An IgE-associated polymorphism in *STAT6* alters NF-κB binding, *STAT6* promoter activity, and mRNA expression

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Background: The IL-4/IL-13 pathway is central for IgE regulation. Signal transducer and activator of transcription 6 (STAT6) is the major transcription factor within this pathway. *STAT6* polymorphisms were recently associated with elevated total IgE levels in a genome-wide association study.
Objective: This study aimed to assess biological mechanisms by which an IgE-associated genetic variation in *STAT6* may potentially influence gene expression.
Methods: *STAT6* intron 2 carrying either the wild-type C or the polymorphic T allele of the putatively causal single nucleotide polymorphism rs324011 was cloned into *STAT6* promoter vectors to investigate their influence on *STAT6* promoter activity by *in vitro* luciferase assays. Transcription factor binding depending on rs324011 was examined by electrophoretic mobility shift assays in Jurkat T cells and primary $CD4^+$ T cells. Allele-specific *STAT6* gene expression of 3 splice variants was studied *ex vivo* by real-time PCR in 239 individuals.
Results: *STAT6* intron 2 acts as a silencer regulatory element. The polymorphic T allele at rs324011 (in linkage disequilibrium with the genome-wide association signal and consistently associated with elevated IgE levels in 3 previous studies) increases *STAT6* promoter activity significantly *in vitro* ($P < .00001$) and gene expression of *STAT6* splice variants *ex vivo* ($P < .01$) compared with the wild-type C allele. These effects correlate with the creation of a novel, T-allele–specific binding site for the transcription factor nuclear factor-κB in T cells.
Conclusion: The consistently replicated effects of genetic variance in *STAT6* on IgE regulation may be explained in part by allele-specific alterations in nuclear factor-κB binding at rs324011 and consecutive changes in *STAT6* gene expression. (J Allergy Clin Immunol 2009;124:583-9.)

Key words: *STAT6, NF-κB binding, regulatory element, mRNA gene expression, splice variants*

Signal transducer and activator of transcription (STAT)–6 is known to be a key mediator in the activation of the IL-4/IL-13 pathway essential for the class-switch to IgE. STAT6 affects the development of atopic diseases in many ways, and the Janus tyrosine kinase-STAT signaling pathway is involved in numerous physiologic events that are dysregulated in asthma.[1-3] It was shown that *STAT6*-deficient mice failed to develop bronchial hyperreactivity and airway eosinophilia after allergen provocation.[4,5]

Three major genetic determinants of IgE regulation have recently been identified by a genome-wide association study and replications in white populations.[6] The *STAT6*-associated polymorphism rs12368672 relates to one of the genome-wide association signals. It is in strong linkage disequilibrium ($r^2 = 0.8$) with a single nucleotide polymorphism (SNP) in *STAT6* intron 2 (rs324011; see this article's Fig E1 in the Online Repository at www.jacionline.org), which had been associated with elevated IgE levels in 3 independent previous populations.[7-9] The same SNP in intron 2 strongly influenced IgE regulation and asthma susceptibility in combination with other polymorphisms of the IL-4/IL-13 pathway.[10]

Although STAT6 is a central transcription factor in an important immunological pathway, few data on *STAT6* gene regulation and even less information on genetically determined changes in *STAT6* gene function are available. A putative functional role of rs324011 was previously suggested by the high degree of phylogenetic conservation of the SNP harboring region among apes and primates.[8] *In silico* sequence analysis implied that the polymorphic T allele of rs324011 may create a novel nuclear factor-κB (NF-κB) transcription factor binding site not detectable with the C allele. Thus, in the current study, the functional role of intron 2 *per se* was analyzed. Also investigated was how polymorphism rs324011 located in this intronic region may affect *STAT6* regulation, explaining the highly consistent associations with IgE across different populations.

METHODS

STAT6 reporter constructs

Three pGL3-basic vector constructs containing *STAT6* promoters of different sizes, pBP78 (5643 bp), pBP88 (2588 bp), and pBP86 (1081bp), were

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Abbreviations used
EMSA: Electrophoretic mobility shift assay
GMR: Geometric mean ratio
kb: Kilo base pair
NF-κB: Nuclear factor-κB
PARSIFAL: Prevention of Allergy-Risk factors for Sensitization In Children related to Farming and Anthroposophic Lifestyle
PMA: Phorbol 12-myristate 13-acetate
PO: Polymorphic allele
SNP: Single nucleotide polymorphism
SP: Specificity protein
STAT: Signal transducer and activator of transcription
WT: Wild-type allele

generously provided by Dr B. Patel.[11] To study the effects of *STAT6* intron 2, 1287 bp of *STAT6* intron 2 was cloned 5′3′ or 3′5′ into *STAT6* promoter constructs (pBP78, pBP86) downstream of the luciferase gene (see this article's Fig E2 in the Online Repository at www.jacionline.org). More detailed information is available from the authors on request. Site-directed mutagenesis was performed to insert the polymorphic T allele of rs324011 (Stratagene, La Jolla, Calif; 5′-CATGAGTGGTGGTGGGGACAGTCCCTAGGAGGGCTA TC-3′). The respective sequence of *STAT6* promoters and intron 2 was confirmed by resequencing.

Transient transfection experiments using *STAT6* reporter constructs

Jurkat T cells (8×10^6) were transiently cotransfected with each *STAT6* reporter construct (4 μg; N = 10) and pRL-TK (10 ng; Promega, Madison, Wis) by square-wave electroporation with 1 pulse (50 ms; 200 V; Genpulser-Xcell; Biorad, Hercules, Calif). Cells were left in medium or stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin (12.5 ng/mL; 0.25 μmol/L) 3 hours after transfection and harvested 24 hours after transfection (N = 10). Luciferase activity was quantified as relative light units by using the Dual-Luciferase Assay according to the manufacturer's protocol (Promega, Madison, Wis).

Electrophoretic mobility shift assay

Nuclear extract was prepared from Jurkat T cells (http://www.dsmz.de) and primary $CD4^+$ T cells as previously described.[12] Primary $CD4^+$ T cells were isolated, applying the $CD4^+$ T-cell isolation kit (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) after retaining PBMCs from whole blood by using the density-gradient centrifugation (Ficoll-Paque; Amersham, Piscataway, NJ). Double-stranded probes (100 ng; Table I) carrying either the wild-type C or the polymorphic T alleles of rs324011were end-labeled as previously described.[12] Each binding reaction included 5 μg nuclear extract, 1x binding buffer,[12] and 0.5 μg/reaction Poly(dI-dC)-Poly(dI-dC). Supershift and competition experiments were performed by adding either the transcription factor specific antibody (4 μg) or unlabeled probes in a 100-fold molar excess before adding the labeled probe.

Ex vivo mRNA expression study of *STAT6* splice variants

Gene expression of STAT6 splice variants (non-STAT6d/STAT6e, STAT6d, STAT6e) was measured in PBMCs obtained from children from the Swiss branch of the cross-sectional Prevention of Allergy-Risk factors for Sensitization In Children related to Farming and Anthroposophic Lifestyle (PARSIFAL) population.[13] DNA and RNA were available for 239 children in sufficient quantities. The genotype of rs324011 for each individual was determined by restriction fragment length polymorphism. Isolation of RNA and reverse transcription of RNA were performed as previously described.[14] Primers and probes for 3 STAT6 isoforms (non-STAT6d/STAT6e, STAT6d, STAT6e) and the endogenous control (18SrRNA) were designed with the primer design software Primer Express (Applied Biosystems, Foster City, Calif) and purchased from Microsynth (Balgach, Switzerland; Table II). Real-time PCR (*Taq*Man) was carried out with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Data with the comparative (ΔΔCt) method were analyzed according to the manufacturer's instructions (Applied Biosystems, Foster City, Calif). Statistical analyses were performed with SAS 9.1.3 (SAS Institute, Cary, NC). Geometric mean ratios (GMRs) and 95% CIs were calculated in a multiple logistic regression analysis.

RESULTS

Intron 2 silences *STAT6* promoter activity

Because SNP rs324011 is located in an intronic region of *STAT6* with unknown function, we first investigated the general influence of intron 2 on *STAT6* promoter activity. Only then could the role of rs324011 on *STAT6* promoter activity be studied. *STAT6* promoter constructs (pBP78, pBP88, pBP86[11]) of different sizes (5.6kb, 2.6kb and 1.1kb) were used to identify the minimal region necessary for maximal *STAT6* transcription in Jurkat T cells. Consistent with a previous report,[11] our data indicated that the minimal *STAT6* promoter pBP86 (1.1kb) had the strongest transcriptional activity (15-fold upregulation) after T-cell stimulation (see this article's Fig E3 in the Online Repository at www.jacionline.org). The largest promoter construct pBP78 (5.6 kb) led to a 5-fold increase in comparison with the pGL3-basic vector.

Next, luciferase activity of pBP86 (1.1 kb) and pBP78 (5.6 kb) containing intron 2 downstream of the luciferase gene was assessed by using the wild-type C allele (corresponding to pBP86_wild-type allele [WT] or pBP78_WT). *STAT6* intron 2 acted as a silencing element on the pBP86 promoter, indicated by a significant drop in luciferase activity (Fig 1, *A*). A similar decline of the *STAT6* promoter activity was obtained for pBP78_WT in the presence of intron 2 (Fig 1, *B*).

Alleles of SNP rs324011 influence *STAT6* promoter activity *in vitro*

Allele-specific effects were assessed with *STAT6* vectors pBP86 and pBP78 also carrying the polymorphic T allele of rs324011 in intron 2 (corresponding to pBP86_polymorphic allele [PO] and pBP78_PO, Fig 1, *A and B*). The activity of the *STAT6* promoter pBP78 was strongly influenced by the presence of polymorphic T allele (pBP78_PO) significantly increasing the luciferase signal compared with pBP78_WT. Very similar allele-specific effects were achieved when intron 2 was clone 3′5′ into the respective site of the pBP78 vector downstream of the luciferase gene (data not shown).

Allele-specific NF-κB binding to rs324011 in intron 2 in T cells

Previous *in silico* transcription factor binding analyses suggested that NF-κB[8] and specificity protein (SP)–1 may bind to rs324011 within *STAT6* intron 2, whereas the binding of NF-κB was predicted to be allele-specific (Fig 2). Because altered transcription factor binding may explain the observed increase in *STAT6* promoter activity, electrophoretic mobility shift assay (EMSA) experiments were conducted with nuclear extract from the human Jurkat T-cell line to examine DNA/protein interactions of these transcription factors at the respective *STAT6* site.