Alanine-scanning mutagenesis of human signal transducer and activator of transcription 1 to estimate loss- or gain-of-function variants

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Background: Germline heterozygous mutations in human signal transducer and activator of transcription 1 *(STAT1)* can cause loss of function (LOF), as in patients with Mendelian susceptibility to mycobacterial diseases, or gain of function (GOF), as in patients with chronic mucocutaneous candidiasis. LOF and GOF mutations are equally rare and can affect the same domains of STAT1, especially the coiled-coil domain (CCD) and DNA-binding domain (DBD). Moreover, 6% of patients with chronic mucocutaneous candidiasis with a GOF *STAT1* mutation have mycobacterial disease, obscuring the functional significance of the identified *STAT1* mutations. Current computational approaches, such as combined annotation-dependent depletion, do not distinguish LOF and GOF variants.

Objective: We estimated variations in the CCD/DBD of STAT1.

Methods: We mutagenized 342 individual wild-type amino acids in the CCD/DBD (45.6% of full-length STAT1) to alanine and tested the mutants for STAT1 transcriptional activity.

Results: Of these 342 mutants, 201 were neutral, 30 were LOF, and 111 were GOF mutations in a luciferase assay. This assay system correctly estimated all previously reported LOF mutations (100%) and slightly fewer GOF mutations (78.1%) in the CCD/DBD of STAT1. We found that GOF alanine mutants occurred at the interface of the antiparallel STAT1 dimer, suggesting that they destabilize this dimer. This assay also precisely predicted the effect of 2 hypomorphic and dominant negative mutations, E157K and G250E, in the CCD of STAT1 that we found in 2 unrelated patients with Mendelian susceptibility to mycobacterial diseases.

Conclusion: The systematic alanine-scanning assay is a useful tool to estimate the GOF or LOF status and the effect of heterozygous missense mutations in *STAT1* identified in patients with severe infectious diseases, including mycobacterial and fungal diseases. (J Allergy Clin Immunol 2017;140:232-41.)

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Signal transducer and activator of transcription (STAT) 1 is a latent cytoplasmic transcription factor belonging to the STAT family. STAT1 is phosphorylated on tyrosine 701 (Y701) by the Janus kinases when a cytokine or growth factor binds to its receptor, allowing STAT1 dimerization. STAT1 can form a homodimer, which is known as IFN-γ activation factor (GAF), after stimulation by IFN-γ, IFN-α/β, or IL-27. GAF translocates to the nucleus and binds to specific DNA sequences known as gamma-activating sequences (GASs) in the promoters of interferon-stimulated genes to induce their transcription.[1] STAT1 also forms a heterotrimer after stimulation by IFN-α/β or IFN-λ, consisting of STAT1, STAT2, and interferon regulatory factor (IRF) 9, which is known as interferon-stimulated gene factor 3 (ISGF3). ISGF3 binds the interferon-stimulated response element, initiating gene transcription.[1] The deactivation of STAT1 is mediated by a nuclear phosphatase thought to be TC45, which dephosphorylates STAT1, allowing its release into the cytoplasm.[2,3] In human subjects STAT1 plays a nonredundant role in IFN-α/β, IFN-γ, IL-27, and IFN-λ signaling.[4]

Inborn errors in human STAT1-based immunity cause 4 types of immune deficiency: (1) autosomal recessive (AR) complete STAT1 deficiency, (2) AR partial STAT1 deficiency, (3) autosomal dominant (AD) STAT1 deficiency, and (4) AD STAT1 gain of activity.[4] Biallelic loss-of-function (LOF) mutations have been identified in patients with complete and partial AR STAT1 deficiency. These patients experience life-threatening viral infections (especially herpes virus infections) because they lack the STAT1-dependent response to IFN-α/β (and perhaps IFN-λ) signaling and experience mycobacterial susceptibility because they lack the response to IFN-γ (and perhaps IL-27).[5] Partial AR STAT1 deficiency is a milder form, and therefore patients with this disorder have mild viral and mycobacterial diseases and impaired but not abolished responses to IFN-α/β, IFN-γ, IL-27, and IFN-λ signaling.[6-8] Heterozygous LOF *STAT1* mutations cause Mendelian susceptibility to mycobacterial diseases (MSMD), which is attributable to the impairment of IFN-γ signaling, and mutations have been identified in the DNA-binding domain (DBD), SH2 domain, and transactivation domain (Fig 1, *A*).[9-14] Heterozygous gain-of-function (GOF) mutations underlie chronic mucocutaneous candidiasis (CMC) and have been most commonly identified in the coiled-coil domain (CCD) and the DBD (Fig 1, *A*).[15-49]

LOF and GOF mutations can affect the same domains in STAT1, obscuring the behavior of a particular mutation based solely on its location. Therefore we systematically investigated the effects of alanine substitutions in the CCD and DBD (CCD/DBD) of STAT1, screening 342 alanine mutants (in a total of 750 residues) with a GAS reporter assay after IFN-γ stimulation. All the LOF MSMD-causing mutations and most (78.1%) GOF CMC-related mutations previously identified in the CCD/DBD were correctly identified in the alanine mutants, suggesting that this technique can be used to establish a reference library of STAT1 variants. Our results clearly demonstrate that the majority of GOF mutations are located at the interface of the antiparallel STAT1 dimer, probably disrupting the dimerization of antiparallel STAT1 structures. We confirmed our results by identifying 2 germline heterozygous hypomorphic and dominant negative mutations in the CCD of STAT1 in patients with MSMD.

Abbreviations used

AD:	Autosomal dominant
AR:	Autosomal recessive
CADD:	Combined annotation-dependent depletion
CMC:	Chronic mucocutaneous candidiasis
DBD:	DNA-binding domain
dbSNP:	Single Nucleotide Polymorphism Database
ExAc:	Exome Aggregation Consortium
GAF:	IFN-γ activation factor
GAS:	Gamma-activating sequence
GOF:	Gain of function
IRF:	Interferon regulatory factor
ISGF3:	Interferon-stimulated gene factor 3
LOF:	Loss of function
MSC:	Mutation significance cutoff
MSMD:	Mendelian susceptibility to mycobacterial diseases
STAT:	Signal transducer and activator of transcription
WT:	Wild-type

METHODS

Functional assay based on systematic alanine-scanning mutagenesis

A vector from which to express HaloTag STAT1 was obtained from the Kazusa cDNA/ORF clone collection (FHC013013). The codons of the vector-encoding residues from L136 to F487 of STAT1, except the 10 alanines (A188, A230, A246, A254, A267, A401, A402, A415, A469, and A479), were individually substituted with GCC, the codon most frequently encoding alanine in human subjects, by using site-directed mutagenesis. The activities of the mutants were measured with a luciferase reporter assay with the pGL4.24 vector (Promega, Madison, Wis) driven by 5 tandem IRF-derived GAS elements (TTCCCCGAA; IRF1 reporter plasmids). Detailed methods of the luciferase reporter assay and the other experiments are available in the Methods section in this article's Online Repository at www.jacionline.org.

Case

Detailed case reports of kindreds A, B, and C (Fig 1) are available in the Methods section in this article's Online Repository. Briefly, 8 patients from 3 unrelated families are included in this study. Six patients received BCG at infancy, and 5 of the 6 had BCGitis. Multifocal osteomyelitis was observed in 5 patients, including 4 with BCGitis. Two patients who did not receive BCG had a history of tuberculosis. One patient who does not have an obvious history of BCGitis had intracranial granuloma with *Mycobacterium avium* complex detectable by means of PCR from brain biopsy specimens. The penetrance of STAT1 mutation was not complete. One family member in kindred A who has no history of BCG vaccination turned out to have STAT1 mutation based on the results of a familial study.

RESULTS

Functional assay based on systematic alanine-scanning mutagenesis

Alanine-scanning mutagenesis is a widely used technique in the determination of the catalytic or functional role of protein residues. We systematically investigated the effects of alanine substitutions in the CCD/DBD of STAT1 with a GAS reporter assay after IFN-γ stimulation. We generated 176 alanine mutants