

FCER2 (CD23) Asthma-Related Single Nucleotide Polymorphisms Yields Increased IgE Binding and Egr-1 Expression in Human B Cells

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Abstract

CD23 is the low-affinity Fc receptor for IgE. When expressed on B cells, CD23 appears to play a role in regulation of IgE synthesis. Polymorphisms within FCER2, the gene encoding CD23, have been associated with atopy, increased risk of exacerbations in patients with asthma, and high serum IgE levels. A single-nucleotide polymorphism (rs2228137) present in exon 4 of FCER2 encodes a nonsynonymous amino acid change (R62W) and is the subject of the present analysis. Human B cell stable transfectants were established to characterize the functional relevance of the R62W SNP. We demonstrate that CD23b-R62W-expressing human B cells bind IgE with greater affinity than wild-type cells and display differences in kinetics of CD23-mediated ERK1/2 activation that may be responsible for the increased levels of Egr-1 mRNA observed after stimulation through CD23. Finally, the R62W SNP

seems to alter the tertiary or quaternary structure of CD23 because in the absence of N-glycosylation the CD23b-R62W-expressing cells appear to be less sensitive to endogenous proteases. These observations may have implications in mechanisms responsible for the atopic phenotypes observed in patients with asthma who possess this genotype.

Keywords: B cells; IgE receptor; asthma; polymorphism

Clinical Relevance

Delineation of the mechanism by which a single nucleotide polymorphism influences the onset of asthma could potentially guide selection of therapeutic regimens for affected individuals.

CD23, the low-affinity Fc receptor for immunoglobulin E (IgE), plays a critical role in IgE regulation (1, 2). It is expressed on several cell types, including monocytes/macrophages, eosinophils, follicular dendritic cells, intestinal epithelial cells, B cells (1), and airway smooth muscle cells (3). CD23 appears to have several roles in IgE-mediated immunity. These include mediating the production of proinflammatory cytokines by monocytes (4), transporting IgE and allergen-IgE complexes through gastrointestinal epithelium (5, 6), presentation of antigen-IgE complexes to T_H2 cells (7), and regulating IgE synthesis by B cells (1, 2). In addition to being present on the cell surface, CD23 is cleaved into soluble

fragments by endogenous proteases (8, 9) and released from the cell. In B cells, CD23 has been proposed to participate in positive- and negative-feedback mechanisms in the regulation of IgE synthesis (1, 2). It has been suggested that soluble CD23 is responsible for the up-regulation of IgE synthesis and that intact CD23 remaining on the membrane is responsible for the down-regulation of IgE (2). In response to stimulation with IL-4 plus anti-CD40, human B cells undergo class switching and begin to secrete IgE. In that process, germline C ϵ transcripts appear before initiation of switch recombination and thus before final gene rearrangement where the mature C ϵ transcript is made (10). However, when CD23 is cross-linked

using monoclonal anti-CD23 at the time human B cells are stimulated with IL-4 plus anti-CD40, germline C ϵ RNA expression (11) and total IgE production are diminished (11, 12), implying negative regulation by CD23 when it is occupied by IgE and the cognate antigen is encountered.

It has been suggested that polymorphisms within FCER2, the gene encoding CD23, could influence the ability of CD23 to control IgE synthesis (13) by influencing the tertiary and quaternary structure of the CD23 molecule. A link has been observed between genetic variations in FCER2, atopy, and high serum IgE levels (14). Single-nucleotide polymorphisms (SNPs) in FCER2 have been associated with increased risk of exacerbations in patients

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with asthma treated with inhaled corticosteroids (15), but the functional relevance of these SNPs in FCER2 remains unknown. One such SNP (rs2228137) encodes a nonsynonymous amino acid change (R62W). Nonsynonymous SNPs occur in the coding regions of genes. A missense SNP results in a substitution of an amino acid and therefore is likely to change the structure and function of the protein. As an example, the N40D polymorphism in the extracellular N-terminal region of the human mu opioid receptor enhances receptor function. Higher binding affinity and potency to the agonist β -endorphin were seen in the genetic variant compared with wild-type cells (16).

CD23 is a type II integral membrane protein with a single transmembrane region, and thus the carboxy-terminal region is extracellular and the amino-terminal domain is intracytoplasmic (17). The R62W SNP, which is located in exon 4 of the FCER2 gene (18), is in the extracellular segment of the protein close to the transmembrane domain. We have previously shown that stable human B-cell transfectants expressing the R62W SNP have increased IL-4R expression compared with wild-type CD23b transfectants after stimulation through CD23 (19). This suggests that the SNP conferred a change in the role of IgE regulation by CD23.

In the present study, we show that the CD23 R62W SNP binds IgE with higher affinity and displays differences in kinetics of ERK1/2 activation, resulting in increased expression of the transcription factor Egr-1, which regulates B-cell proliferation and development (20). We propose that the R62W SNP is functionally relevant and influences the ability of CD23 to regulate IgE synthesis by human B cells.

Materials and Methods

Cell Culture

The human Burkitt's lymphoma-derived B cell line DG75 does not express Fc receptors, including CD23 (21). Stable transfectants expressing wild-type CD23b-R62 or its genetic variant CD23b-R62W (19) were maintained in RPMI1640 (American Type Culture Collection, Manassass, VA) supplemented with 10% FBS (Hyclone, Thermo Scientific, Rockford, IL), 2 mM L-glutamine (Invitrogen, Life Technologies, Carlsbad, CA), 100 U/ml

penicillin (Invitrogen), and 100 μ g/ml streptomycin (Invitrogen) plus 5 μ g/ml blasticidin (Invitrogen). At indicated times, 30 to 50 $\times 10^6$ cells/ml were treated at 37°C with 10 μ g/ml of anti-CD23 (Ancell, Bayport, MN) or mouse IgG (Ancell) and cross-linked with 10 mg/ml rabbit anti-mouse IgG (Southern Biotech, Birmingham, AL). In some experiments, 1 $\times 10^6$ cells/ml were treated with 0.8 μ g/ml tunicamycin (Sigma-Aldrich, St. Louis, MO) for 48 hours at 37°C or with 0.5 mg/ml trypsin (Promega, Madison, WI) for 60 minutes at 4°C. In trypsin-treated cells, the reaction was terminated by the addition of 0.2 volumes of FBS containing 1.5 mg soybean trypsin inhibitor (EMD Millipore, Billerica, MA). Cells were extensively washed and analyzed for expression of membrane CD23.

Flow Cytometry

Expression of membrane CD23 was monitored by flow cytometry (Attune; Applied Biosystems, Life Technologies) using allophycocyanine (APC)-conjugated anti-CD23 or APC-isotype matched control antibody (BioLegend, San Diego, CA). Transfectants were checked every 2 to 3 weeks for the level of CD23 expression as described (19) because the cells were not clonally derived. Once the levels began to diminish, new aliquots were thawed and used for study. The level of ADAM10 (CD156c) was assessed using phycoerythrin-conjugated anti-ADAM10 (BioLegend).

Soluble CD23

Soluble CD23 was quantified using an sCD23-EASIA kit (Invitrogen) according to the manufacturer's instructions. Absorbance was read at 450 and 490 nm using the Synergy HT microplate reader equipped with KC4 software (Biotek, Winooski, VT).

IgE Binding Assay

Purified human IgE protein (Fitzgerald Industries, Acton, MA) and BSA (Sigma-Aldrich) for use as control for binding irrelevant protein were conjugated to APC using the DyLight Microscale Antibody Labeling Kit (Pierce, Thermo Scientific). The degree of labeling per mole of protein was calculated for IgE and BSA spectrophotometrically according to kit instructions. One million cells per milliliter were incubated on ice with various concentrations of IgE-APC as indicated or with 90 nM BSA-APC in TBS buffer (10 mM Tris-HCl [pH 7.3], 140 mM NaCl, 2 mM CaCl_2 , 1 mM MgCl_2). After 2 hours, cells were washed extensively with TBS buffer and analyzed by flow cytometry. Mean fluorescence intensity (MFI) was used as a measure of the amount of IgE-APC or BSA-APC bound by the cells. Specific binding to IgE was determined by subtraction of BSA-APC MFI from IgE-APC MFI. Binding affinities were determined using nonlinear regression analysis (GraphPad Prism, San Diego, CA).

Western Blotting

Preparation of whole cell lysates, SDS-PAGE, and protein blotting were performed

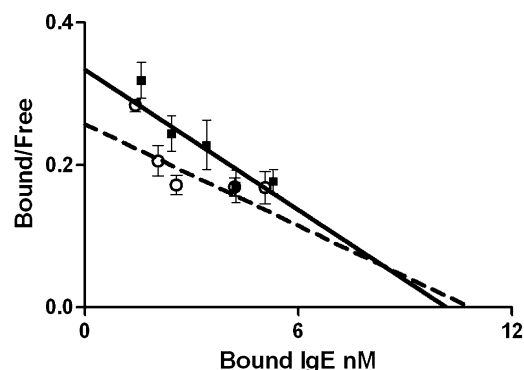


Figure 1. Scatchard analysis of IgE binding showing higher IgE binding affinity in CD23b-R62W cells. CD23b-R62 (circles) and CD23b-R62W (squares) cells were incubated at 4°C with BSA-allophycocyanine (APC) or various concentrations of human IgE-APC. After 2 hours, cells were washed four times, and IgE bound was quantified by flow cytometry. Each point represents the mean $\pm$ SEM of four independent experiments.

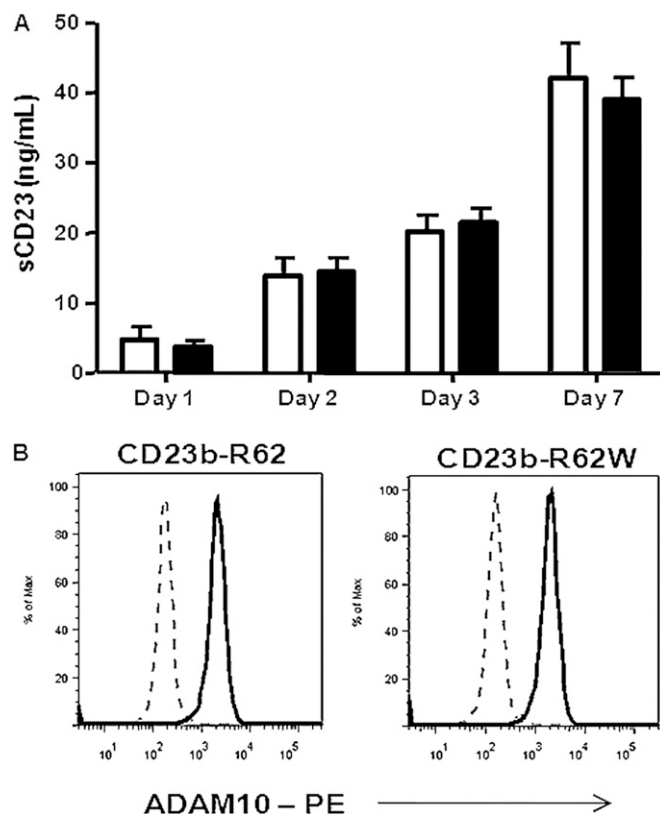


Figure 2. Comparable levels of soluble CD23 and ADAM10 expression. (A) Culture supernatant fluids were harvested from CD23b-R62 (open bars) and CD23b-R62W (closed bars) cells at various days after initiation of culture and quantified for soluble CD23 by ELISA. Data represent the mean $\pm$ SEM of four independent experiments. (B) CD23b-R62 and CD23b-R62W cells were stained with phycoerythrin-conjugated anti-human ADAM10 (solid line) or isotype control (stippled line), and surface expression was assessed by flow cytometry. Data are representative of three independent experiments.

as previously described (22). Antibodies were purchased from Cell Signaling Technology (Danvers, MA). Proteins were visualized by enhanced chemiluminescence (LumiGlo; Cell Signaling Technology). Densitometric measurements were performed using the Alpha Innotech FluorChem Imaging System (San Leandro, CA).

RNA Extraction, cDNA Preparation, PCR Arrays

Cytoplasmic RNA extraction and cDNA synthesis and quantification were performed as previously described (19). cDNA was used for the Human Signal Transduction Pathway Finder RT² Profiler PCR Array (SABiosciences/Qiagen, Valencia, CA) as previously described (19).

Results

Higher IgE Binding Affinity by R62W

The R62W SNP, located on exon 4 of the FCER2 gene (18), is in the extracellular

segment of the protein close to the transmembrane domain. It has been suggested that variations in the CD23 molecule as a result of a SNP could adversely affect the role of CD23 in the control of IgE synthesis (13), possibly by influencing the tertiary and quaternary structure of the molecule. We established stable human B-cell transfectants expressing wild-type CD23b-R62 or the genetic variant CD23b-R62W (19). Although the IgE binding domain of CD23 is located within the lectin head near the carboxy terminus of the molecule and the R62W SNP is located near the cytoplasmic membrane, we sought to determine if the R62W SNP could influence the ability of CD23 to bind IgE. Figure 1 shows by Scatchard plot analysis that the CD23b-R62W transfectants bound more IgE than wild-type CD23b-R62 cells. K_a values were 2.8×10^7 and 3.7×10^7 , respectively. These results suggest that the polymorphism affected the ability of CD23 to bind IgE

such that the R62W SNP could retain more IgE using its greater affinity.

Soluble CD23 Is Not Affected by the SNP

Membrane CD23 can be cleaved into soluble fragments (sCD23) by membrane-associated metalloproteases. ADAM10 has been identified as the predominant sheddase responsible for generating sCD23 in human B cells (9). One of the cleavage sites of the protease is close (15 aa) to the R62W SNP. To determine if the ability to release sCD23 was affected by the R62W SNP, culture supernatant fluids were collected from CD23b-R62- and CD23b-R62W-expressing cells at various days after initiation of culture and quantified for sCD23. The expression of ADAM10 was also evaluated by flow cytometry. Figure 2 illustrates that there was no difference in the generation of sCD23 (Figure 2A) and that both transfectants expressed comparable amounts of ADAM10 (MFI values of 27,985 and 25,426 for CD23b-R62 and CD23b-R62W cells, respectively) (Figure 2B). These results suggest that, despite the proximity to the cleavage site of the proteases, the R62W SNP did not influence the ability of ADAM10 or other metalloproteases to cleave membrane CD23 into its soluble fragments.

Influence of Tunicamycin on Membrane CD23

It has been suggested that N-glycosylation can stabilize and protect proteins from proteolytic enzymes (23). CD23 has a single N-glycosylation site adjacent to the R62W SNP, and in the presence of tunicamycin, an N-glycosylation inhibitor, less membrane CD23 was expressed by the human B cell line RPMI 8866 (24). This suggested that perhaps more sCD23 was generated due to increased access of proteolytic enzymes in the absence of N-glycosylation. We assessed the expression of membrane and soluble CD23 in nontreated and tunicamycin-treated cells by flow cytometry and ELISA, respectively. A significant difference ($P < 0.001$) as assessed by MFI was seen in the amount of membrane CD23 expressed between tunicamycin-treated CD23b-R62 and R62W cells. In tunicamycin-treated wild-type CD23b-R62 cells, there was an 87% mean decrease ($n = 8$) in the amount of membrane CD23 expressed compared with nontreated cells (Figure 3A). In

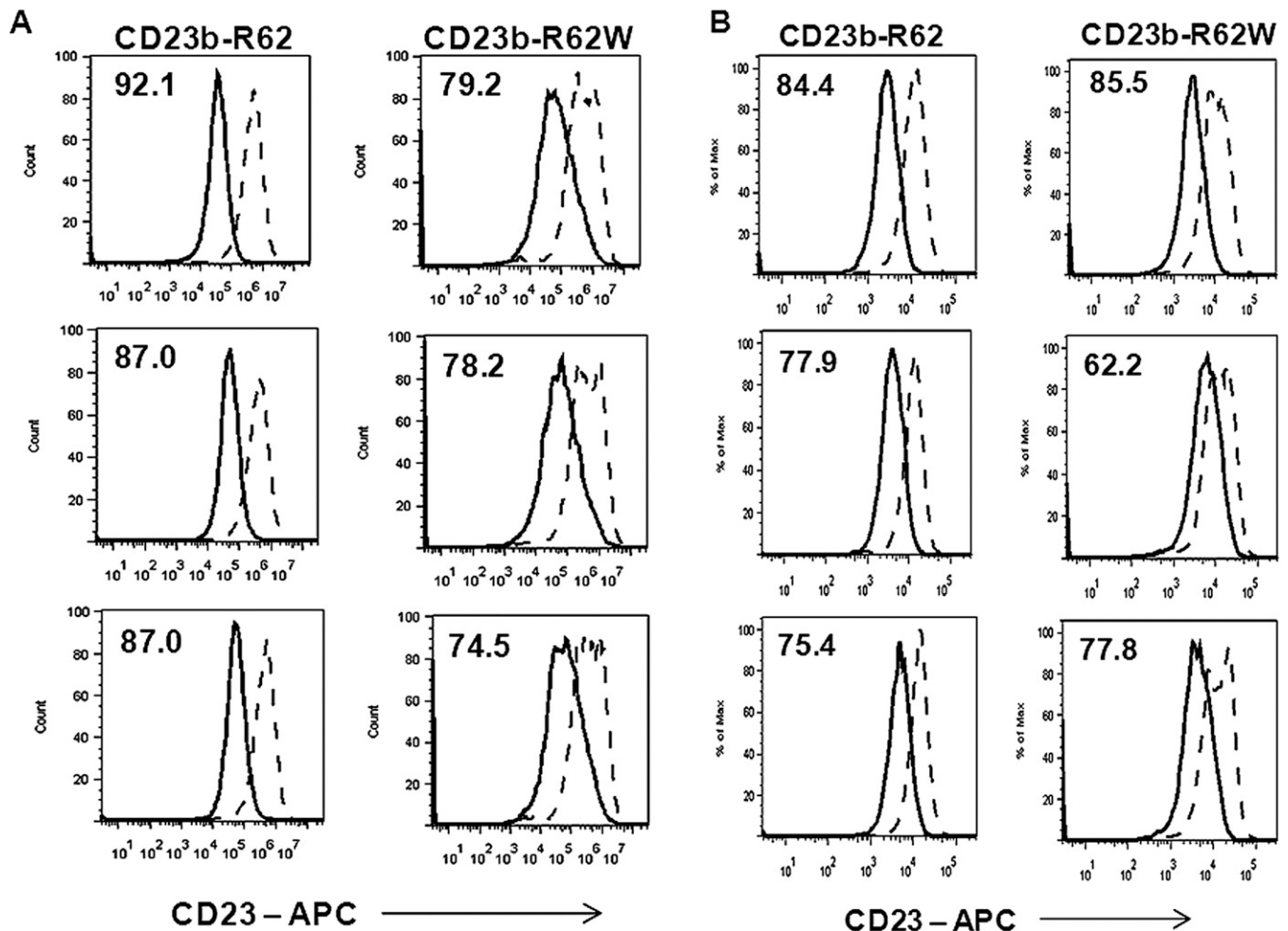


Figure 3. R62W cells are resistant to proteases in the absence of N-glycosylation. (A) CD23b-R62 and CD23b-R62W cells were left nontreated (stippled line) or treated for 48 hours with 0.8 μ g/ml tunicamycin (solid line). Cells were analyzed by flow cytometry after staining with anti-CD23 conjugated to APC. Three experiments are shown. (B) CD23b-R62 and CD23b-R62W cells were left nontreated (stippled line) or treated for 60 minutes with 0.5 mg/ml trypsin (solid line), after which soybean trypsin inhibitor was added. Cells were analyzed by flow cytometry after staining with anti-CD23 conjugated to APC. Three experiments are shown. Values shown in the upper left hand corner represent the percentage decrease in surface CD23 expression after treatment.

contrast, only a 76% mean decrease ($n = 8$) in membrane CD23 was observed in tunicamycin-treated CD23b-R62W cells compared with nontreated cells (Figure 3A). This difference was reflected also in the amount of sCD23 secreted by the cells. The level of sCD23 secreted by wild-type CD23-R62 cells was similar in nontreated ($22.36 \text{ ng/ml} \pm 0.50$) and tunicamycin-treated cells ($20.93 \text{ ng/ml} \pm 0.23$; $n = 3$). In contrast, a significant difference in the level of sCD23 was seen between nontreated ($17.67 \text{ ng/ml} \pm 0.31$) and tunicamycin-treated R62W cells ($11.42 \text{ ng/ml} \pm 0.25$; $P < 0.001$ [$n = 3$]). However, when the cells were treated with trypsin, a serine protease, the mean percentage decrease in membrane CD23 expression was comparable between wild type (78%)

and genetic variant (75%) (Figure 3B). These results suggest that in the absence of N-glycosylation the R62W SNP may influence the access of the proteases to membrane CD23.

ERK1/2 Phosphorylation

Because the CD23b-R62W transfectants appeared to bind more IgE, we sought to determine if this increased binding is reflected in differences in signaling into the cell. We have previously shown that ERK1/2 is activated in primary human B cells after ligation through CD23 (22). Kinetic differences in ERK1/2 phosphorylation between wild type CD23b-R62 and CD23b-R62W cells were observed after stimulation through CD23 (Figure 4). In wild-type CD23b-R62 cells, ERK1/2 phosphorylation

was seen within 2 minutes of stimulation and was diminished by 10 minutes (Figure 4A), whereas in the genetic variant CD23b-R62W, ERK1/2 phosphorylation occurred within 2 minutes of stimulation but was sustained such that there appeared to be a greater degree of phosphorylation of ERK1/2 after 10 minutes of stimulation (Figure 4B). These results suggest that the sustained activation of ERK1/2 phosphorylation observed in the CD23b-R62W genetic variant may be associated with increased IgE binding rather than with a difference in the amount of CD23 expressed on the cells. The mean fluorescence intensities of membrane CD23 were 34,909 and 33,222 for CD23b-R62 and CD23b-R62W, respectively. Differences in the kinetics of ERK1/2 activation have also

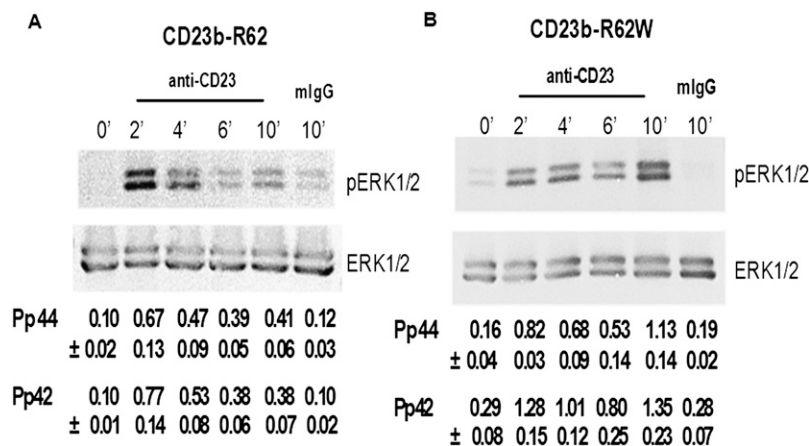


Figure 4. Kinetic differences in ERK1/2 activation. ERK1/2 phosphorylation in CD23b-R62 (A) and CD23b-R62W (B) cells left nontreated or stimulated with anti-CD23 or mouse IgG (mIgG) (control) for the indicated times. Cell lysates were analyzed by Western blotting. Blots were probed with anti-phosphoERK1/2 (pERK1/2) and then stripped and reprobed with anti-ERK1/2 (loading control). Values represent the ratio of pERK1/2 to total ERK1/2 and represent an average of three independent experiments $\pm$ SEM.

been seen when the cells are stimulated with IgE/anti-IgE (data not shown).

Egr-1 Activation Is Greater in the R62W Variant

Because we observed differences in the kinetics of ERK1/2 activation, we sought to determine whether differences in gene expression among the transfectants occurred after ligation through CD23. Using the Human Signal Transduction Pathway Finder RT² Profiler PCR array, which profiles the expression of 84 key genes representative of 18 different signal transduction pathways, we observed that the gene encoding the transcription factor Egr-1 was differentially expressed in the transfectants (Figure 5). The CD23b-R62W

genetic variant expressed a 16-fold increase ($P = 0.05$) in Egr-1 mRNA levels compared with nonstimulated wild-type CD23b-R62. These results suggest that stimulation through CD23 in the genetic variant leads to increased expression of the transcription factor Egr-1.

Discussion

CD23, the low-affinity receptor for IgE, has been proposed to play a critical role in IgE regulation (1, 2). It is expressed on several cell types, including smooth muscle cells, monocytes/macrophages, and B cells (1). Monoclonal antibody to CD23 was shown to interfere with IgE synthesis in

human B cells stimulated with IL-4 plus anti-CD40 (11, 12), and the target of inhibition appeared to be at the germline C ϵ transcription level (11), indicating that CD23 interfered with class switch to mature C ϵ RNA.

SNPs have been identified in the FCER2 gene that encodes CD23. The genetic variants are associated with increased asthma severity, decreased treatment response, and increased total serum IgE levels (15). We established stable human B cell transfectants expressing wild-type CD23b-R62 and the genetic variant R62W SNP (rs2228137) located in exon 4 of the FCER2 gene. We demonstrated previously an increase in IL-4R expression associated with the genetic variant when cells were stimulated through CD23 (19). We proposed that up-regulation of the IL-4R would facilitate signaling through IL-4 and favor class switch to increased synthesis of IgE. Thus, the genetic variant conferred a change by which CD23 regulated IgE production.

In the present study, we have elucidated further differences in the properties of cells expressing the genetic variant CD23b-R62W compared with cells expressing wild-type CD23b-R62. We have shown that the genetic variant consistently binds more IgE with greater affinity than wild-type cells. The K_a values were calculated to be 3.7×10^7 and 2.8×10^7 , respectively. These association constants were in the range of values calculated for IgE binding to CD23 in the Epstein-Barr virus-transformed cell line L-KT9 (25), U937 cells (26), and CD23-transformed COS cells (27). We did not expect to observe a dramatic increase in the binding affinity of IgE by the genetic variant because it is unlikely that a single amino acid substitution could drastically affect the overall tertiary structure of the receptor. Recently the crystal structure of IgE bound to CD23 revealed that only one IgE molecule could bind a single CD23 trimer allowing two membrane CD23 trimers or two membrane IgE molecules to be cross-linked (28). In the former case, this interaction would lead to down-regulation of IgE and the latter to up-regulation of IgE (2). We do not know if the CD23b-R62W SNP can form a trimer in the same manner as wild-type CD23b-R62. If the CD23b-R62W SNP is unable to form a trimer or if the structure of the trimer is different from wild-type CD23b-R62, then this could account for the increased IgE binding observed in the genetic variant.

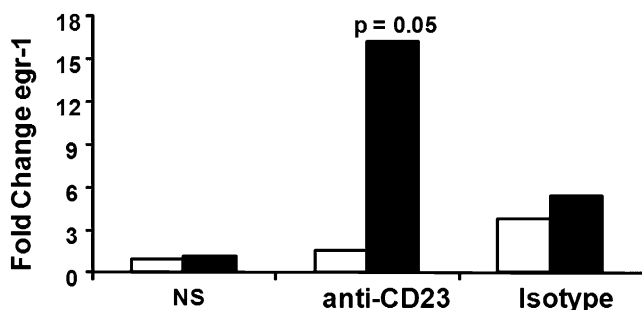


Figure 5. Egr-1 mRNA levels are induced in CD23b-R62W cells. CD23b-R62 (open bars) and CD23b-R62W (closed bars) cells were left nonstimulated (NS) or were stimulated with anti-CD23 or isotype control. After 6 hours, RNA was extracted and cDNA synthesized for use in PCR array analysis. Results were normalized relative to nonstimulated CD23b-R62 transfectants from three independent experiments. Statistically significant differences (P values) were determined by Student's t test.



Figure 6. Protein prediction model based on I-Tasser. Predicted protein structure of CD23b-R62 (yellow) and CD23b-R62W (teal) based on I-Tasser using the amino acid sequence of the extracytoplasmic domains of CD23b-R62 and CD23b-R62W. R62 and R62W are highlighted in pink and purple, respectively. Image generated by PyMol.

We also observed differences in the kinetics of ERK1/2 activation when the cells were stimulated through CD23. Although phosphorylation of ERK1/2 occurred within 2 minutes of stimulation of wild-type or genetic variant, ERK1/2 phosphorylation was sustained in the genetic variant such that the level of ERK1/2 phosphorylation was greater in the genetic variant after 10 minutes of stimulation. Activation of ERK1/2 can lead to the activation of several transcription factors, including Egr-1 (29, 30). By PCR array analysis, we showed that Egr-1 mRNA levels were significantly increased ($P = 0.05$) in the genetic variant compared with wild type after stimulation through CD23. Using the TFSEARCH program (31), we identified an Egr-1 consensus binding site in the IL-4R α promoter region. Thus, it is possible that the increased level of Egr-1 mRNA observed in the genetic variant may

contribute to the increased expression of the IL-4R that we have previously reported in the genetic variant (19).

Recently it was reported that CD23-R62W was resistant to enzymatic cleavage by the metalloproteinase ADAM 33 (18). Human embryonic kidney-derived 293-F cells were cotransfected with plasmids expressing CD23-R62W and ADAM33. No soluble CD23 was detected in 293-F cells coexpressing ADAM33 and the R62W SNP. Several metalloproteinases are capable of cleaving membrane CD23 into its soluble fragments, but it appears that the metalloproteinase ADAM10 is the physiological CD23 sheddase (9). We assessed the expression of ADAM10 by flow cytometry in the B cell transfectants and found that the two cell strains expressed comparable levels of ADAM10. Furthermore, we did not observe a difference in the quantity of soluble CD23

secreted between the two cell strains after 3 days of culture. It is possible that the differences in the resistance of the R62W SNP to cleavage by the metalloproteinases may be inherent to the cell origin and regulation of expression of ADAM10 and other potential sheddases.

N-glycosylation of proteins stabilizes proteins from proteases (23). The R62W SNP is adjacent to the N-glycosylation site within CD23 and is close (15 aa) to the cleavage site for ADAM10. We observed that in the absence of N-glycosylation the ability of the endogenous proteases to cleave CD23 into its soluble fragments is hindered in cells expressing the genetic variant CD23b-R62W compared with wild-type CD23b-R62. Thus, it is possible that the tryptophan substitution may prevent access to the cleavage site by altering the tertiary or quaternary structure of CD23. Molecular modeling (32, 33) of the extracytoplasmic domains of the wild-type CD23b-R62 and the genetic variant CD23b-R62W has predicted changes in the helical structure of the stalk region of CD23 close to and 56 residues away from the R62W SNP (Figure 6). We do not know the impact of the R62W SNP on CD23 trimerization. Studies are underway to determine if the R62W SNP influences the ability of CD23 to form trimers on the cell surface.

In the present study we continue to elucidate the functional relevance of the R62W SNP. In cells expressing the genetic variant, the ability to bind more IgE may result in prolonged ERK1/2 activation, which leads to increased expression of the transcription factor Egr-1. Increased Egr-1 may result in increased expression of IL-4R that would favor increased IgE production. Studies are under investigation to determine if these observations occur in primary B cells from patients with atopy expressing the R62W genotype. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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