Signal transduction pathways triggered by the FcεRIIb receptor (CD23) in human monocytes lead to nuclear factor-κB activation

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Background: Alveolar macrophages play a key role in the initiation of the inflammatory reaction of allergic asthma. Alveolar macrophages and peripheral blood monocytes are activated when IgE/allergen immune complexes bind to the CD23 receptor, which leads to the production of inflammatory cytokines.
Objective: We sought to investigate the molecular mechanisms regulating this early inflammatory response. We have focused on the study of the signal transduction pathways triggered by CD23 in human monocytes and the promonocytic cell line U937.
Methods: CD23 was cross-linked in human monocytes and U937 cells with IgE immune complexes. Surface expression of CD23 was determined by FACS analysis. Transcription factor activation and gene transcription were studied by gel-shift assays and Northern blot analysis, respectively. IκBα phosphorylation and degradation was analyzed by Western blot.
Results: Nuclear factor (NF)-κB is the main transcription factor involved in the gene activation that follows CD23 cross-linking in monocytes. CD23-induced NF-κB is a heterodimer composed of p65/p50 subunits. NF-κB nuclear translocation is secondary to the phosphorylation and subsequent degradation of the NF-κB inhibitory molecule IκBα. Tyrosine kinase–dependent, and not protein kinase C–dependent, pathways mediate CD23-triggered NF-κB activation but do not participate in the direct phosphorylation of IκBα. IκBα degradation and NF-κB nuclear translocation correlate with transcriptional activation of the inflammatory cytokines TNF-α and IL-1β.
Conclusions: NF-κB is the main transcription factor involved in the signal transduction pathway of CD23 in monocytes. (J Allergy Clin Immunol 1999;104:376-87.)

Key words: *CD23, FcεRI, IgE, signal transduction, nuclear factor-κB, IκB*

Abbreviations used

AP-1:	Activation protein-1
AM:	Alveolar macrophage
CREB:	cAMP response element binding factor
DMSO:	Dimethyl sulfoxide
DTT:	Dithiothreitol
GαHIgE:	Goat anti-human IgE
HEPES:	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
NF:	Nuclear factor
PE:	Phycoerythrin
PKC:	Protein kinase C
P-Tyr:	Antiphosphotyrosine antibody
SP-1:	Promoter selective transcription factor
TK:	Tyrosine kinase

Allergic asthma is characterized by inflammation of the bronchial epithelium with an increase of alveolar macrophages (AMs) and infiltration of eosinophils. AMs are the initial cells to encounter the allergen in the airway by processing and presenting it to the T lymphocyte. This AM/T-cell interaction results in activation of many other leukocytes and in consequent damage to the bronchial epithelium.[1] AMs express the low-affinity receptor for IgE (FcεRII), also called CD23. CD23 specifically binds IgE-allergen complexes and plays an important role in the pathogenesis of IgE-mediated immunity.[2]

Two types of FcεRII have been described in humans, FcεRIIa and FcεRIIb, which are encoded by the same gene but are distinctively regulated.[3] FcεRIIa is constitutively expressed in mature B cells and seems to be involved in B-cell growth and differentiation, antigen presentation, and downregulation of IgE synthesis.[4] FcεRIIb is primarily expressed on circulating monocytes, AMs, B lymphocytes, and eosinophils of atopic individuals, whereas its expression is very low or undetectable in these cells from normal subjects. Surface expression of FcεRIIb is upregulated by IL-4 and IL-13 at the transcriptional level.[5] FcεRIIb seems to be important in the effector phase of IgE-mediated immunity, playing a role in cell activation. The binding of IgE/allergen complexes to CD23 induces cross-linking of this receptor and triggers the release of proinflammatory mediators and cytokines from monocytes.[6,7] These molecules are essential in the development of the allergic reaction that results in bronchoconstriction, chronic airway inflammation, and airway hyperresponsiveness, which are characteristic of asthma. In addition, monocytes and other antigen-presenting cells were recently shown to express FcεRI, the high-affinity receptor for IgE.[8] Cross-linking of FcεRI on monocytes induces both an increase of intracellular calcium and the release of prostaglandins.[9]

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Supported by a grant from the Mayo Foundation.
Received for publication Jan 29, 1999; revised Apr 1, 1999; accepted for publication Apr 3, 1999.
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0091-6749/99 $8.00 + 0 **1/1/99117**

Little is known about the signal transduction pathways triggered by CD23 cross-linking. Increased levels of cyclic adenosine monophosphate have been noted in B lymphocytes, monocytes, and the promonocytic cell line U937 after CD23 cross-linking. This is accompanied by increases in intracellular calcium in B lymphocytes, but not in monocytes or U937 cells.[10]

Nuclear factor-κB (NF-κB) is a transcription factor that activates transcription of genes that mediate immune functions. NF-κB is rendered inactive in the cytoplasm of most nucleated cells by the inhibitory protein IκB. On cell activation, IκB is phosphorylated and degraded, allowing NF-κB to be released. Free NF-κB translocates to the nucleus where it binds specific decameric sequences present in the promoter of multiple genes.[11] Several pathways that lead to IκB phosphorylation at different sites of the molecule have been described.[12-14]

Glucocorticoids are effective anti-inflammatory drugs that are widely used for the treatment of asthma. Glucocorticoids have been shown to exert immunosuppressive action in part by inducing IκB gene transcription and consequently inhibiting NF-κB–mediated transcriptional activation.[15] In addition, many of the genes induced in AMs after CD23 cross-linking are regulated by NF-κB.[11] Therefore NF-κB is a likely candidate to mediate the signal transduction pathway triggered by the allergen/IgE activation of CD23 in AMs. Preliminary data has shown that CD23 cross-linking by a specific mAb or by IgE-dinitrophenyl complexes can induce activation of NF-κB in U937 cells[16] and rat macrophages,[17] respectively. In addition, recent studies have reported the importance of NF-κB in airway inflammation in asthma.[18,19]

Elucidating the molecular mechanisms of CD23 signal transduction in human monocytes is important to understand the pathogenesis of atopic diseases. Therefore we have investigated the transcription factors and their regulatory molecules, which are targets of CD23 activation. We demonstrate that NF-κB and IκBα are the main molecules involved in the signal transduction of FcεRIIb in monocytes and the promonocytic cell line U937. We describe the pathway leading to IκBα phosphorylation and subsequent NF-κB activation triggered by FcεRIIb.

METHODS

Cell lines and culture conditions

The promonocytic cell line U937 was obtained from the American Type Tissue Collection and maintained in culture in RPMI media supplemented with 5% heat-inactivated FBS (Intergen). Monocytes were purified from human buffy coats by Ficoll-Hypaque gradient separation followed by adherence to plastic tissue culture flasks overnight in RPMI media with 10% human AB serum (Gibco). Nonadherent cells were removed, and the adherent population was incubated in the same media for an additional 48 hours. U937 cells stably transfected with the α-, β-, and γ-chains of FcεRI (U937-FcεRαβγ) were kindly provided by J. P. Kinet (Beth Israel Deaconess Medical Center, Boston, Mass) and maintained in culture in RPMI media with 10% FBS. All culture media contained 100 U/mL penicillin/streptomycin and 2 mmol/L L-glutamine.

Induction and analysis of CD23 and FcεRI expression

U937 cells grown at a density of 0.25×10^6 cells/mL or human monocytes adhering to plastic tissue culture flasks were incubated with 10 ng/mL recombinant human IL-4 (R&D Systems) for 48 hours. Surface CD23 expression was analyzed in a flow cytometer (FACScan, Becton Dickinson) by using phycoerythrin (PE)-conjugated anti-CD23 or the PE isotype-matched control antibody (Becton Dickinson) by standard procedures. Surface expression of FcεRI was analyzed by staining the cells with mAb 15.1 directed to the α-chain of FcεRI[8] or an isotype control followed by incubation with a PE-conjugated rabbit anti-mouse antibody (Sigma Chemicals).

Reverse transcription polymerase chain reaction

Total RNA was obtained from U937 cells, monocytes, or U937-FcεRIαβγ by the RNAzol method, following the manufacturer's recommendations (Tel-Test Inc). cDNA was generated with 10 μg of RNA, 20 ng/mL oligo-dT, 0.5 μL AMV-reverse transcriptase (Boehringer Mannheim), 0.1 μL Rnase inhibitor (Boehringer Mannheim), and 2 mmol/L dNTP in a volume of 10 μL. RT-PCR was performed by means of standard PCR with 1 μL of cDNA and the following primers: 5′-ATGGCTCCTGCCATGGAA-3 and 5′-GTTGTTTTTGGGGTTTGGCT-3′ for FcεRIα and 5′-CAGAACGGCCGATCTCCAGCCCAAGATG-3′ and 5′-GCCAAAGAAGAATATGACCGCATCTATTCTAAAG-3′ for FcεRIγ.

Cell stimulation

CD23 cross-linking. Cells were incubated at 5×10^6 cells/mL in 6-well plates with 20 μg/mL affinity-purified monoclonal human IgE (Fitzgerald Industries Int) for 1 hour at 37°C. The cells were then centrifuged and resuspended in fresh media containing 20 μg/mL affinity-purified goat anti-human IgE antibody (GαHIgE, Fitzgerald Industries Int) at 37°C for various times.

TNF-α stimulation. U937 cells were incubated at 5×10^6 cells/mL in 6-well plates with media containing 10 ng/mL human TNF-α (Genzyme) for various times.

Pervanadate stimulation. U937 cells (10^6) were resuspended in 200 μL of RPMI media containing 0.5% BSA and incubated at 37°C for 5 minutes with 10 μL of pervanadate (2 μmol/L $NaVO_4$, 0.2% H_2O_2). The reaction was stopped with 1 mL of ice-cold stop solution (10 mmol/L Tris, 50 mmol/L NaCl, 5 mmol/L EDTA, 30 mmol/L $Na_4P_2O_7$, and 50 mmol/L NaF). The cells were lysed with 20 μL of lysis buffer (10 mmol/L Tris, 50 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L NaF, 30 mmol/L $Na_4P_2O_7$, 100 μmol/L $NaVO_4$, 1% Triton X-100, and 2 mmol/L phenylmethylsulfonyl fluoride) at 0°C for 10 minutes.

Nuclear extraction and gel mobility shift assay

Nuclear protein was extracted by using a modification of the method of Dignam et al.[20] Briefly, 10^7 cells were washed with buffer A (10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid [HEPES], 1.5 mmol/L $MgCl_2$, and 10 mmol/L KCl). Cells were then lysed with buffer B (buffer A containing 0.1% Nonidet P-40) for 4 minutes at 0°C and washed with buffer A. The nuclear pellet was resuspended in 20 μL of buffer C (20 mmol/L HEPES, 25% glycerol, 0.42 mol/L NaCl, 1.5 mmol/L $MgCl_2$, and 0.2 mmol/L EDTA) and incubated at 4°C for 30 minutes in a rotator. After centrifugation, the supernatant was diluted with 40 μL of buffer D (20 mmol/L HEPES, 20% glycerol, 0.05 mol/L KCl, and 0.2 mmol/L EDTA) and stored at –70°C. All buffers contained 0.5 mmol/L phenylmethylsulfonyl fluoride, and buffers B, C, and D also contained 0.5 mmol/L

dithiothreitol (DTT), 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 2 μg/mL pepstatin. Proteins were quantified by using the Bio-Rad protein assay. For mobility shift assays, 3 μg of protein extract was incubated with (γ^{32}P)adenosine triphosphate–labeled double stranded oligodeoxynucleotide probe at room temperature for 15 minutes with 3 μL of binding buffer and 1 μg of polydIdC (Pharmacia), as previously described.[21] The binding buffers used were the following: 100 mmol/L HEPES, 300 mmol/L KCl, 20% Ficoll, 0.05% NP-40, and 0.5 mg/mL BSA for the NF-κB reaction; 60% glycerol, 60 mmol/L HEPES, 300 mmol/L KCl, 25 mmol/L $MgCl_2$, 20 mmol/L Tris-HCl, and 25 mmol/L EDTA for the activation protein-1 (AP-1) reaction; 50 mmol/L HEPES, 30 mmol/L $MgCl_2$, 250 mmol/L KCl, and 2.5 mmol/L DTT for the CAMP responsive element binding factor (CREB) reaction; and 50 mmol/L Tris-HCl, 250 mmol/L NaCl, 25% glycerol, 5 mmol/L EDTA, 0.5 % NP-40, and 5 mmol/L DTT for the promoter selective transcription factor-1 (SP-1) reaction. In some reactions the nuclear proteins were incubated with polyclonal anti-p50, anti-p65, anti-p52, or anti-cRel antibodies (Santa Cruz Biotechnology Antibodies) for 5 minutes at room temperature before the radiolabeled probe was added. The binding reaction was analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel and visualized by autoradiography. DNA binding competition was assessed by preincubating the extract with a 40-fold excess of unlabeled oligonucleotide. The oligonucleotides used in the binding reaction corresponded to the NF-κB binding sequence present within the enhancer of the HIV long terminal repeat (5′-ACAAGGGACTTTCCGCTGGGGACTTTCCAGGGA-3′) or the AP-1, SP-1, or CREB consensus binding sequences (Promega). The double-stranded probe was end-labeled with (γ^{32}P)dATP by using polynucleotide kinase.

Cytosolic extracts, immunoprecipitation, and Western blots

Cytosolic proteins were obtained from the supernatant of the cells lysed with buffer B (see above). In some experiments 200 μg of cytosolic proteins were immunoprecipitated with an ammonium sulfate–precipitated rabbit anti-human IκBα antibody linked to protein G sepharose (Gibco) and rabbit anti-human FcεRIγ antibody (kindly provided by P. Leibson, Mayo Clinic, Rochester, Minn) linked to protein A agarose (Gibco) at 4°C for 2 hours. In other experiments 20 μg of crude extract cytosolic proteins were used. Proteins were denatured by boiling, separated by SDS 10% to 12% PAGE, and transferred to Immobilon-P membranes (Millipore) by standard procedures. Recombinant IκBα (rIκBα) was produced after cloning the complete cDNA of human IκBα into a pGEX-GST system. Immunoblotting was done with polyclonal rabbit anti-human IκBα (obtained by immunizing rabbits with rIκBα) or with anti-phosphotyrosine antibody (P-Tyr; 4G10 mAb, Upstate Biotechnology) and visualized by using an enhanced chemoluminescence Western blotting detection kit (Amersham). To ensure equal amounts of protein loading, Western blot membranes were also blotted with an affinity-purified polyclonal rabbit anti-human β-actin antibody (Sigma). In some experiments 50 μg/mL of a calpain I inhibitor (ALLN, Boehringer Mannheim) or 3 μmol/L of the protein kinase C (PKC) inhibitor bisindolylmaleimide I (GF 109203X, Calbiochem) were added to the cell cultures for 1 hour before CD23 cross-linking. In some experiments 4 μmol/L of the tyrosine kinase (TK) inhibitor herbimycin (Calbiochem) reconstituted in 1.7 mmol/L dimethyl sulfoxide (DMSO) was added to the cell cultures 18 hours before the CD23 cross-linking. In the experiments in which herbimycin was used, 1 mmol/L of the tyrosine phosphatase inhibitor NaVO4 (Sigma) was added to the lysis buffer (buffer B).

Northern blot analysis

Total RNA was extracted from 10^7 cells by using the RNAzol method, according to the manufacturer's recommendations (Tel-Test Inc). Ten micrograms of RNA were electrophoresed in a 5% formaldehyde 1% agarose gel and transferred overnight to a Hybond-N^+ membrane (Amersham). Equal amounts of RNA were loaded in each well, as assessed by ethidium bromide staining of the gel. The membrane was hybridized with an (α^{32}P)-labeled probe for 2 hours, washed with standard saline citrate buffer (0.3 mol/L NaCl and 0.03 mol/L Na citrate), and autoradiographed. The probes were obtained by amplifying 315 and 400 nucleotide long regions of the human *IL-1β* and *TNF-α* genes, respectively, by a PCR reaction with cDNA from U937 cells and specific oligonucleotides (IL-1: 5′-GACACATGGGATAACGAGGC-3′ and 5′-GGGATCTACACTCTCCAGCTG-3′; TNF-α: 5′-TAGCCCATGTTGTAGCAAACCC and 5′-TCGGCAAAGTCGAGATAGTC-3′). The probes were labeled with (α^{32}P)dCTP by using a random primer DNA labeling kit (Pharmacia) and purified over G-50 quick spin columns (Boehringer Mannheim).

RESULTS

NF-κB is the major transcription factor involved in FcεRIIb signal transduction

To investigate the signal transduction pathway or pathways activated after FcεRIIb receptor cross-linking that lead to the transcription of inflammatory cytokines, we first focused on the study of transcription factors that are known to be involved in the synthesis of such cytokines.

$CD23^+$ monocytes and U937 cells were generated by treating peripheral blood monocytes and U937 cells with IL-4, as previously described.[5] Although both U937 cells and human monocytes express a basal level of surface CD23 (Fig 1, *b* and *f*, respectively), its expression increased after 48 hours of IL-4 treatment, as assessed by FACS analysis (Fig 1, *d* and *h*).

Surface expression of FcεRI was also assessed by FACS with an antibody directed against the α-chain of this receptor (antibody 15.1). Neither U937 nor human monocytes expressed surface FcεRI (Fig 2, *b* and *f*), even after treatment with IL-4 (Fig 2, *d* and *f*). As a positive control, we used U937 cells stably transfected with FcεRIαβγ, which expressed high levels of FcεRIα (Fig 2, *h*). To further analyze the presence of FcεRI in U937 cells and monocytes, RT-PCR was performed to amplify mRNA encoding the α- and γ-chains of this receptor (Fig 3). Although monocytes expressed both FcεRIα and FcεRIγ (Fig 3, *lane 3*), we were unable to detect any FcεRIα in U937 cells before or after IL-4 treatment (Fig 3, *lanes 1* and *2*, respectively). As expected, U937 cells had significant amounts of FcεRIγ because this chain is shared with the FcγR (Fig 3, *lanes 1* and *2*).

The lack of a significant role for FcεRI in U937 cell activation by IgE immune complexes was also demonstrated by immunoprecipitation and phosphorylation analysis of FcεRIγ. As shown in Fig 4, FcεRIγ immunoprecipitated from cytoplasmic extracts of IL-4–treated U937 cells was not phosphorylated after stimulation with IgE immune complexes *(lane 2)* compared with cells stimulated with pervanadate *(lane 4)*.

Having ruled out the involvement of FcεRI in the activation of U937 cells after stimulation with IgE immune complexes, we then proceeded to study the role of CD23 on these cells and human monocytes.

To characterize the activation of transcription factors

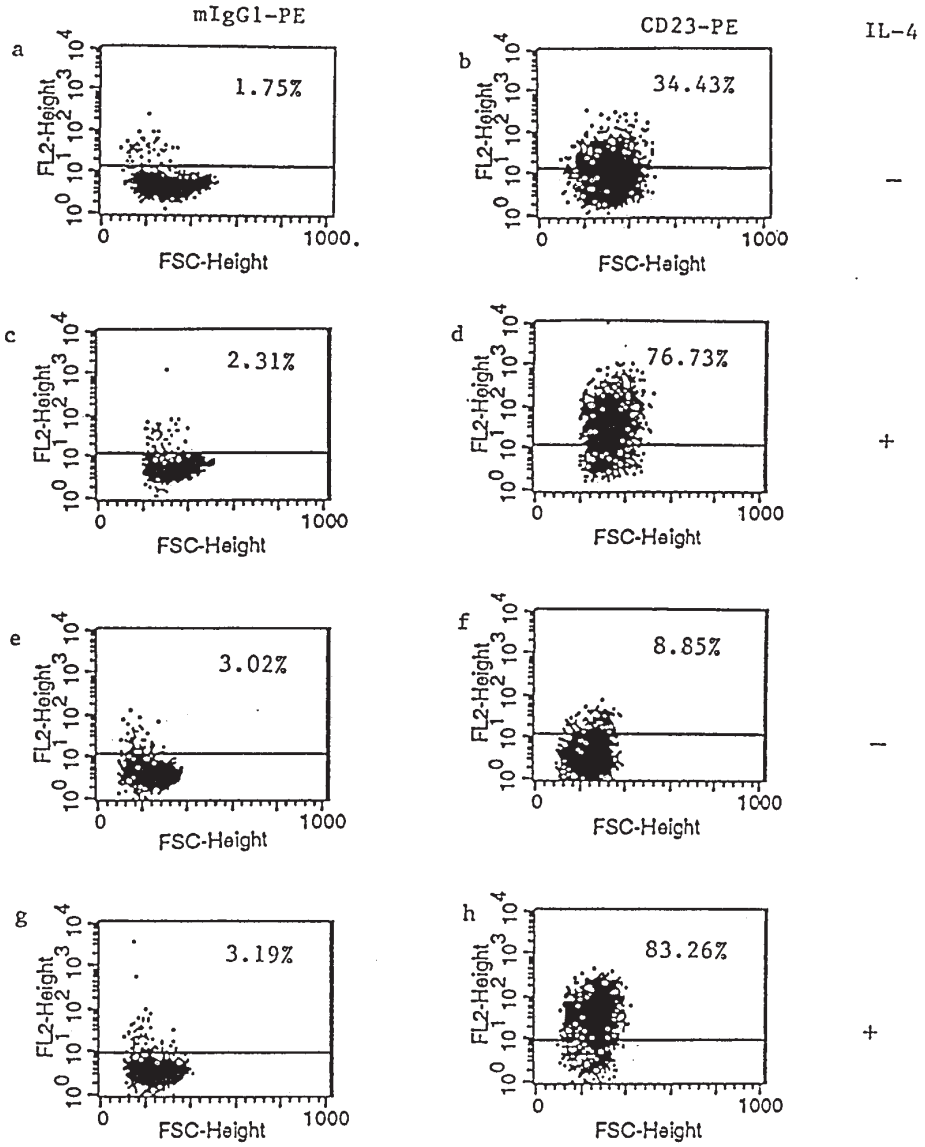

FIG 1. IL-4 induction of CD23 in U937 cells and monocytes. Flow cytometric analysis of U937 cells (**a** to **d**) or human monocytes (**e** to **h**) with PE-conjugated CD23 (**b**, **d**, **f**, and **h**) or isotype-matched IgG1 (**a**, **c**, **e**, and **g**) is shown. Cells were cultured for 48 hours in the presence (**c**, **d**, **g**, and **h**) or absence (**a**, **b**, **e**, and **f**) of IL-4. The numbers in the upper quadrants indicate percentage of positive cells. *FSC,* Forward scatter.

after CD23 cross-linking, we analyzed the DNA-binding properties of nuclear extracts from stimulated cells. Gel mobility shift assays indicated that cross-linking of CD23 in U937 cells induced the nuclear translocation of the transcription factor NF-κB (Fig 5, *A*, *lane 4*) but did not modify the binding activity of the transcription factors SP-1, AP-1, and CREB (Fig 5, *B*). The amount of NF-κB that translocated to the nucleus after CD23 cross-linking was much greater in U937 cells treated with IL-4 than in nontreated cells (Fig 5, *A*, *lanes 4* and *2*, respectively), suggesting a direct correlation between NF-κB activation and the level of CD23 expression on U937 cells (Fig 1). IgE sensitization was necessary for CD23 cross-linking because the GαHIgE by itself was insufficient to induce NF-κB activation (Fig 5, *A*, *lane 10*). The CD23-activated NF-κB complex was similar to the NF-κB complex induced by TNF-α treatment of the cells (Fig 5, *A*, *lane 11*) and was composed of a p65/p50 heterodimer, as indicated by the disappearance of the NF-κB band after incubation with anti-p65 or anti-p50 antibodies (Fig 5, *A*, *lanes 5* and *6*, respectively). Incubation with anti-cRel or anti-p52 antibodies did not interfere with the CD23-triggered NF-κB (Fig 5, *A*, *lanes 8* and *9*, respectively). The induced NF-κB band was completely inhibited by the addition of a 40-fold excess of unlabeled probe (Fig 5, *A*, *lane 7*), indicating specific binding.

A similar analysis performed with nuclear extracts obtained from human monocytes indicated that CD23

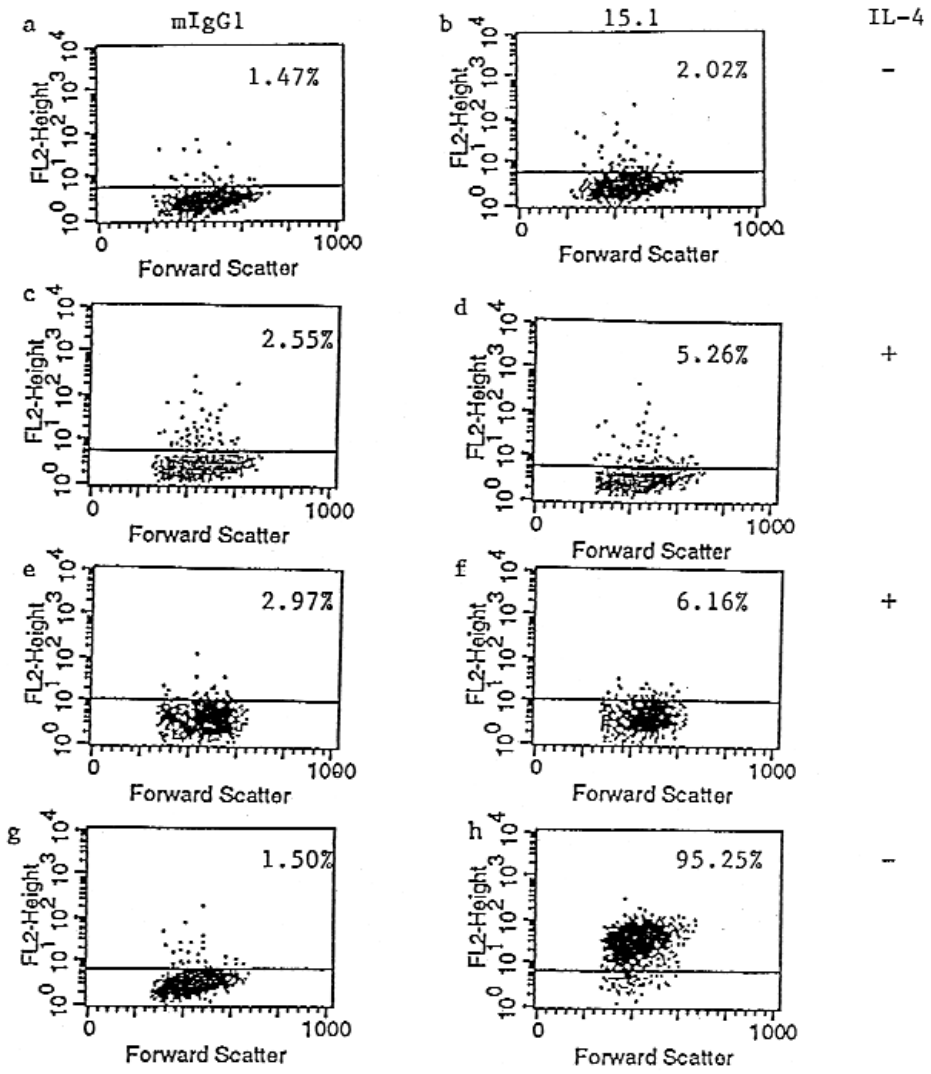

FIG 2. Analysis of surface FcεRI expression on U937 and monocytes. Flow cytometric analysis of U937 cells (**a** to **d**), human monocytes (**e** and **f**), or U937-FcεRIαβγ (**g** and **h**) with mAb 15.1 (**b**, **d**, **f**, and **h**) or isotype-matched IgG1 (**a**, **c**, **e**, and **g**) and PE-conjugated rabbit anti-mouse antibody is shown. Cells were cultured for 48 hours in the presence (**c**, **d**, **e**, and **f**) or absence (**a**, **b**, **g**, and **h**) of IL-4. The numbers in the upper quadrants indicate percentage of positive cells.

cross-linking of these cells induced NF-κB nuclear translocation (Fig 5, *C*, *lane 3*), whereas stimulation of monocytes with the GαHIgE secondary antibody without prior sensitization with IgE did not induce NF-κB translocation (Fig 5, *C*, *lane 6*), indicating that these results are not a nonspecific effect from the GαHIgE. The induced band was also upshifted by incubation with anti-p65 and anti-p50 antibodies (Fig 5, *C*, *lanes 4* and *5*, respectively).

To confirm the lack of involvement of FcεRI in mediating the NF-κB signal, U937 cells were treated with 10 μg/mL of antibody 15.1 for 45 minutes followed by stimulation with IgE and GαHIgE as above. Antibody 15.1 has been shown to block IgE binding at this concentration by binding to the IgE site in the FcεRIα.[8] Fig 5, *D*, shows that NF-κB induced by IgE/ GαHIgE *(lane 2)* was not inhibited by preincubation with antibody 15.1 *(lane 3)*. This data again confirms that NF-κB activation seen after IgE immune complex stimulation is not mediated by FcεRI.

Overall, these results indicate that CD23 cross-linking on U937 cells and monocytes results in selective activation of the transcription factor NF-κB.

The activation of NF-κB after CD23 cross-linking correlates with the induction of transcription of inflammatory monokines

To study whether the CD23-induced NF-κB translocation correlated with the transcriptional activation of inflammatory monokines, Northern blots of RNA obtained from human monocytes before or after CD23 cross-linking

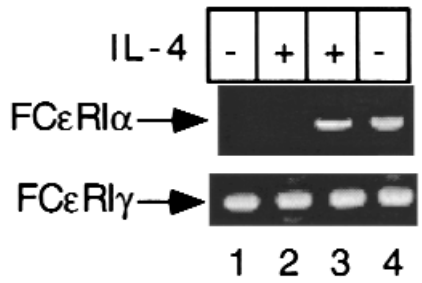

FIG 3. Analysis of mRNA FcεRI expression on U937 cells and monocytes. RT-PCR of RNA from untreated *(lane 1)* or IL-4–treated U937 cells *(lane 2)*, IL-4 treated monocytes *(lane 3)*, or U937-FcεRIαβγ *(lane 4)* with primers for FcεRIα and FcεRIγ is shown.

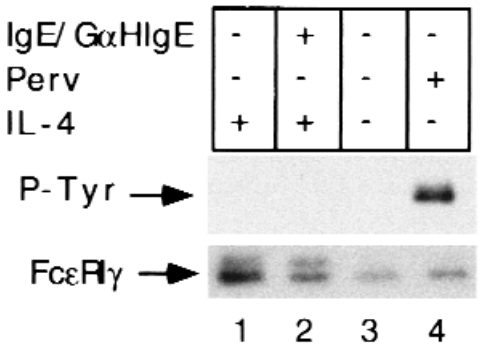

FIG 4. Analysis of FcεRIγ phosphorylation in U937 cells. Western blotting of FcεRIγ immunoprecipitated from cytosolic extracts from U937 cells (*lanes 1* and *2*) or U937-FcεRIαβγ (*lanes 3* and *4*) immunoblotted with P-Tyr antibody *(upper panel)* or anti-FcεRIγ *(lower panel)* is shown. IgE/GαHIgE indicates CD23 cross-linking with IgE sensitization for 1 hour followed by treatment with GαHIgE for 2 minutes *(lane 2)*. *Perv* indicates treatment with pervanadate for 2 minutes as described in the Methods section *(lane 4)*.

were performed. Monocytes were treated with IL-4 for 48 hours, and total RNA was obtained after 2 hours of CD23 cross-linking. The results shown in Fig 6 suggest that CD23 cross-linking induced transcription of both TNF-α and IL-1β genes *(lane 3)*, demonstrating the functional relevance of NF-κB activation in the transcription of the NF-κB-dependent genes TNF-α and IL-1β.

Phosphorylation of IκBα precedes NF-κB activation triggered by CD23 cross-linking

Because NF-κB is anchored in the cytoplasm by a number of inhibitory proteins from the IκB family, we first focused our studies to investigate whether modifications of IκBα occurred after CD23 cross-linking.

By using Western blot analysis and immunoblotting of IκBα in cytosolic extracts, we demonstrate that the CD23-induced nuclear translocation of NF-κB in U937 cells and monocytes tightly correlates with the disappearance of cytoplasmic IκBα (Fig 7, *A*, *lane 4* and Fig 7, *B*, *lane 2*). These results indicate that NF-κB activation triggered by CD23 cross-linking results in the specific degradation of IκBα in the cytoplasm, which has previously been shown to be mediated by the proteosome.[12] IκBα degradation was greater in U937 cells treated with IL-4 than in untreated cells (Fig 7, *A*, *lanes 4* and *2*, respectively), once more confirming a correlation between the degree of surface CD23 expression and the activation of signal transduction pathways leading to IκBα degradation (Fig 1).

Because the degradation of IκBα in the proteosome is preceded by specific phosphorylation modifications,[12] we attempted to inhibit the proteolytic machinery of the proteosome with ALLN, a calpain I inhibitor, to investigate whether CD23 activation leads to a slower migrating form of IκBα. IL-4–treated U937 cells were either sensitized or unsensitized with IgE in the presence or absence of the proteosome inhibitor ALLN. The cells were then stimulated with GαHIgE to induce CD23 cross-linking followed by lysis at different times. Western blots were performed to detect IκBα in cytosolic extracts. Immunoblotting was also done with β-actin to indicate that equal amounts of protein were analyzed for each sample. Our results in Fig 8 show that IκBα was degraded as early as 10 minutes after CD23 cross-linking in the absence of ALLN (*lanes 2* to *4*). However, a slower migrating form of IκBα could be detected 10 minutes after CD23 cross-linking in cells treated with ALLN *(lane 9)*. The kinetics of CD23-induced IκBα phosphorylation and degradation was slower than that triggered by TNF-α, which was detected as early as 1 minute after treatment *(lane 6)*.

These results indicate that phosphorylation of IκBα occurs after CD23 cross-linking and is followed by rapid degradation of the molecule.

CD23-induced phosphorylation of IκBα is indirectly mediated by TK-dependent signal transduction pathways

Recent studies have demonstrated that IκBα phosphorylation is dependent on specific serine kinases and TKs that act at different domains on the molecule. In addition, PKC and TK have also been shown to participate in upstream signal transduction pathways that lead to IκBα phosphorylation.[13-15] Taking advantage of specific pharmacologic inhibitors available to block PKC and TK, we have investigated whether these kinases play a role in the signal transduction of CD23 in U937 cells. U937 cells were pretreated with PKC- or TK-specific inhibitors before CD23 cross-linking and IκBα protein levels were analyzed by Western blot and immunoblotting. As shown in Fig 9, *A*, degradation of IκBα triggered by CD23 cross-linking *(lane 3)* was not affected by the PKC inhibitor GF 109203X *(lane 4)* but was completely blocked by the TK inhibitor herbimycin *(lane 8)*. GF 109203X was shown to be active in inhibiting the phosphorylation of IκBα triggered by phorbol myristate acetate and ionomycin in Jurkat T cells (results not shown). In addition, translocation of NF-κB triggered by CD23 cross-linking in U937 cells was also completely inhibited by herbimycin (Fig 9, *B*, *lanes 3* and *4*), as demonstrated by gel shift assays. These results suggest

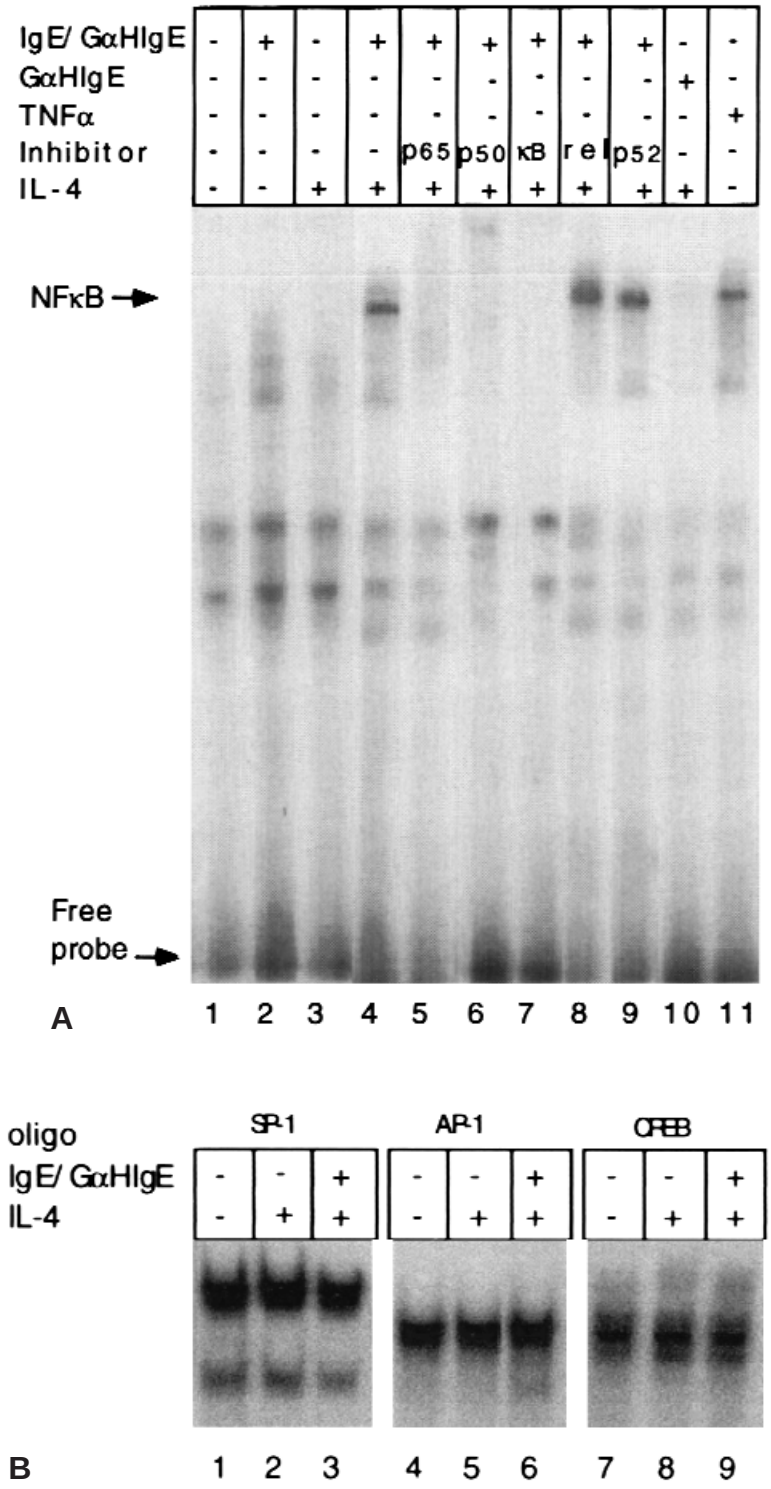

FIG 5. Activation of NF-κB by CD23 cross-linking. Gel mobility shift assays with nuclear extracts from U937 cells (**A**, **B**, and **D**) or monocytes (**C**) are shown. *IgE/GαHIgE* indicates CD23 cross-linking with IgE sensitization for 1 hour followed by treatment with GαHIgE for 30 minutes. *GαHIgE* indicates treatment with GαHIgE for 30 minutes without presensitization with IgE. *TNFα* indicates treatment with TNF-α for 30 minutes (**A**, *lane 11*). *p65, p50, rel*, and *p52* are antibodies to NF-κB subunits that were added before incubation with the radiolabeled probe (**A** and **C**). *κB* indicates competition with a 40-fold excess of unlabeled probe (**A**, *lane 7*). *15.1 Ab* indicates incubation with this antibody for 45 minutes before treatment with IgE and GαHIgE (**D**, *lane 3*). Radiolabeled probes corresponded to the DNA binding sequences for NF-κB (**A**, **C**, and **D**), SP-1 (**B**, *lanes 1* to *3*), AP-1 (**B**, *lanes 4* to *6*), or CREB (**B**, *lanes 7* to *9*).

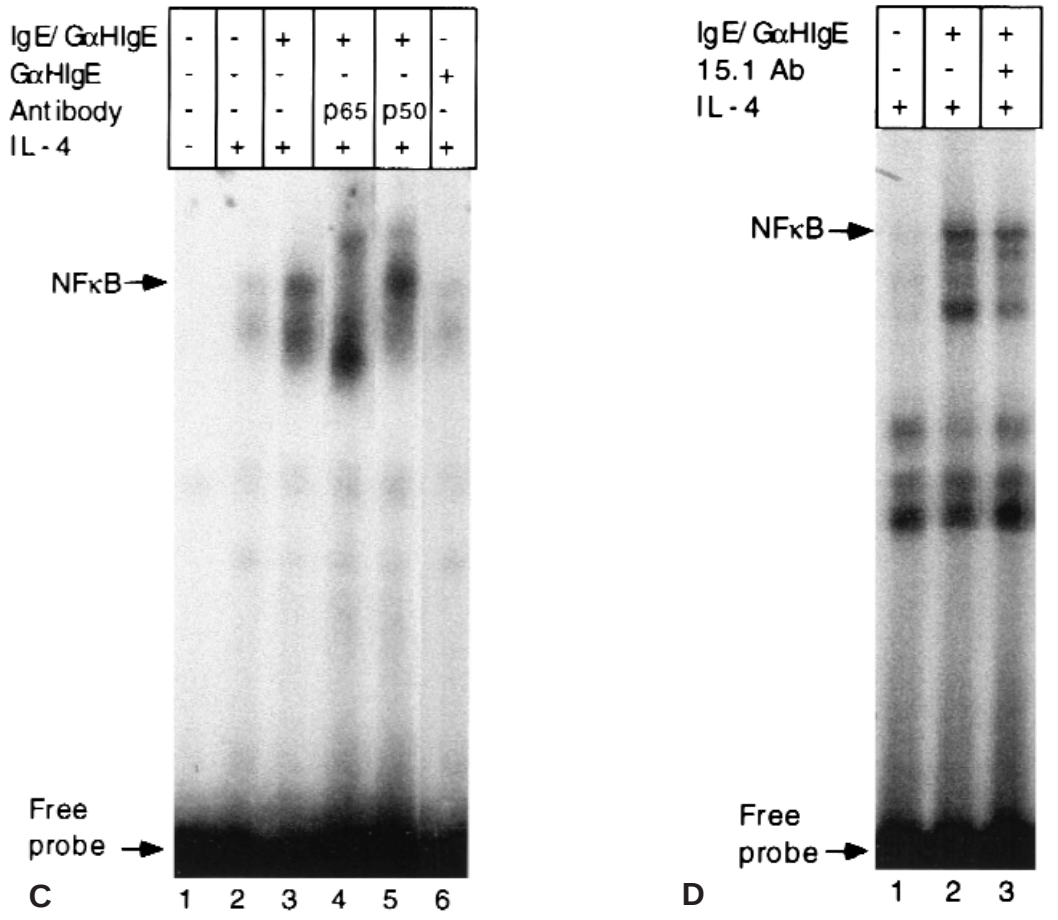

FIG 5. CONT.

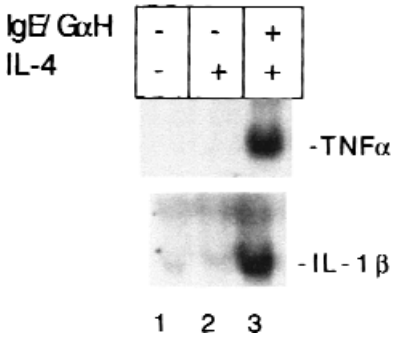

FIG 6. CD23 cross-linking induces gene transcription in monocytes. Northern blot analysis of total RNA from human monocytes hybridized with probes for human IL-1β or TNF-α is shown. *IgE/GαH* indicates CD23 cross-linking with IgE sensitization for 1 hour followed by treatment with GαHIgE for 2 hours.

that CD23-induced IκBα phosphorylation is mediated by TK and is independent of the PKC pathway.

To verify that herbimycin inhibition of IκBα degradation was not caused by an effect on CD23 expression, surface CD23 was measured by FACS in IL-4–induced U937 cells either treated or untreated with herbimycin for 18 hours. Our results demonstrated no difference in CD23 expression on herbimycin-treated cells compared with control cells (not shown), indicating a role of TK in the signal transduction pathway initiated by CD23.

To determine whether the target of the CD23-induced TK activity was IκBα, IκBα was immunoprecipitated from cytosolic extracts of U937 cells after CD23 cross-linking with or without ALLN. As shown in Fig 10, Western blot followed by anti-P-Tyr immunoblotting did not show the presence of phosphorylated tyrosine residues at the position at which IκBα migrates in the immunoprecipitated samples (*lanes 1* to *4*, *upper panel*). The P-Tyr antibody was shown to detect phosphotyrosine residues in whole cell lysates of control pervanadate-treated U937 cells (*lane 6*, *upper panel*). IκBα immunoblotting of the membrane previously used for P-Tyr immunoblotting revealed the presence of a slower migrating hyperphosphorylated form of IκBα induced after CD23 cross-linking in the ALLN-treated cells (*lanes 3* and *4*, *lower panel*). As expected, IκBα was not detected in cells after CD23 cross-linking in the absence of ALLN (*lane 2*, *lower panel*). These results indicate that IκBα is not directly phosphorylated in tyrosine residues after CD23 cross-linking and suggest the key role of a TK in the

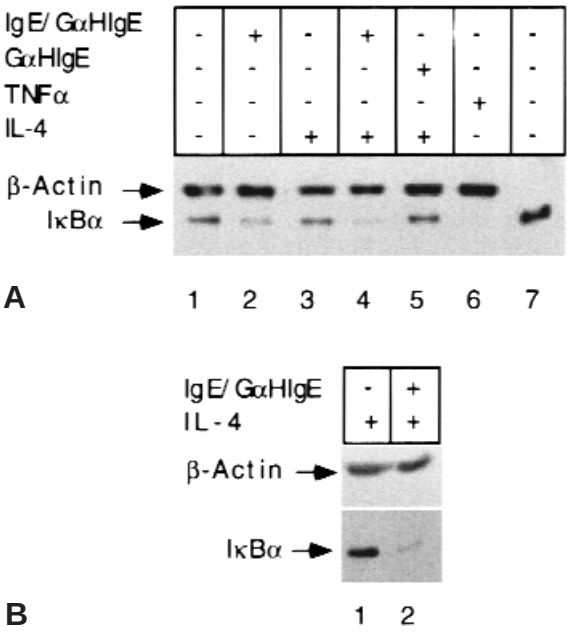

FIG 7. Degradation of IκBα by CD23 cross-linking. Western blots of cytosolic proteins from U937 cells **(A)** or monocytes **(B)** immunoblotted with antibodies to IκBα and β-actin are shown. *IgE/GαHIgE* indicates CD23 cross-linking with IgE sensitization for 1 hour followed by treatment with GαHIgE for 30 minutes. *GαHIgE* indicates treatment with GαHIgE for 30 minutes without presensitization with IgE (**A**, *lane 5*). *TNFα* indicates treatment with TNF-α for 30 minutes (**A**, *lane 6*). Recombinant IκBα was added (**A**, *lane 7*) as a molecular weight marker.

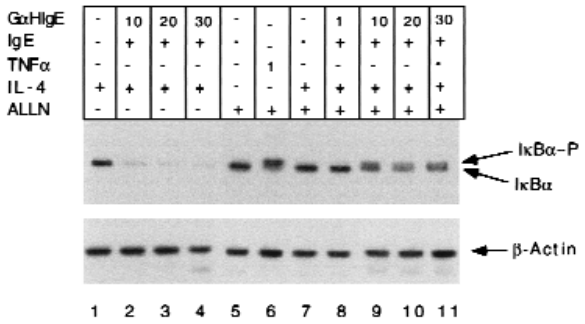

FIG 8. Phosphorylation of IκBα induced by CD23 cross-linking. Western blot analysis of cytosolic proteins from U937 cells immunoblotted with antibodies to IκBα and β-actin is shown. Cells were sensitized with IgE for 1 hour, and CD23 cross-linking was performed by incubation with GαHIgE for the indicated times (in minutes). TNF-α treatment was done for 1 minute *(lane 6)*. *Arrows* indicate the migration of the phosphorylated form of IκBα *(IκBα-P)*, unphosphorylated IκBα, and β-actin.

CD23-initiated signal transduction pathway that leads to IκBα phosphorylation and hence NF-κB activation.

DISCUSSION

Our results suggest a new model in the activation of monocytes through their CD23 receptor (Fig 11). Monocytes from atopic individuals express increased amounts of CD23 on their surface.[2] The CD23 upregulation is probably the result of increased production of IL-4 and IL-13 secondary to a T_{H1}/T_{H2} imbalance in allergic diseases.[5] Atopic subjects also have higher levels of circulating IgE than normal control subjects. IgE is not only present in the circulation but also bound to its low-affinity receptor CD23. When the individual is exposed to the relevant allergen, it binds to the IgE that is attached to the CD23 on the surface of monocytes, inducing the cross-linking of these receptors. CD23 cross-linking induces TK activity that phosphorylates a cytoplasmic molecule yet to be identified. This results in phosphorylation of IκBα in the cytoplasm of monocytes. Phosphorylated IκBα then dissociates from the p65/p50 NF-κB complex and is suitable for degradation in the cellular proteosome. Free NF-κB translocates to the nucleus and binds κB sequences in the promoter of genes such as IL-1β, TNF-α, and other inflammatory cytokines. Activated monocytes then secrete these gene products, which have been shown to be NF-κB activators and can act in an autocrine manner to perpetuate the immunologic reaction.

Our results indicate that NF-κB is the main transcription factor involved in the signal transduction of FcεRIIb. We have analyzed the nuclear translocation of other transcription factors, such as SP-1, AP-1, and CREB, which potentially could be implicated in the activation of genes expressed in AMs during an allergic reaction. We have not been able to demonstrate activation of any of the above transcription factors after CD23 cross-linking under the same conditions that activate NF-κB. The importance of NF-κB activation in the pathogenesis of asthma is supported by recent in vivo studies. Yang et al[19] have shown an essential role of NF-κB in the induction of eosinophilia in allergic airway inflammation in mice. In addition, Hart et al[18] demonstrated significant NF-κB activation in induced sputum and bronchial biopsy specimens of patients with asthma.

Recent reports have shown evidence of surface expression of the high-affinity receptor for IgE (FcεRI) on human monocytes and other antigen-presenting cells from atopic individuals.[8,9] FcεRI on monocytes seems to play a role in antigen presentation, although it might also be involved in cell activation.[9] We were unable to detect any expression of FcεRI on U937 cells by FACS and RT-PCR analysis. Human peripheral blood monocytes from anonymous donors (unknown atopy history) had mRNA for both chains of FcεRI but did not express this receptor on the surface. Stimulation of U937 cells with IgE immune complexes was not associated with phosphorylation of the γ-chain of FcεRI and induced NF-κB activation that was not inhibited by a blocking antibody to the α-chain of FcεRI. In addition, NF-κB activation was consistently stronger in cells that were treated with IL-4, which upregulates CD23 but not FcεRI. Therefore we conclude that the effects seen after cellular activation with IgE immune complexes are caused by cross-linking of CD23 and not FcεRI.

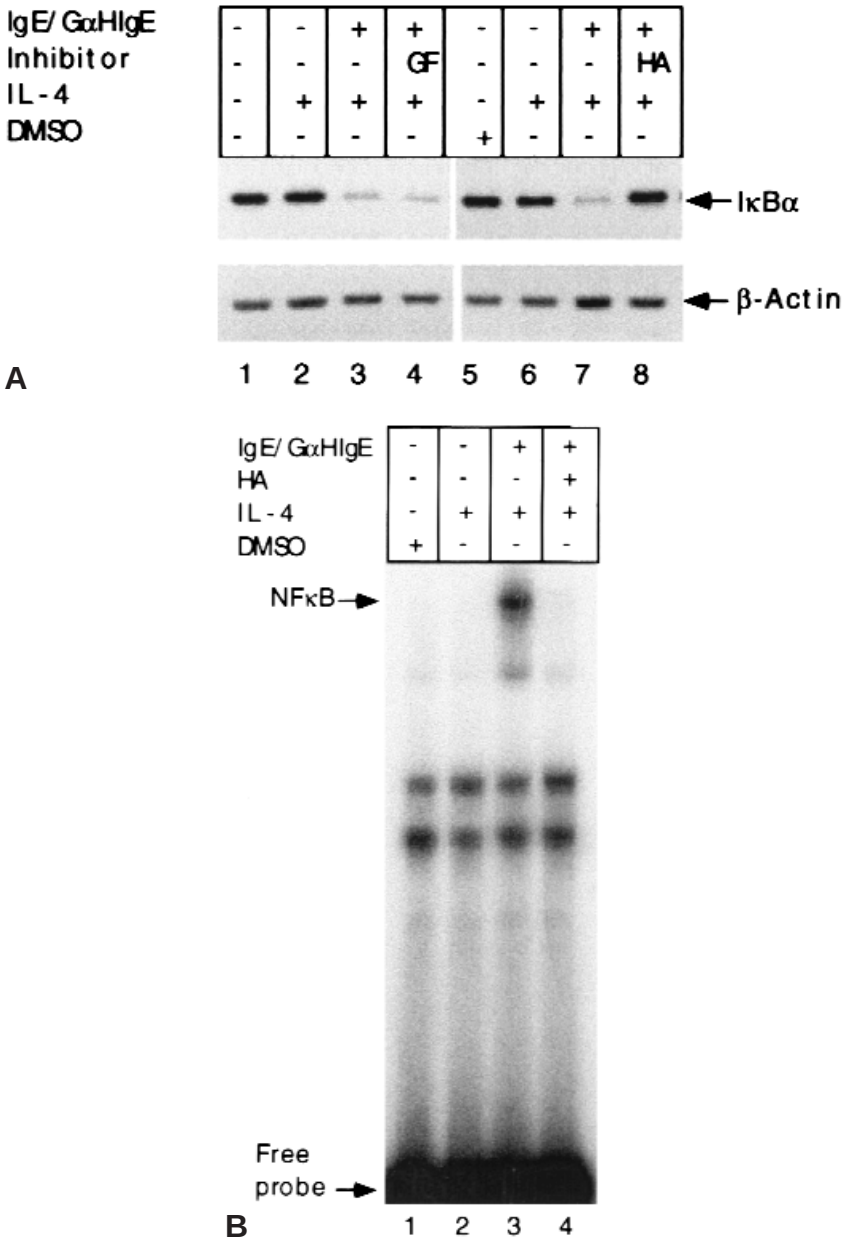

FIG 9. CD23-induced phosphorylation of IκBα is mediated by a TK. **A,** Western blot analysis of cytosolic proteins from U937 cells immunoblotted with antibodies to IκBα and β-actin is shown. *IgE/GαHIgE* indicates CD23 cross-linking with IgE sensitization for 1 hour followed by treatment with GαHIgE for 30 minutes (*lanes 3* and *4*) or 10 minutes (*lanes 7* and *8*). *GF* and *HA* indicate treatment of the cells with the PKC inhibitor GF 109203X or the TK inhibitor herbimycin for 1 or 18 hours, respectively, before CD23 cross-linking. *DMSO* indicates treatment of the cells with DMSO for 18 hours *(lane 5)*. **B,** Gel shift assay of nuclear extracts from U937 cells with a κB probe is shown. *IgE/GαHIgE* indicates CD23 cross-linking with IgE sensitization for 1 hour followed by treatment with GαHIgE for 10 minutes (*lanes 3* and *4*). *HA* indicates treatment with herbimycin for 18 hours *(lane 4)*. *DMSO* indicates treatment of the cells with DMSO for 18 hours *(lane 1)*.

Several NF-κB inhibitory proteins have been described that keep NF-κB inactive in the cytoplasm.[11] We show here that IκBα is the molecule targeted during the signal transduction of CD23 in monocytes and U937 cells. Different phosphorylation sites exist in IκBα. The classical sites are serines in the amino terminus of the molecule that are phosphorylated by identified kinases.[12] Recent reports indicate that IκBα can also be phosphorylated at tyrosine residues.[13,14] However, a physiologic role for Tyr-phosphorylated IκBα has not been found yet. The FcεRIIb molecule does not contain tyrosines in the intracytoplasmic domain that could be a substrate for TK. Therefore it is likely that binding of the ligand to the FcεRIIb receptor induces activation of a TK that phos-

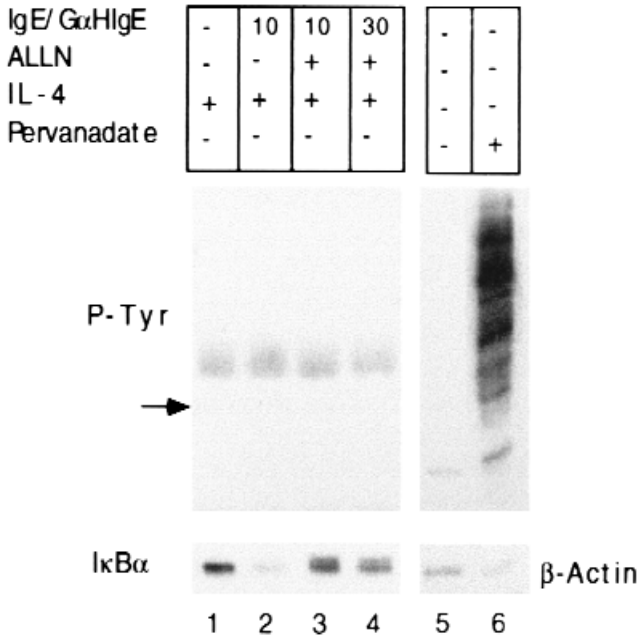

FIG 10. IκBα phosphorylation induced by CD23 cross-linking is not located in tyrosine residues. Western blot analysis of cytosolic proteins from U937 cells immunoprecipitated with anti-IκBα antibody (*lanes 1* to *4*) or U937 whole cell extracts (*lanes 5* and *6*) is shown. The membrane was blotted with anti-phosphotyrosine (*P-Tyr*; *lanes 1* to *6*, *upper panels*), anti-IκBα (*lanes 1* to *4*, *lower panel*) or β-actin (*lanes 5* and *6*, *lower panel*) antibodies. *Arrow* indicates the position where IκBα should migrate. *IgE/GαHIgE* indicates CD23 cross-linking with IgE sensitization for 1 hour followed by treatment with GαHIgE for the indicated time (in minutes). ALLN, IL-4, and pervanadate treatments were performed as described in the Methods section.

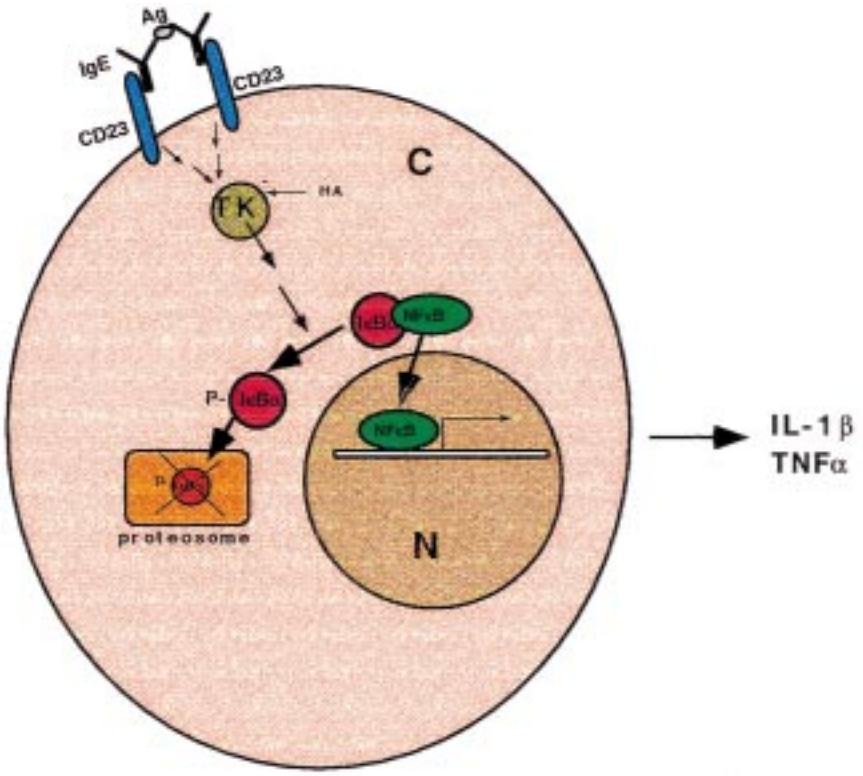

FIG 11. Diagram of the proposed mechanism of CD23 signal transduction. *Ag,* Antigen; *HA,* herbimycin A; *P,* phosphorylated protein; *C,* cytoplasm; *N,* nucleus. The minus symbol denotes inhibitory action.

phorylates a mediator protein involved in the signal transduction. We have shown here that this protein is not IκBα but another as yet unidentified molecule. The TK-mediated phosphorylation of this unknown target is essential for the induction of IκBα phosphorylation and subsequent NF-κB activation.

Our model provides data in U937 cells and peripheral blood monocytes. However, recent reports have shown the importance of CD23 activation in AMs from asthmatic individuals.[22]

These data provide useful tools for the future development of drugs that inhibit the CD23-mediated inflammatory response and could be used in the treatment of patients with asthma and other allergic diseases.

We thank all the members of Dr C. Paya's laboratory for helpful discussions. We also thank Dr J. P. Kinet for kindly providing the U937-FcεRIαβγ cells and mAb 15.1 and Dr P. Leibson for providing the anti-FcεRIγ antibody and for helpful discussion.

REFERENCES

1. Campbell AM. Bronchial epithelial cells in asthma. Allergy 1997;52:483-9.
2. Williams J, Johnson S, Mascali JJ, Smith H, Rosenwasser LJ, Borish L. Regulation of low affinity IgE receptor (CD23) expression on mononuclear phagocytes in normal and asthmatic subjects. J Immunol 1992;149:2823-9.
3. Yokota A, Kikutani H, Tanaka T, Sato R, Barsumian EL, Suemura M, et al. Two species of human Fce receptor II (FceRII/CD23): tissue-specific and IL-4-specific regulation of gene expression. Cell 1988;55:611-8.
4. Mudde GC, Bheekha R, Bruijnzeel-Koomen CAFM. Consequences of IgE/CD23-mediated antigen presentation in allergy. Immunol Today 1995;16:380-3.
5. Vercelli D, Jabara HH, Lee B, Woodland N, Geha RS, Leung DYM. Human recombinant interleukin 4 induces FcεR2/CD23 on normal human monocytes. J Exp Med 1988;167:1406-16.
6. Gosset P, Tsicopoulos A, Wallaert B, Joseph M, Capron A, Tonnel A. Tumor necrosis factor alpha and interleukin-6 production by human mononuclear phagocytes from allergic asthmatics after IgE-dependent stimulation. Am Rev Respir Dis 1992;146:768-74.
7. Dugas N, Vouldoukis I, Bécherel P, Arock M, Debré P, Tardieu M, et al. Triggering of CD23b antigen by anti-CD23 monoclonal antibodies induces interleukin-10 production by human macrophages. Eur J Immunol 1996;26:1394-8.
8. Maurer D, Fiebiger E, Reininger B, Wolff-Winiski B, Jouvin MH, Kilgus O, et al. Expression of functional high affinity immunoglobulin E receptors (Fc epsilon RI) on monocytes of atopic individuals. J Exp Med 1994;179:745-50.
9. Takenara M, Tanaka Y, Anan S, Yoshida H, Ra C. High affinity IgE receptor-mediated prostaglandin E2 production by monocytes in atopic dermatitis. Int Arch Allergy Immunol 1995;108:247-53.
10. Paul-Eugene N, Kolb JP, Abadie A, Gordon J, Delespesse G, Sarfati M, et al. Ligation of CD23 triggers cAMP generation and release of inflammatory mediators in human monocytes. J Immunol 1992;149:3066-71.
11. Barnes PJ, Karin M. Nuclear factor-κB—a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997;336:1066-71.
12. Régnier CH, Song HY, Gao X, Goeddel DW, Cao Z, Rothe M. Identification and characterization of an IκB kinase. Cell 1997;90:373-83.
13. Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EBM, Mueller-Dieckmann C, et al. Tyrosine phosphorylation of IκB-α activates NF-κB without proteolytic degradation of IκB-α. Cell 1996;86:787-98.
14. Singh S, Darnay BG, Aggarwal BB. Site-specific tyrosine phosphorylation of IκBα negatively regulates its inducible phosphorylation and degradation. J Biol Chem 1996;271:31049-54.
15. Marx J. How the glucocorticoids suppress immunity. Science 1995;270:232-3.
16. Quaaz F, Sola B, Issaly F, Kolb JP, Davi F, Mentz F, et al. Growth arrest and terminal differentiation of leukemic myelomonocytic cells induced through ligation of surface CD23 antigen. Blood 1994;84:3095-104.
17. Bayón Y, Alonso A, Sanchez Crespo M. Immunoglobulin-E/dinitrophenyl complexes induce nitric oxide synthesis in rat peritoneal macrophages by a mechanism involving CD23 and NF-κB activation. Biochem Biophys Res Commun 1998;242:570-4.
18. Hart LA, Krishnan VL, Adcock IM, Barnes PJ, Chung KF. Activation and localization of transcription factor, nuclear factor-κB, in asthma. Am J Respir Crit Care Med 1998;158:1585-92.
19. Yang L, Cohn L, Zhang D-H, Homer R, Ray A, Ray P. Essential role of nuclear factor κB in the induction of eosinophilia in allergic airway inflammation. J Exp Med 1998;188:1739-50.
20. Dignam J, Lebovitz R, Roeder R. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 1983;5:1475-89.
21. McElhinny JA, MacMorran WS, Bren GD, Ten RM, Israel A, Paya CV. Regulation of IκBα and p105 in monocytes and macrophages persistently infected with human immunodeficiency virus. J Virol 1995;69:1500-9.
22. Gosset P, Tillie-Leblond I, Oudin S, Parmentier O, Wallaert B, Joseph M, et al. Production of chemokines and proinflammatory cytokines by human alveolar macrophages activated by IgE receptors. J Allergy Clin Immunol 1999;103:289-97.