Molecular Analysis of Human Interleukin-9 Receptor Transcripts in Peripheral Blood Mononuclear Cells

IDENTIFICATION OF A SPLICE VARIANT ENCODING FOR A NONFUNCTIONAL CELL SURFACE RECEPTOR*

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Asthma is a complex inflammatory disorder that is characterized by periodic airway obstruction, wheezing, and bronchial hyperresponsiveness (BHR)1 (1). Clinical studies of asthma have revealed that the pathology of this disease is associated with widespread narrowing of the airways due to edema and infiltration of multiple inflammatory cells into the lung epithelia (2). These cell types include mast cells, eosinophils, B-cells, and TH2-lymphocytes as the predominant cell mediators of this disorder (3, 4). While these inflammatory cells appear to be important, their precise role in producing BHR is unclear and still under investigation. Nevertheless, the response to antigen and subsequent release of chemical inflammatory mediators (histamines, leukotrienes, prostaglandins, etc.) by a number of these cells has been documented (5).

Genetic studies have suggested that BHR is a multifaceted process (6–11). Recently, we have identified interleukin-9 (IL9) as a factor in regulating airway hyperresponsiveness in inbred strains of mice (12). C57BL/6 mice, which show low airway responsiveness, had undetectable levels of IL9 in activated splenocytes and in the lung, while DBA/2 mice, which have airway hyperresponsiveness, expressed robust levels of IL9 in both tissues. A role for IL9 in asthma and allergy has been supported by the findings that it has pleiotropic activities on cell types associated with these diseases such as TH2 lymphocytes, B-cells, mast cells, and eosinophils (13–19). In particular, IL9 has been shown to act as a growth and differentiation factor for mast cells and to enhance the release of IgE from B-cells. These two activities as well as the effect of IL9 on controlling BHR in mice suggest that it and its associated pathway(s) are involved in the pathogenesis of allergy and asthma. Most recently, Holroyd et al.2 have found genetic linkage of asthma and BHR to the IL9 receptor (hIL9R) locus in humans, supporting the notion that this pathway is involved in the disease process in humans.

Because of the genetic linkage of the IL9 receptor locus to allergy and asthma susceptibility, we explored the possibility that variations in hIL9R structure/function or gene expression may exist. Here we report the identification of an abundantly expressed receptor splice variant (referred to as ΔQ receptor) that lacks codon 173. This variant is unable to bind IL9 and transduce its signal to effector cells. These data suggest a mechanism for modulating IL9 signaling in effector cells.

EXPERIMENTAL PROCEDURES

Purification of Human PBMCs and Mitogen Stimulation—Peripheral blood was obtained by consent from 50 unrelated donors. Venipuncture was performed, and blood was collected into vacutainer tubes containing EDTA. An equal volume of PBS (Mg2+/Ca2+-free) was added to whole blood. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll-Paque gradients (Pharmacia Biotech 17-1440-02) (21). Purified cells were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 5 µg/ml phorbol 12-myristate 13-acetate, and 1 µg/ml phytohemagglutinin (Sigma) and incubated at 37 °C in 5% CO2 for 6 days.

Nucleic Acid Isolation and Reverse Transcriptase-PCR—Cytoplasmic

1 The abbreviations used are: BHR, bronchial hyperresponsiveness; IL, interleukin; IL9R, interleukin-9 receptor; hILR, human IL9R; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; FACS, fluorescence-activated cell sorting; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

2 Holroyd, K. J., Martinati, L. C., Trabetti, E., Scherprier, T., Eleff, S. M., Bomer, A. L., Pignatti, P. F., Kiser, M. B., Dragwa, C. R., Hubbard, F., Sullivan, C. D., Grasso, L., Messler, C. J., Huang, M., Hu, Y., Nicolaides, N. C., Buettow, K. H., and Levitt, R. C. (1998) Genomics, in press.
RNA and genomic DNAs were isolated from phorbol 12-myristate 13-acetate/phytohemagglutinin-treated PBMCs as described previously (22). RNAs were reverse transcribed and amplified by PCR as described (23). Primers used to amplify the entire coding region of the hIL9R (GenBank™ accession no. M94747) were as follows: 5′-GCT CGA CCT TGG AGA GTG-3′ (R8; forward), centered on codon 1822 of the cDNA (15, 24).

**Plasmids, Subcloning, and Sequencing Procedures**—For the structural analysis of hIL9R, reverse transcriptase-PCR products containing the entire coding region of the hIL9R from 50 donors were cloned into pCR2.1 T-tailed vectors (Invitrogen). At least 10 recombinant clones from each individual were completely sequenced. Recombinant expression vectors were engineered by subcloning the relevant cDNAs from the original cloning vector as EcoRI fragments into the EcoRI restriction site of the LXXN retroviral vector (25) that contains a neomycin resistance gene as a selectable marker. The GHS (the only combination of codons 344 and 410–418 that has not been reported by others nor observed in our IL9R structure analysis) and G9B (see Fig. 2A) expression plasmids were engineered by first subcloning the GHS and G9B forms of the hIL9R cDNA into the EcoRI site of pZevoSV2 (Invitrogen) to produce pZevoGR8 and pZevoGH9, respectively. The GHS and G9B cDNAs were then engineered by exchanging XmaI fragments of these plasmids (5′ site is located in the pZevoSV2 polylinker, and the 3′ site is located in the pCR2.1 vector) by using Ascl1 and Mlu1 (between nucleotides 2286 and 4114 of the hIL9R cDNA) to generate pZevoGHS and pZevoGR9. Finally, GHS and G9B cDNAs were subcloned as EcoRI fragments from pZevoSV2 into the EcoRI site of LXXN vector. pZevoR9 was obtained by replacing the BstEII/NotI fragment of pZevoGH9 containing base pairs 791–1822 of the hIL9R cDNA with the BstEII/NotI fragment from pBHR6 (26). Successively, the EcoRI/XhoI fragment from pZevoR9 containing the R9 version of the hIL9R cDNA was subcloned into the EcoRI/XhoI site of LXXN. Constructs were sequenced using the ABI Prism DNA sequencing kit (Perkin-Elmer), and reactions were run in a model 377 DNA automated sequencer (ABI Prism, Perkin-Elmer).

**Cell Lines and Cell Culture**—TS1 is an IL9-dependent murine T cell line (15). It was maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.) containing 10% fetal bovine serum in the presence of 25 ng/ml of murine IL9 (R & D Systems, Minneapolis, MN). Mo7e is a cytokine-dependent human megakaryoblastic leukemia cell line that proliferates in response to IL3, granulocyte-macrophage colony-stimulating factor, IL9, or stem cell factor. These cells were maintained in RPMI medium containing 20% fetal bovine serum in the presence of 50 ng/ml human IL9 or 10 ng/ml IL3. COS7 cells were purchased from American Type Culture Collection and maintained in growth medium supplemented with 25 ng/ml murine IL9 (R & D Systems, Minneapolis, MN). Cells were maintained in growth medium supplemented with 25 ng/ml murine IL9 and 0.4 mg/ml G418. Expression of hIL9R was periodically assessed by Western blot.

**Cell Proliferation and Cytokine Stimulation**—Interleukin-9-induced cell proliferation was performed as follows. TS1 transfectants were washed three times with PBS and resuspended in DMEM, 10% fetal bovine serum. Cells were seeded at 105 cells/well in 96-well microplates and grown in the presence of 5 ng/ml recombinant human IL9 or murine IL9. Proliferation was assayed by the addition of 200-fold excess of unlabeled IL9 for 3 h at 4 °C. Cells were layered over 800 μl of a 1.5:1 mixture of dibutyl- and dioctylphalate oils and centrifuged at 900 × g for 7 min, 3500 × g for 7 min, and 3500 × g for 10 min, and 16,000 × g for 20 min at 4 °C. Total cell extracts were obtained by RIPA buffer extraction as described above.

## RESULTS

**Structural Analysis of hIL9R from Human PBMCs**—In order to analyze hIL9R nucleotide structure in randomly ascertained individuals, we established a cohort of 50 unrelated volunteers. These volunteers were asked to report their past medical history as being nonallergic, allergic, or asthmatic. Using this information, we established a cohort of 50 unrelated volunteers. The volunteers were asked to report their past medical history as being nonallergic, allergic, or asthmatic.
Phenotypes of human interleukin-9 receptor (hIL9R) were characterized. The receptor consists of an extracellular region encoded by exons 2–6, a transmembrane region by exon 7, and an intracellular domain by exons 8 and 9. The receptor here on. The hIL9R cDNA, which was originally cloned from the human megakaryoblastic leukemia cell line Mo7e, contained an Arg344 and SER9, as did the megakaryoblastic leukemia cell line UT-7 (24). To prove the consistency of our experimental protocol, we extracted RNA from Mo7e cells, reverse transcriptase-PCR-amplified the full-length receptor cDNA, and cloned it into T-tailed vectors. We analyzed 16 independent cDNAs and found that 6 had the Arg344-SER8 allele, while the remaining 10 contained the published sequence (Arg344-SER9). We also genotyped the human acute myelogenous leukemia cell line KG-1 and found that it was a His344-SER9 homozygote (an allele found in our cohort). At the present time, we do not know whether the Arg344-SER9 allele found in MO7e and UT-7 cells is a rare allele or the result of a mutational event that occurred during transformation in these tumor cells.

One mechanism that can affect gene function is monoallelic expression. One could hypothesize that if this mechanism is involved in altering receptor function, then heterozygous individuals may appear to be homozygous at the transcript level. Of the 23 (out of 50) individuals that appeared to be homozygous at the transcript level, all were homozygous at the genome level, as determined by the codon 310 or 344 polymorphisms located within exon 9 (see above). These data rule out the possibility of an effect on gene expression that is due to an altered regulatory element and also confirm the previous finding that the IL9R gene escapes X inactivation in PBMCs (27).

Further structure/function studies on the receptors containing the various missense changes at codons 310, 344, and 410–417/410–418 were continued in an attempt to gain further insight into the functional activity of these isoforms. In addition, the ΔQ splice variant was also studied because of its prevalence within our cohort in contrast to the other alternative splice isoforms (those lacking exons 3, 4, 5, and 8) that appeared less frequently.

Expression of Human IL9 Receptor Variants in a Murine IL9-dependent Cell Line—Cell lines were generated that expressed the various receptor isoforms in order to study receptor function (see Fig. 2A for details on constructs). Each of the receptor variants was subcloned into the LXSN mammalian expression vector that contains a murine moloney leukemia viral long terminal repeat for constitutive expression and a neomycin resistance gene as a selectable marker. Each construct was then electroporated into the murine TS1 cell line. This cell line was derived from primary murine T-lymphocytes and was found to be dependent on murine IL9 for growth and survival, but it is not responsive to human IL9 (30). Transfectants were then analyzed by Western blot for hIL9R expression. As shown in Fig. 2B, comparable hIL9R expression was found for all isoforms except for GR8, which showed 3-fold less

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**Table I**

| Codon | Nucleotide change | Amino acid change | Frequency |
|-------|-------------------|------------------|-----------|
| 173   | CAG to ACAG       | Gln to Glu        | 0–50      |
| 310   | AGG to GGG        | Arg to Gly        | 76        |
| 344   | CTG to CAT        | Arg to His        | 47        |
| 410–417/410–418 | (AGC)₉ to (AGC)₈ | 9 Ser to 8 Ser     | 47        |

* Determined from an average of 10 cloned transcripts from each of 50 individuals.

* Determined from greater than 100 independent alleles.

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N. Nicolaides, unpublished observation.
expression. Interestingly, we noticed that GR8 protein ran slightly faster than GH9 when a higher electrophoretic resolution could be obtained (Fig. 2C). The addition of human IL9 to the cells did not result in change of the migration pattern between the GR8 and GH9 receptors (lanes 4 and 6). To address the possibility that this migratory difference was due to cell-specific post-translational modifications, we transiently transfected the simian derived COS7 cells with the GR8, GR9, GH8, and GH9 expression plasmids. Cells were grown for 48 h and then harvested for protein and analyzed for IL9 receptor migration by Western blot. In these conditions, GR8 and GH8 ran faster than GH9 and GR9 (data not shown), indicating that the serine repeat, and not the Arg234/His344 polymorphism, was critical for this shift in electrophoretic mobility. Studies are in progress to understand the nature of this differential mobility.

Effect of Human IL9 on Proliferation and Survival of TS1 Cells Expressing Different Forms of hIL9R—TS1 survival and proliferation depend on the presence of murine IL9 and are not supported by the human cytokine. Ectopic expression of the human receptor renders these cells responsive to the human cytokine. Ectopic expression of the receptor isoforms, we investigated whether they differed in their ability to transduce an effective growth signal upon human IL9 stimulation. TS1 transfectants were treated with either murine or human IL9 and plated as described under "Experimental Procedures." As shown in Fig. 3, all transfectants grew in the presence of murine IL9. Additionally, TS1 cells that expressed GR8, GH9, RR9, and GR9 (hereon referred to as TSGR8, TSGH9, TSSRR9, and TSGR9, respectively) responded to human IL9 treatment, while TSGR8 showed a lower proliferative response. This was most likely due to the lower expression of the receptor as compared with the other cell lines (see Fig. 2B, lane 2). Indeed, another set of TSGR8 and TSGH9 transfectants were generated later that expressed equal levels of receptor and showed similar growth rates (data not shown). In contrast, TS1 cells expressing ΔQR8 and ΔQGH9 (hereafter referred to as TSAQR8 and TSAQGH9, respectively) as well as those transfected with vector alone (referred to as TSLXSN) failed to respond to the human ligand. These data indicate that the absence of the glutamate at codon 173 suppressed the ability of the receptor to transduce a growth signal.

Human IL9-activated Signal Transduction Cascade in TS1 Cells Expressing hIL9R Variants—Part of the IL9R signal transduction cascade has been well established (26, 31). The interaction of IL9 with its receptor results in phosphorylation of the receptor itself and the activation of Jak1, Jak3, Stat1, Stat3, Stat5, Irs1, and Irs2 signaling molecules. These previous studies all employed the RR9 form of the receptor (26). We examined whether the GR8 and GH9 receptors varied in their ability to activate these proteins. To rule out possible interference that the endogenous receptor may cause in this analysis, we treated TS1 cells with either murine or human IL9 and analyzed the tyrosine phosphorylation of murine IL9 receptor. As shown in Fig. 4A, the murine IL9 receptor could be immunoprecipitated by an anti-phosphotyrosine antibody from cells treated with murine IL9 but not from cells untreated or treated with human IL9, thus demonstrating the inability of human IL9 to react with the murine IL9 receptor. In a similar experiment, TSGR8 and TSGH9 cells were cytokine-starved for 6 h and then treated for 5 min with human IL9. Since both cell lines express the endogenous murine receptor, murine IL9 treatment was included as an internal control (31). After stimulation, total proteins were extracted; immunoprecipitated using various Jak-, Stat-, and Irs-specific antibodies; and then Western blotted onto nylon membranes. Blots were probed with an anti-phosphotyrosine antibody that specifically detects tyrosine-phosphorylated proteins. TS1 cells grown in the absence of cytokine had undetectable amounts of tyrosine-phosphorylated proteins (Fig. 4B, lanes 1 and 4). Treatment of both TSGR8 and TSGH9 cells with murine IL9 resulted in tyrosine phosphorylation of Jak, Stat, and Irs proteins described above. Representative analyses of these experiments are shown for Jak1, Stat1, and Irs1 in Fig. 4B (lanes 2 and 5). Similarly, human IL9 treatment resulted in an identical profile as in the murine IL9-treated cells, while no differences were observed between the GR8 and GH9 receptors (Fig. 4B, lanes 3 and 6, and data not shown). Analysis of GR8 and GH9 receptor tyrosine phosphorylation found both to be equally phosphorylated after human IL9 treatment (data not shown), thus suggesting that these residues have no detectable role in receptor phosphorylation or phosphorylation of downstream substrates. Next, we examined whether failure of TSAQR8 or TSAQGH9 cells to proliferate in response to human IL9 reflected an altered signal transduction function of these receptors. Again, we analyzed the tyrosine phosphorylation of members of the Irs, Jak, and Stat families in these cell lines upon treatment with either murine or human IL9. Immunoprecipitation-Western blot analysis revealed that neither ΔQR8 nor ΔQGH9 receptors were capable of ligand-induced activation of any Jak, Stat, or Irs family member (Fig. 4C, lanes 3 and 6) as compared with the GH9 receptor that was used as positive control (Fig. 4C, lane 9). Furthermore, human IL9 treatment did not lead to phosphorylation of the receptor itself (data not shown). The TSAQR8 and TSAQGH9 cells treated with murine IL9 showed activation of all proteins tested (Fig. 4C, lanes 2 and 5),
thus demonstrating the integrity of the IL9 signaling cascade in these cells.

Ligand-binding Analysis, Cellular Localization, and Immunoreactivity of the ΔQ Receptor—We have demonstrated that deletion of the glutamine at codon 173 results in loss of hIL9R signaling. This loss of function could be explained by an impaired binding of the ligand to the receptor. To test this hypothesis, we performed ligand-binding analysis on TSLXSN, TSGR8, TSGH9, TSAQGR8, and TSAQGH9 cells using iodinated human IL9 protein (125I-hIL9). As shown in Fig. 5A, TSGR8 and TSGH9 cells were capable of binding 125I-hIL9, whereas binding was undetectable in TSLXSN, TSAQGR8, or TSAQGH9 cells. The TSLXSN, TSGH9, and TSAQGH9 cells were next fractionated in order to assess the cellular localization of the ΔQ receptor. Protein fractions as well as total cell extracts were run in SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and probed with antibodies specific for actin (marker for cytoplasm (32)), proliferating cell nuclear antigen (marker for cytoplasm and nucleus (33)), and hIL9R. As shown in Fig. 5B, actin could be detected only in the cytoplasmic/nuclear fraction and in total extracts. Likewise, most of the proliferating cell nuclear antigen was predominantly localized to the plasma membrane whereas binding was undetectable in TSLXSN, TSAQGR8, or TSAQGH9 cells. The TSLXSN, TSGH9, and TSAQGH9 cells were next fractionated in order to assess the cellular localization of the ΔQ receptor. Protein fractions as well as total cell extracts were run in SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and probed with antibodies specific for actin (marker for cytoplasm (32)), proliferating cell nuclear antigen (marker for cytoplasm and nucleus (33)), and hIL9R. As shown in Fig. 5B, actin could be detected only in the cytoplasmic/nuclear fraction and in total extracts. Likewise, most of the proliferating cell nuclear antigen was present in the cytoplasmic/nuclear fraction, although a small portion could be found in the membrane fraction. In contrast, both GH9 and ΔQGH9 were exclusively detected in the membrane fraction at comparable levels. Longer exposures failed to detect either receptor in the cytoplasmic/nuclear fraction (data not shown), suggesting that both receptor forms are predominantly localized to the plasma membrane.

The finding that the ΔQ receptor is expressed on the cell membrane but is unable to bind the ligand suggested a defect in the binding domain of the ΔQ receptor. To test this hypothesis, COS7 cells were transfected with GR8, GH9, ΔQGR8, or ΔQGH9 (hereafter referred to as COSGR8, COSGH9, COSΔQGR8, and COSΔQGH9, respectively). 48 h after transfection, cells were immunostained using an N-terminal specific anti-hIL9R neutralizing antibody (MAB290) and analyzed by fluorescence microscopy. As shown in Fig. 6A, COSGR8 and COSGH9 (D and F, respectively) cells showed positive staining compared with cells transfected with LXSN vector (COSLXSNI, panel B). In contrast, no staining was apparent in COSΔQGR8 or COSΔQGH9 cells (H and J, respectively), suggesting that this neutralizing antibody failed to recognize the region of the receptor where the binding of the ligand occurs. Since this antibody has been reported to only react with native but not denatured receptor, these data are suggestive of a conformational change in the protein. To confirm that all receptor forms were being expressed, these same cells were fixed, permeabilized, and then stained with a C-terminal (intracellular domain) specific antibody that can detect both native and denatured proteins. In these experiments, COSGR8, COSGH9, COSΔQGR8, and COSΔQGH9 cells (Fig. 6b, panels D, F, H, and J, respectively) showed positive staining, whereas none was observed in COSLXSNI cells (Fig. 6b, panel B), thus demonstrating equivalent intracellular expression of all receptor types. These two antibodies could not be employed for the analysis of TS1 cells because of a high background in these cells. Therefore, we probed TS1 cells expressing the hIL9R variants with another N-terminal monoclonal antibody (AH9R7) that produced less background in immunostaining protocols (data not shown). This antibody was then used on the various TS1 cell lines, and staining was analyzed via FACS (Fig. 7A). Fluorescence remained at background levels in TSLXSN, TSAQGR8, and TSAQGH9 cells. Therefore we probed TS1 cells expressing the hIL9R variants with another N-terminal monoclonal antibody (AH9R7) that produced less background in immunostaining protocols (data not shown). This antibody was then used on the various TS1 cell lines, and staining was analyzed via FACS (Fig. 7A). Fluorescence remained at background levels in TSLXSN, whereas TSGR8 and TSGH9 cells showed a significant higher signal over background, indicating that the receptor was expressed and present on the cell surface. In contrast, TSAQGR8 and TSAQGH9 cells did not show any positive signal. These data were corroborated by the use of another monoclonal N-terminal antibody (AH9R1) (data not shown). Interestingly, these additional antibodies detect native receptor but not denatured receptor forms, further suggesting a conformational change in the N terminus of the ΔQ receptor.

To further support this notion, we used the same antibodies employed in the FACS and immunohistochemistry studies described above to immunoprecipitate the native receptor from total proteins extracted from TSLXSN, TSGH9, or TSAQGH9 cells. The C-terminal antibody was able to precipitate both the GH9 and ΔQGH9 receptors (Fig. 7B), while the N-terminal antibodies AH9R2 (top), AH9R7 (middle), and MAB290 (bottom) could only precipitate GH9 receptor. These data strongly support the hypothesis that a major conformation change occurs in the ΔQ protein that in turn results in the inability of the receptor to efficiently bind its ligand and induce a cellular response.

4 Santa Cruz Biotechnology, Inc., personal communication.
DISCUSSION

The interleukin-9 pathway has been implicated in the pathogenesis of asthma (12, 19). This study was undertaken in an attempt to identify hIL9R structure variants that may explain the linkage to this locus in determining resistance or susceptibility to asthma and/or allergy. The data presented here describe the identification of several hIL9R variants. We identified numerous nucleotide changes within the intracellular coding region of the receptor (codons 310, 344, and 410–418) that did not affect proliferation or signal transduction induced by hIL9R (26, 31, 34). Furthermore, analysis of these variants in assays used to assess IL9-induced gene activation and anti-apoptosis activity (as described in Ref. 26) again failed to identify any functional differences among these isoforms (data not shown). However, these experiments do not preclude the involvement of these residues in receptor signaling functions for other IL9-induced biological processes not evaluated here, such as cell type-specific differentiation, apoptosis, or induced gene expression. Several studies have shown that IL9 has a direct role on the differentiation of blood precursors (35) and mast cells (16, 17), while others have demonstrated its stimulatory activity on B-lymphocytes (13, 14). In addition to cell type specificity, these receptor variants may also exert a differential activity on IL9-induced gene expression. The reporter assay used in our studies employed a reporter element that is dependent upon Stat activation. The effect of these receptor variants has not yet been tested on Stat-independent, IL9-induced gene expression. The finding that IL9 receptor mutants, which abolish Stat1, Stat3, or Stat5 activation, under certain circumstances are still able to induce cellular proliferation suggests that IL9 receptor may signal through motifs other than Jak and Stat domains. A differential phosphorylation of the SER8 and SER9 receptor variants could account for the electrophoretic pattern seen in these receptors and might serve as a regulatory mechanism in signaling events such as gene expression. In addition, two human transformed cell lines have been found to contain receptor variants (Arg344-SER9) not found in the transcripts analyzed in this study. These alleles may have evolved from mutational event(s) that occurred during transformation in which the activation of IL9R is required for clonal expansion. This possibility is supported by the finding that IL9 overexpression is associated with

FIG. 4. IL9-induced activation of Jak, Stat, and Irs proteins by hIL9R variants. A, human IL9 does not induce phosphorylation of murine IL9 receptor. TS1 cells were starved for 6 h and then treated for 5 min with no cytokine (−), murine IL9 (m), or human IL9 (h). Cell extracts were immunoprecipitated with an antibody specific for tyrosine-phosphorylated (α-PY) proteins, blotted, and probed with an anti-murine IL9 receptor antibody (α-mIL9R). B, GR8 and GH9 receptors activate Jak, Stat, and Irs proteins upon IL9 binding. TSGR8 and TSGH9 cells were treated as in A, and lysates were immunoprecipitated using antibodies specific for various Jak, Stat, and Irs proteins as indicated. Immunoblots were first probed with an anti-phosphotyrosine antibody (α-PY) to detect only tyrosine-phosphorylated proteins, stripped and reprobed with the same antibody used for the immunoprecipitation. C, ΔQ receptor does not activate Jak, Stat, and Irs proteins upon IL9 treatment. TSΔQGR8, TSΔQGH9, and TSGH9 cells were treated as in A, and proteins were immunoprecipitated and immunoblotted as in B. I.P.: α-, antibody used for immunoprecipitation.

FIG. 5. ΔQ receptor is localized in the plasma membrane but does not bind hIL9. A, binding analysis of 125I-labeled hIL9 on TSLXSN, TSGR8, TSGH9, TSΔQGR8, and TSΔQGH9 cells. Specific binding (dotted bar) is compared with nonspecific binding (black bar) in the presence of a 200-fold excess of unlabeled human IL9. B, localization of GH9 and ΔQGH9 receptors. TSLXSN, TSGH9, and TSΔQGH9 cells were lysed, and cytoplasmic/nuclear and membrane fractions were isolated. Protein fractions and total protein extracts were run in SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Immobilized proteins were sequentially reacted with antibodies specific for hIL9R, actin, and proliferating cell nuclear antigen (PCNA).

5 J.-C. Renauld, unpublished observations.
T-cell lymphomas in IL9 transgenic mice (36).

Several alternative splice variants were also identified from our analyses. One class of alternative splice variants potentially encodes for soluble receptors due to alternatively spliced out exon 3, 4, or 5. These splice variants bear frameshifts and premature stop codons that in turn truncate the predicted polypeptide upstream of the residues encoded by exon 7, which encodes for the transmembrane domain. An alternative splice deletion of exon 8 was also identified that results in a putative membrane-bound form of receptor lacking the intracellular signaling domain sequences. These alternative splice forms may all be involved in serving as IL9 antagonists, as is the case of other cytokine receptors (such as IL4 and IL5 receptors), although the binding affinity of these receptors is greatly reduced (37–39). In addition to the splice variants described above, the abundant ΔQ transcript was also identified. It was found expressed in 47 out of 50 individuals and ranged up to 50% of the total transcript within individual samples. The encoded protein was found to be present at the cell surface when transfected into a murine T-cell line but was unable to bind its known ligand. The cause for its decreased ligand affinity appears to be due to a conformational change as determined by the fact that it lacked reactivity to three different N-terminal antibodies that only recognize the native receptor. A potential role of the ΔQ receptor may be to down-regulate IL9 receptor function.

Negative regulation via alternative splicing has been previously documented for the human growth hormone and the thyroid hormone receptors in controlling molecular signaling of the wild type versions of these proteins (40, 41). Alternatively, a dominant negative model can be proposed. As demonstrated by studies on IL4 and IL6 receptors, ligand-induced homodimerization of hemopoietin receptor subunits may serve to co-approximate receptor-bound protein tyrosine kinases such as the members of the Jak family (42, 43). This dimerization then results in trans-phosphorylation and activation of Jak and receptor subunits, which in turn initiate the

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6 L. Grasso, M. Huang, C. D. Sullivan, C. J. Messler, M. B. Kiser, C. R. Dragwa, K. J. Holroyd, J.-C. Renaud, R. C. Levitt, and N. C. Nicolaides, unpublished observations.

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**FIG. 6. Immunofluorescence micrographs of COS7 cells expressing wild type or ΔQ receptors.** a, cells were transiently transfected with LXSN (A and B), GR8 (C and D), GH9 (E and F), ΔQGR8 (G and H), or ΔQGH9 (I and J) constructs. 48 h after transfection, cells were sequentially incubated with MAB290 neutralizing antibody (N-terminal specific) and anti-mouse IgG Texas Red-conjugated antibody (B, D, F, H, and J) and fixed as described under “Experimental Procedures.” Cells were counterstained with 4,6-diamidino-2-phenylindole (A, C, E, G, and I) to visualize every cell in the photographed field. b, same as in a except that cells were first fixed/permeabilized and then incubated with a C-terminal specific antibody (sc698) followed by incubation with anti-rabbit IgG Texas Red-conjugated antibody. Bar, 10 μm.
signal transduction cascade. The IL9 receptor could undergo a similar dimerization. The co-expression of the nonfunctional ΔQ and wild type receptor subunits might lead to the formation of a complex incapable of eliciting an effective trans-phosphorylation and activation of the protein-tyrosine kinases. Since ligand binding seems to be required for subunit dimerization and the ΔQ receptor lacks this property, this conjecture cannot be supported at the present time. However, it would be worthwhile to determine the effect of the ectopic co-expression of these receptors to address this hypothesis. These complex studies are now being attempted.

The aim of this study was to assess the variability in hIL9R structure in PBMCs derived from a cohort of unrelated individuals because of the recent findings that the hIL9R locus is linked to asthma and allergy susceptibility. In this report, we have described the identification of multiple structural changes within the hIL9R transcript. While no biological differences were found in the receptors bearing amino acid substitutions, a clear difference was found in the ΔQ splice variant. A further ΔQ transcript was found to be statistically less in the allergic and/or asthmatic group as compared with the non-allergic group (18.3 and 28.7%, respectively (p < 0.05)). However, we believe that studies on additional populations will be required to further validate the significance of these results. We are currently analyzing the hIL9R locus for genomic changes that may influence the overall rate of ΔQ splicing and account for the genetic linkage data. Nucleotide sequences within distal and proximal intronic sequences as well as in 5'-flanking sequences have been documented to influence splicing (20, 45). The identification of a polymorphism that might affect the ΔQ splicing will grant us the use of a more quantitative genetic approach to study large populations of nonallergic and allergic and/or asthmatic individuals. This will allow us a better evaluation of the potential association of this receptor type with asthma and/or allergy.

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