

THE ASSOCIATION OF ATOPY WITH A GAIN-OF-FUNCTION MUTATION IN THE α SUBUNIT OF THE INTERLEUKIN-4 RECEPTOR

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ABSTRACT

Background Atopic diseases are very common, and atopy has a strong genetic predisposition.

Methods Using single-strand conformation polymorphism analysis and DNA sequencing, we searched for mutations in the α subunit of the interleukin-4 receptor that would predispose persons to atopy. We examined the prevalence of the alleles among patients with allergic inflammatory disorders and among 50 prospectively recruited adults. Subjects with atopy were identified on the basis of an elevated serum IgE level (≥ 95 IU per milliliter) or a positive radioimmunosorbent test in response to standard inhalant allergens. The signaling function of mutant interleukin-4 receptor α was examined by flow cytometry, binding assays, and immunoblotting.

Results A novel interleukin-4 receptor α allele was identified in which guanine was substituted for adenine at nucleotide 1902, causing a change from glutamine to arginine at position 576 (R576) in the cytoplasmic domain of the interleukin-4 receptor α protein. The R576 allele was common among patients with allergic inflammatory disorders (found in 3 of 3 patients with the hyper-IgE syndrome and 4 of 7 patients with severe atopic dermatitis) and among the 50 prospectively recruited adults (found in 13 of 20 subjects with atopy and 5 of 30 without atopy; $P=0.001$; relative risk of atopy among those with a mutant allele, 9.3). The R576 allele was associated with higher levels of expression of CD23 by interleukin-4 than the wild-type allele. This enhanced signaling was associated with a change in the binding specificity of the adjacent tyrosine residue at position 575 to signal-transducing molecules.

Conclusions The R576 allele of interleukin-4 receptor α is strongly associated with atopy. This mutation may predispose persons to allergic diseases by altering the signaling function of the receptor. (N Engl J Med 1997;337:1720-5.)

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ATOPY is characterized by the formation of IgE antibody in persons with a genetic predisposition, who respond with immediate hypersensitivity on exposure to specific allergens. Atopy is common, affecting up to 40 percent of populations of Western societies,¹⁻³ and it underlies the development of allergic diseases in susceptible persons. Although environmental factors such as exposure to antigens have an important role in the development of allergic diseases,⁴⁻⁶ there is a

strong genetic predisposition.^{7,8} Recently, a number of atopy susceptibility genes have been identified. Some of these, such as those for interleukin-4⁹⁻¹¹ and several HLA class II molecules,^{8,12,13} regulate the production of IgE, whereas others, such as *Fc ϵ RI β* ,¹⁴ mediate IgE effector functions.

The production of IgE is initiated by the interaction of antigen-presenting B cells with antigen-specific type 2 T helper cells.¹⁵ Cytokines secreted by type 2 T helper cells, including interleukin-4 and interleukin-13, act at their respective receptors to activate germ-line transcription from the epsilon heavy-chain gene locus and, together with signals delivered by the B-cell-surface molecule CD40, induce isotype switching from mu to epsilon.¹⁶ The interleukin-4 receptor is composed of two subunits: a 140-kd α subunit, which binds interleukin-4 and transduces its growth-promoting and transcription-activating functions,^{17,18} and a γ c subunit, common to several cytokine receptors, which amplifies signaling of interleukin-4 receptor α .¹⁹⁻²¹ There is evidence to suggest that interleukin-4 receptor α is also a component of the interleukin-13 receptor.²²

The central part played by interleukin-4 receptor α in regulating the production of IgE prompted us to investigate whether it is targeted by gain-of-function mutations or deletions that would enhance receptor signaling and hence precipitate atopy.

METHODS

Study Subjects

The hyper-IgE syndrome and severe atopic dermatitis were diagnosed in three and seven patients, respectively, according to standard criteria.²³⁻²⁵ Patients with severe atopic dermatitis had chronic inflammation affecting 20 percent or more of their skin area and recurrent superficial infections. Fifty healthy, unrelated adults were prospectively recruited among employees of medical centers affiliated with Washington University School of Medicine. The single exclusion criterion was a history of immunotherapy, which may influence the results of tests used to identify subjects with atopy. Subjects were designated as having atopy on the basis of in vitro correlates of immediate hypersensitivity: an elevated serum IgE level (≥ 95 IU per milliliter) or a positive radioimmunosorbent test (score, $\geq 3+$) in response to one or more of the

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following inhalant allergens: the common mold *Alternaria alternata*, the house-dust mite *Dermatophagoides pteronyssinus*, and cat dander.²⁶ The IgE assays and radioimmunosorbent test were performed with kits purchased from Pharmacia Diagnostics (Columbus, Ohio). Informed consent was obtained from all adult participants in these studies and, in the case of children, from parents or legal guardians.

Cell Lines

Isolation of peripheral-blood mononuclear cells and derivation of B-cell lines transformed by Epstein-Barr virus (EBV) were carried out according to standard methods. The EBV-negative Burkitt's lymphoma cell line BJAB was a kind gift of Dr. Elliott Kieff (Harvard Medical School, Boston). Cells were cultured in RPMI medium supplemented with 10 percent fetal bovine serum and maintained at 37°C in an atmosphere of 5 percent carbon dioxide.

Single-Strand Conformation Polymorphism Analysis and Sequencing

Complementary DNA (cDNA) was derived from EBV-transformed cell lines or peripheral-blood mononuclear cells and analyzed by single-strand conformation polymorphism (SSCP) as previously described²⁷ with synthetic primers (Integrated DNA Technologies, Coralville, Iowa). A nested polymerase chain reaction (PCR) was used to amplify nucleotides 1840 to 2125 of interleukin-4 receptor α cDNA, with 5'GCCACACTGGAAGA-ATTGTCTTAC/3 (sense) and 5'TTTTGGGGTCTGGCTTG-AG3' (antisense) as the outer primer pair and 5'CCGAAATGTC-CTCCAGCATG3' (sense) and 5'CCAGTCCAAAGGTGAACAA-GGGG (antisense) as the inner primer pair. Direct PCR sequencing was done with the fmol sequencing system from Promega (Madison, Wis.).

Peptide Conjugation, Binding Assays, and Immunoblotting

The following synthetic peptides corresponding to the sequences flanking amino acids Y575 and Y603 of interleukin-4 receptor α were used: wild-type unphosphorylated Y575 (NH₂-SAPTSGYQEFVHAVE-COOH), wild-type phosphorylated Y575 (NH₂-SAPTSG(pY)QEFVHAVE-COOH), mutant phosphorylated Y575 (NH₂-SAPTSG(pY)REFVHAVE-COOH), and phosphorylated Y603 (high-affinity Stat-6 site; NH₂-SSGEEG(pY)-KPFQDLIP-COOH) (all from Quality Controlled Biochemicals, Hopkinton, Mass.). The peptides were coupled to Affigel 10 beads (BioRad Laboratories, Hercules, Calif.) at a ratio of 3 mg of peptide per milliliter of beads.

To assess the binding of cellular proteins to synthetic interleukin-4 receptor α peptides, 20 μ l of peptide-conjugated beads was incubated with BJAB-cell lysates (2×10^7 cells) and then analyzed for the presence of peptide-associated Stat-6 or SHP-1 by immunoblotting with specific antiserum.²⁸ Rabbit antihuman Stat-6 antiserum was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.); the rabbit anti-SHP-1 antiserum has been described previously.²⁹ To assess the binding of recombinant SHP-1 protein fragment (positions 1 to 219)²⁹ to interleukin-4 receptor α peptides, 100 ng of this fragment was incubated with the indicated peptide-conjugated beads, and bound protein was detected by immunoblotting.

Flow-Cytometric Analysis of the Expression of CD23

Peripheral-blood mononuclear cells were cultured for 48 hours in the presence of 33 ng of recombinant human interleukin-4 per milliliter (R&D Systems, Minneapolis) and in its absence, and the cells were then stained with fluorescein isothiocyanate-conjugated murine antihuman CD23 monoclonal antibody (Coulter Immunology, Hialeah, Fla.). Monocytic cells present in the respective samples of peripheral-blood mononuclear cells were identified by flow cytometry on the basis of forward-scatter and side-scatter characteristics and analyzed for the expression of CD23.

Statistical Analysis

The data were analyzed by the two-tailed version of Fisher's exact test with Primer of Biostatistics software for Apple Macintosh.³⁰

RESULTS

Identification of an Interleukin-4 Receptor α Allele That Segregates with Atopy

There are phenotypic similarities between patients with the hyper-IgE syndrome and interleukin-4 transgenic mice, including very high IgE levels, allergic inflammation, and osteoporosis.³¹⁻³³ However, the patients have normal or decreased production of interleukin-4 in vitro,^{34,35} suggesting the presence of mutations that affect the function of interleukin-4 receptor α .

We screened the full-length interleukin-4 receptor α cDNA of three patients with the hyper-IgE syndrome for mutations by SSCP analysis of sequential cDNA segments spanning about 300 bp each. All three patients had an identical alteration (as compared with controls) in the SSCP profile of one cDNA segment, spanning nucleotides 1840 to 2125 (Fig. 1A). This SSCP profile is consistent with a heterozygous configuration and predicts the presence of a point mutation or a small deletion in the affected allele. The affected cDNA segment was amplified by PCR and subjected to direct PCR sequencing, a method that allows the sequences of both alleles to be visualized simultaneously. The results of sequence analysis of cDNA of one patient with the hyper-IgE syndrome and one control subject are shown in Figure 1B: the patient has a missense mutation in which guanine is substituted for adenine at nucleotide 1902 (according to the interleukin-4 receptor α nucleotide sequence of Idzerda et al.¹⁷), causing a change from glutamine to arginine residue at position 576 (R576) of the protein (including the signal peptide).^{17,18} The sequence from the patient had both wild-type and mutant nucleotides, confirming the presence of heterozygosity. The same substitution was also found in the two other patients with hyper-IgE syndrome (data not shown). Analysis of the parents of these patients revealed that the R576 allele was inherited in a classic mendelian fashion (data not shown).

The phenotype of the hyper-IgE syndrome overlaps that of severe atopic dermatitis, in that both are characterized by allergic inflammatory skin disease and high IgE levels. We therefore examined seven patients with severe atopic dermatitis for the presence of the mutant interleukin-4 receptor α allele. Three were found to be heterozygous and one was homozygous for the R576 allele, indicating that this allele is not restricted to patients with the hyper-IgE syndrome but may also involve patients with different allergic inflammatory conditions (Table 1).

To establish the relation of the R576 allele with

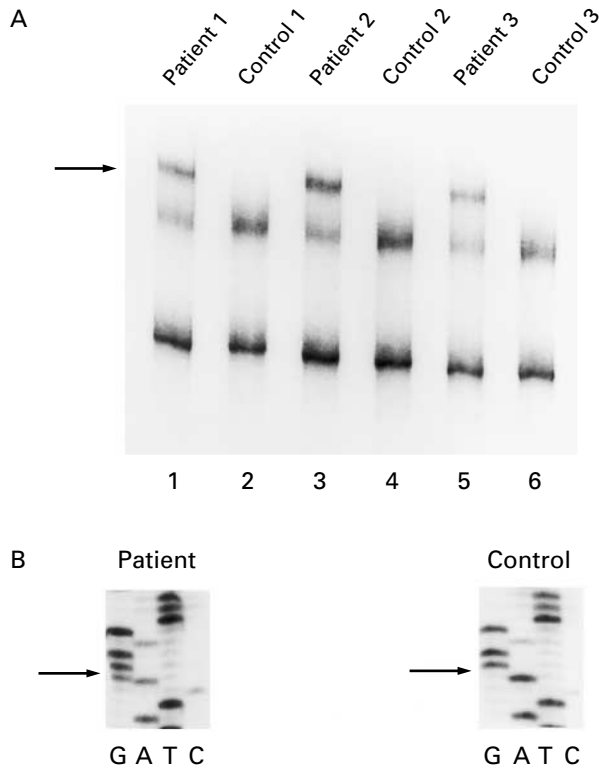


Figure 1. Identification of a Mutant Interleukin-4 Receptor α Allele.

Panel A shows the results of SSCP analysis of a fragment of interleukin-4 receptor α cDNA spanning nucleotides 1840 to 2125 in three patients with hyper-IgE syndrome (lanes 1, 3, and 5) and three controls (lanes 2, 4, and 6). The mutant allele manifests as an extra band in the samples from the patients (arrow). Panel B shows a missense mutation identified at position 1902 of the interleukin-4 receptor α cDNA sequence. Direct PCR sequencing of cDNA of one patient with the hyper-IgE syndrome reveals both adenine and guanine at nucleotide 1902 (arrow), a finding consistent with the presence of one wild-type and one mutant allele. Only adenine is present at the equivalent position in a cDNA sequence from a control subject (arrow).

TABLE 1. FREQUENCY OF INTERLEUKIN-4 RECEPTOR α ALLELES IN SUBJECTS WITH ATOPY AND THOSE WITHOUT ATOPY.*

GROUP	No. OF SUBJECTS	Allele Frequency		
		QQ	RQ	RR
		number		
Patients with hyper-IgE syndrome	3	0	3	0
Patients with severe atopic dermatitis	7	3	3	1
Adults with atopy†	20	7	12	1
Adults without atopy†	30	25	4	1

*QQ denotes homozygosity for the wild-type allele, RQ heterozygosity, and RR homozygosity for the mutant allele.

†The presence of the mutant allele was associated with a relative risk of atopy of 9.3 ($P=0.001$ by Fisher's exact test, two-tailed).

atopy, we studied their cosegregation in a prospectively chosen sample of 50 unrelated adults (100 chromosomes). Subjects with atopy were identified on the basis of the presence of an elevated serum IgE level or evidence of a specific IgE antibody response to one or more of the following allergens: *A. alternata*, *D. pteronyssinus*, and cat dander. Twenty subjects were identified as atopic, representing a percentage of the study group — 40 percent — that is in agreement with published reports of the prevalence of atopy in large populations.¹⁻³ Among the 20 subjects with atopy, 13 had the R576 allele, as compared with only 5 of the 30 subjects without atopy ($P=0.001$) (Table 1). The presence of the R576 allele carried a calculated relative risk of atopy of 9.3. The overall allelic frequency of R576 in the population of 100 chromosomes was 20 percent (35 percent in the group with atopy and 10 percent in the group without atopy, $P=0.004$). Overall, these results suggested a strong association of the R576 allele with atopy.

Association of the R576 Allele with Enhanced Responsiveness to Interleukin-4

We investigated the molecular basis of the association of the Q576R substitution with atopy by examining the signaling functions of the wild-type and mutant interleukin-4 receptor α alleles. Binding of interleukin-4 to its receptor induces the expression of the low-affinity IgE receptor (CD23) on B cells, monocytes, and macrophages.³⁶ We therefore used flow cytometry to assess the capacities of wild-type and mutant alleles to mediate the induction by interleukin-4 of the expression of CD23 in peripheral-blood mononuclear cells. Interleukin-4 induced higher levels of CD23 expression (by a mean [\pm SE] factor of 2.17 ± 0.14) in peripheral-blood mononuclear cells from R576 heterozygotes than from wild-type homozygotes (six subjects) (Fig. 2). The level of expression of CD23 was also higher in peripheral-blood mononuclear cells from R576 homozygotes (by a mean factor of 3.07 ± 0.62) than from wild-type homozygotes (two subjects). Enhanced expression of CD23 was noted in R576-positive cells from both subjects with atopy and those without atopy, and it was not due to the presence of higher levels of the mutant allele, as determined by flow cytometry (data not shown). These results suggest that the signaling function of the R576 allele is greater than that of the wild-type allele.

Association of SHP-1 with Interleukin-4 Receptor α Alleles

Position 575 is occupied by a tyrosine residue (Y575) that on phosphorylation recruits phosphotyrosine-binding effector molecules.³⁷ This suggested that the substitution of R for Q at position 576 may influence the interaction of phosphorylated Y575 with signaling intermediates.

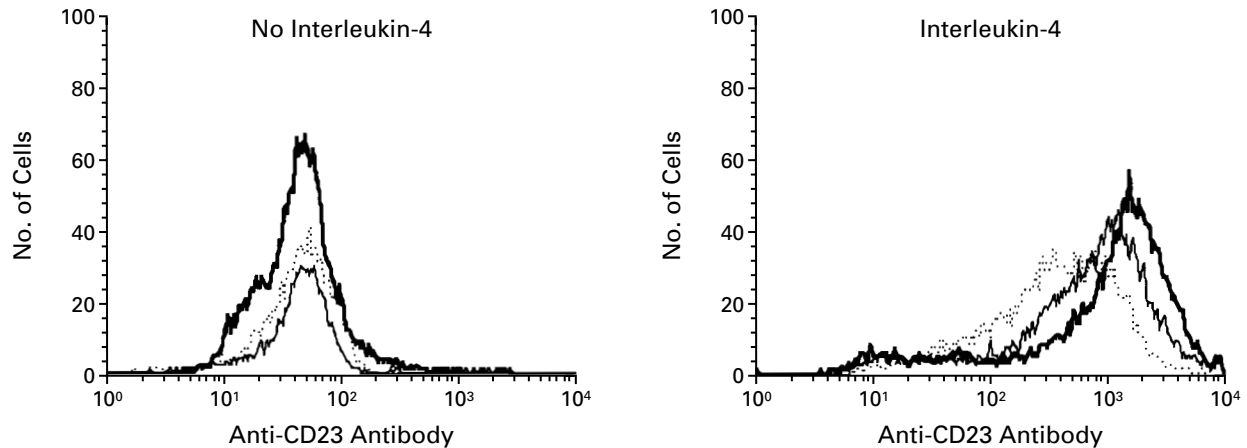


Figure 2. Effect of the R576 Allele on the Expression of CD23.

Peripheral-blood mononuclear cells homozygous for the wild-type Q576 allele (dotted line), heterozygous for R576 (thin line), or homozygous for R576 (thick line) were incubated in the absence and in the presence of interleukin-4 and then examined for the expression of CD23 with flow cytometry.

We focused on the effect of the Q576R mutation on the binding to phosphorylated Y575 of two molecules implicated in interleukin-4 receptor signaling: Stat-6,³⁸ a transcription factor that mediates gene induction by interleukin-4, and SHP-1,³⁹ a phosphotyrosine phosphatase involved in signal termination by means of cytokine receptors, including interleukin-4 receptor.⁴⁰⁻⁴⁵ Binding assays were performed in which synthetic peptides spanning phosphorylated Y575 and harboring either the wild-type or mutant 576 residue were incubated with lysates of BJAB, a Burkitt's lymphoma B-cell line, which contain both Stat-6 and SHP-1. A wild-type peptide with an unphosphorylated Y575 residue was used as a negative control, and a phosphotyrosyl peptide spanning the interleukin-4 receptor α high-affinity Stat-6-binding site at position Y603 was used as a positive control.³⁸ Binding of Stat-6 was similar with the wild-type and mutant phosphorylated Y575 peptides (Fig. 3A, lanes 2 and 3, respectively). The phosphorylated Y603 peptide (lane 4 in Fig. 3A) precipitated levels of Stat-6 that were twice as high as those precipitated by the wild-type and mutant phosphorylated Y575 peptides, in agreement with the fact that Stat-6 has a higher affinity for phosphorylated Y603 than phosphorylated Y575.³⁸ In contrast, virtually no Stat-6 was precipitated by the unphosphorylated wild-type Y575 peptide (lane 1 in Fig. 3A), which is consistent with the requirement for phosphotyrosine residues for Stat-6 binding.

In contrast to Stat-6, SHP-1 bound much better to the wild-type phosphorylated Y575 peptide than to the mutant peptide (Fig. 3A, lanes 2 and 3, respectively). Densitometric analysis revealed that wild-type phosphorylated Y575 peptide bound approxi-

mately twice as much SHP-1 as the mutant phosphorylated peptide (Fig. 3). Wild-type unphosphorylated Y575 and phosphorylated Y603 peptides were ineffective binders (Fig. 3A, lanes 1 and 4, respectively), which is consistent with the specificity of SHP-1 for phosphorylated Y575.

To determine whether SHP-1 associates with phosphorylated Y575 directly, we used a purified recombinant protein fragment of SHP-1, which corresponds to amino acids 1 to 219 of the native protein and contains the two SH2 domains of SHP-1. As shown in Figure 4, the recombinant protein fragment selectively bound to the wild-type phosphorylated Y575 peptide (lane 3), a finding consistent with the occurrence of direct binding, but it bound only minimally to the mutant phosphorylated Y575 (lane 4), wild-type unphosphorylated Y575 (lane 2), and phosphorylated Y603 peptides (lane 5).

DISCUSSION

We found that an allele for the α subunit of the interleukin-4 receptor segregates with atopy. The interleukin-4 receptor α allele that we identified contains an arginine in lieu of a glutamine at position 576. This allele was common in patients with the hyper-IgE syndrome and severe atopic dermatitis as well as in prospectively recruited adults with atopy. It is inherited in a classic mendelian fashion and appears to be widespread, suggesting that it may contribute to the pathogenesis of a variety of allergic diseases.

A majority of subjects identified as carrying a single copy of the mutant allele were found to have atopy, suggesting a dominant effect. However, the finding that some carriers of the R576 allele, including

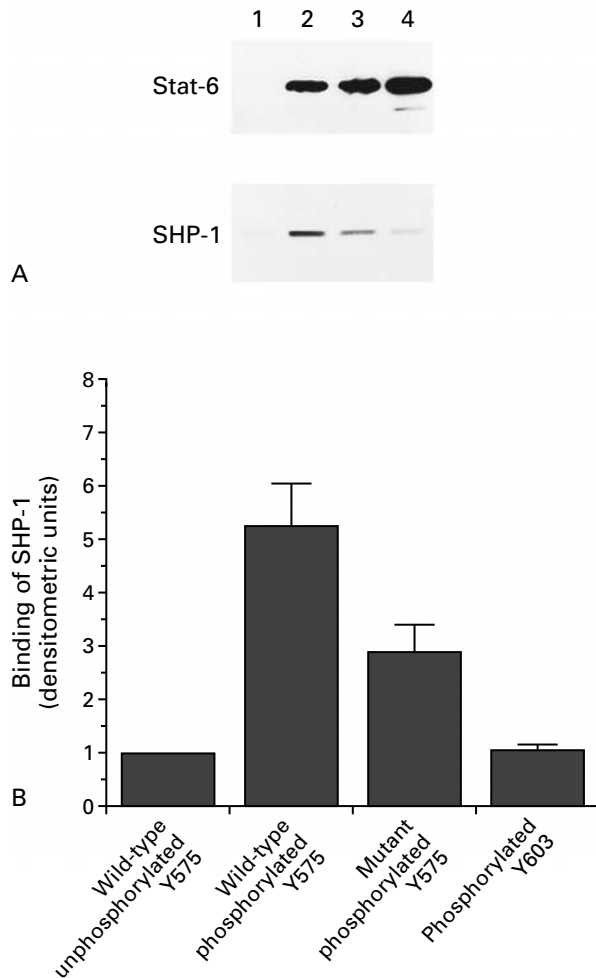


Figure 3. Association of SHP-1 with Allelic Y575 Phosphopeptides. In Panel A, lysates of BJAB cells were incubated with the following agarose-coupled synthetic peptides: wild-type unphosphorylated Y575 (lane 1), wild-type phosphorylated Y575 (lane 2), mutant phosphorylated Y575 (lane 3), and phosphorylated Y603 (lane 4). Bound proteins were detected by immunoblotting with antibodies to Stat-6 or SHP-1. Panel B shows the results of densitometric analysis of SHP-1 binding in four independent experiments. Values are means +SE relative to wild-type unphosphorylated Y575, which was assigned an arbitrary value of 1.

one who was homozygous, were not atopic indicates that the penetrance of this allele may be modified by other factors. These may include distinct genetic loci that impart susceptibility to or protection from atopy and environmental factors such as the level and duration of exposure to allergens.⁴⁻⁶

The Q576R mutation is associated with enhanced signaling of interleukin-4 receptor, an effect that may underlie the predisposition of this allele to atopy. The substitution of arginine for glutamine at position 576 alters the binding profile of the adjacent

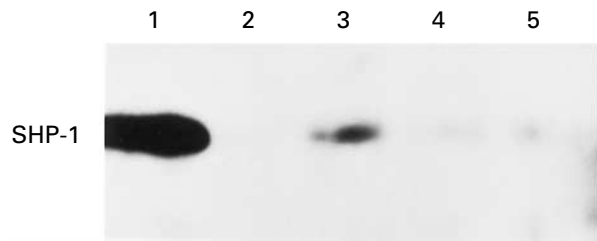


Figure 4. Binding of a Recombinant SHP-1 Fragment to Allelic Y575 Phosphopeptides.

In this experiment, 100 ng of recombinant SHP-1 protein fragment was incubated with the following agarose-coupled synthetic peptides: wild-type unphosphorylated Y575 (lane 2), wild-type phosphorylated Y575 (lane 3), mutant phosphorylated Y575 (lane 4), and phosphorylated Y603 (lane 5). The bound protein was visualized by immunoblotting with antibodies to SHP-1. In lane 1, 100 ng of recombinant SHP-1 was used as a positive control.

phosphorylated tyrosine residue, as is evidenced by the decreased binding of the phosphotyrosine phosphatase SHP-1 in the presence of the mutation. SHP-1 dephosphorylates regulatory phosphotyrosine residues and has been implicated in termination of signaling by means of cytokine receptors.^{40-43,46} In the case of the interleukin-4 receptor signaling pathway, candidate intermediate molecules that may be regulated by SHP-1 include Stat-6, Janus protein kinases (Jak), and phosphatidylinositol 3-kinase.⁴⁴

Decreased binding of SHP-1 to phosphorylated Y575 may be causally related to the altered signaling function of the mutant allele. A precedent for a disease resulting from the failure of receptor regulation by SHP-1 is familial erythrocytosis. Patients with this disorder have deletions in the carboxy-terminal segment of the cytoplasmic domain of the erythropoietin receptor.^{47,48} The deleted sequence contains binding sites for SHP-1, and the resultant lack of SHP-1 regulation results in exaggerated receptor signaling.⁴¹

It should be emphasized that although decreased binding of SHP-1 to phosphorylated Y575 may provide an explanation for the association of the R576 allele with atopy, other mechanisms may be involved. These include alteration in the binding to phosphorylated Y575 of other, as yet unidentified, signaling intermediates, leading to exaggerated signaling. We also cannot rule out the presence in the interleukin-4 receptor gene of additional mutations, not detected by our screening, that predispose persons to atopy either independently of or in synergy with the Q576R mutation. Lastly, the R576 allele may segregate with a mutation in another molecule, such as a signaling intermediate, which fosters atopy. Discrimination between these alternative mechanisms will require further investigation.

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