose-dependent inhibition curve (Fig 5, *A*). Western blotting of cell lysates with anti-Flag antibody demonstrates that WT IκBα and IκBαW11X were expressed comparably (Fig 5, *B*). Note that expression of IκBα in 293T cells transfected with 30 ng of the constructs was less than the limit of detection by means of Western blotting with anti-Flag. Taken together, these data indicate that IκBαW11X is not a dominant-negative mutant but rather a persistence-of-function mutant that results in functional NF-κB haploinsufficiency.

## DISCUSSION

We describe a female patient with ED-ID associated with a novel heterozygous nonsense mutation in the IκBα gene that gives rise to a truncated protein that lacks a portion of the N-terminus of IκBα. The mutant protein is not phosphorylated or degraded after IL-1 receptor signaling. As a result, it sequesters NF-κB and results in functional NF-κB haploinsufficiency.

To our knowledge, the patient we have described is the first reported female patient with ED-ID. She has several of the classical features of ED, including skin, teeth, and hair abnormalities and inability to sweat.[24] She had recurrent infections and exhibited immunologic defects common to patients with ED-ID, which included lymphocytosis, impaired specific antibody responses to polysaccharide antigens, increased serum IgA levels (see Table E1 in the Online Repository at www.jacionline.org), and impaired response to TLR ligands (Fig 1).[10,18]

A heterozygous nonsense mutation (G32A) introduced a stop codon at position 11 in IκBα in the patient (Fig 2). This might have allowed translation initiation from the second or fourth methionine codons. We indeed demonstrated the presence of an N-terminally truncated protein in the patient's cells. The exact start site that is used to

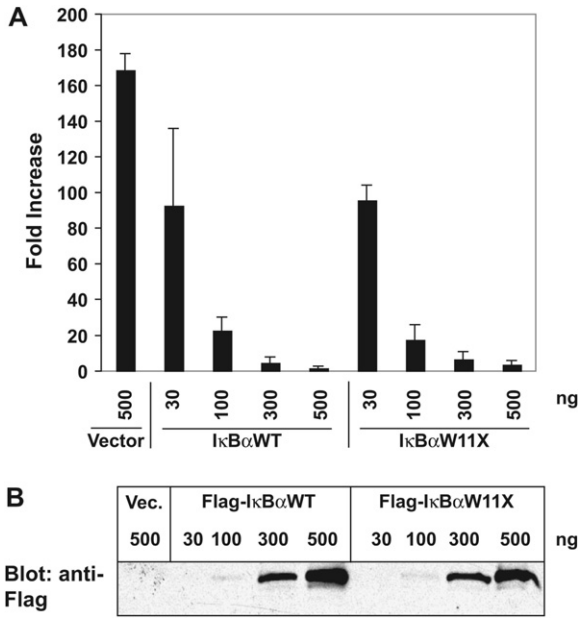


**FIG 5.** IκBαW11X does not exert a dominant-negative effect. **A**, HEK293T cells were cotransfected with Flag-WT IκBα or Flag-IκBαW11X and 100 ng of NF-κB–luciferase reporter construct and pRL TK-Renilla reporter construct and then stimulated with media or 20 ng/mL TNF-α for 6 hours. Fold activation of luciferase activity was calculated relative to unstimulated HEK293T cells transfected with 500 ng of vector DNA. Results were normalized for transfection efficiency by using Renilla. Data are expressed as the mean ± SD of 3 experiments. **B**, Anti-Flag Western blot of cellular lysates.

generate this protein remains to be determined. The mutant IκBαW11X protein is not phosphorylated or degraded after IL-1 receptor engagement (Fig 2). This resulted in retention in the cytosol of approximately half of p65 after stimulation and in a corresponding reduction in the nuclear translocation of p65 (Figs 3 and 4).

The immunodeficiency in our patient is less severe than that in the 2 patients previously described with an S32I mutation in IκBα. These 2 patients had a hyper-IgM syndrome with high serum IgM and low serum IgG levels, absent specific antibody responses to both protein and polysaccharide antigens, and absent T-cell proliferation to antigens. Both patients underwent bone marrow transplantation because of the severity of their recurrent infections.[10,11] In contrast, our patient has normal serum IgG levels, good antibody responses to the protein antigen tetanus toxoid, and normal T-cell proliferation to antigens. More importantly, she has been in relatively good health while receiving intravenous immunoglobulin infusions and to date has not had the mycobacterial infections that commonly occur in patients with ED-ID.

The milder clinical phenotype of this patient compared with the 2 male patients with the S32I mutation in IκBα is likely explained by the milder impairment of NF-κB activation in our patient, although differences in the genetic background might also have contributed. Both the S32I and IκBαW11X mutations prevent ligand-induced phosphorylation and degradation of the mutant IκBα and therefore are persistence-of-function mutants. However, in the case of the S32I mutation, TNF-α–induced phosphorylation and degradation of the product of the normal IκBα allele is barely detectable.[10] In contrast, IL-1–induced phosphorylation and degradation of the product of the normal IκBα allele is intact in our patient (Fig 2). Consistent with these observations, TNF-α–stimulated activation of NF-κB in fibroblasts from the patient with the S32I mutation was barely detectable, whereas IL-1–stimulated activation of NF-κB in fibroblasts from our patient was roughly half that of the healthy control subject, as determined by means of scanning densitometry of NF-κB nuclear translocation and EMSA data (Fig 4).

A dominant-negative effect of the S32I mutation was demonstrated in transfection studies in which the capacity of S32I IκBα and WT IκBα to inhibit a TNF-α–driven NF-κB–luciferase reporter gene expression was compared. The S32I mutant exerted a dominant-negative effect because it was much more potent than WT IκBα in inhibiting reporter gene expression.[10] These results suggest that in addition to being a persistence-of-function mutant, the S32I mutant functions as a dominant-negative mutant. This is possibly because the S32I mutant, which has an intact signal response domain, might compete with WT IκBα for IKK and perhaps function as an irreversible inhibitor of IKK. In contrast, IκBαW11X exerted no detectable dominant-negative effect because its effect was comparable with that of WT IκBα (Fig 5). Because this mutant lacks a portion of the N-terminus of IκBα, it might not effectively compete for IKK. In future experiments we plan to transfect IκBα$^{-/-}$ murine embryonic fibroblasts with WT IκBα and IκBαW11X to confirm that IκBαW11X functions as a persistence-of-function mutant. This will have the advantage of using cells without endogenous IκBα.

In summary, the novel IκBαW11X mutant we describe in this report, which does not undergo normal ligand-induced degradation and which impairs NF-κB activation through persistence of function (eg, retention of NF-κB), simply results in functional NF-κB haploinsufficiency. The fact that functional NF-κB haploinsufficiency was associated with ED-ID strongly suggests that normal ectodermal development and immune function are stringently dependent on NF-κB, in that they might require more than half of normal NF-κB activity.



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