GPRA and the asthma locus on chromosome 7p14-p15

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IV) **Pulkkinen V***, Haataja R*, Hannelius U, Helve O, Pitkänen OM, Karikoski R, Rehn M, Marttila R, Lindgren CM, Hästbacka J, Andersson S, Kere J, Hallman M, Laitinen T. G protein-coupled receptor for asthma susceptibility associates with respiratory distress syndrome. Annals of Medicine 2006;38. In press.

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In addition some unpublished data are presented.

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ABBREVIATIONS

AAA1, asthma associated alternatively spliced gene 1
AD, atopic dermatitis
ADAM, a disintegrin and a metalloproteinase
AHR/BHR, airway/bronchial hyperresponsiveness
BAL, bronchoalveolar lavage
β-AR, adrenergic receptor
BPD, bronchopulmonary dysplasia
cAMP/cGMP, cyclic adenosine/guanosine monophosphate
CD, cluster of differentiation
cDNA, complementary deoxyribonucleic acid
CYFIP2, cytoplasmic fragile X mental retardation protein -interacting protein 2 gene
CysLTR, cysteinyl leukotriene receptor
DHFR, dihydrofolate reductase
DPP10, dipeptidyl peptidase 10
FCV, forced vital capacity
FEV_1, forced expiratory volume in one second
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
GMP/GDP/GTP, guanosine mono/di/triphosphate
GPCR, G protein-coupled receptor
GPRA, G protein-coupled receptor for asthma susceptibility, official name NPSR1
GST, glutathione S-transferase
HLA, human leukocyte antigen
IBD, inflammatory bowel disease
IgE, immunoglobulin E
IL, interleukin
LPS, lipopolysaccharide
LT, leukotriene
MS, multiple sclerosis
mRNA, messenger ribonucleic acid
NPS, neuropeptide S
OVA, ovalbumin
PBMC, peripheral blood mononuclear cell
PG, prostaglandin
PHF, plant homeodomain finger protein
RACE, rapid amplification of cDNA ends
RDS, respiratory distress syndrome
RT-PCR, reverse-transcriptase polymerase chain reaction
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMC, smooth muscle cell
SNP, single nucleotide polymorphism
TBS/PBS, Tris/phosphate-buffered saline
TDT, transmission disequilibrium test
Th, T helper cell
TM, transmembrane
TXA_2, thromboxane A2

A. ABSTRACT

The basis of this work was the identification of a genomic region on chromosome 7p14-p15 that strongly associated with asthma and high serum total immunoglobulin E (IgE) in a Finnish founder population from Kainuu. Using a hierarchical genotyping approach the linkage region was narrowed down until an evolutionary collectively inherited 133-kb haplotype block was discovered. The results were confirmed in two independent data sets: Asthma families from Quebec and allergy families from North-Karelia. In all the three cohorts studied, single nucleotide polymorphisms tagging seven common gene variants (haplotypes) were identified. Over half of the asthma patients carried three evolutionary closely related susceptibility haplotypes as opposed to approximately one third of the healthy controls. The risk effects of the gene variants varied from 1.4 to 2.5.

In the disease-associated region, there was one protein-coding gene named *GPRA* (G Protein-coupled Receptor for Asthma susceptibility alias GPR154 and NPSR1) which displayed extensive alternative splicing. Only the two isoforms with distinct intracellular tail sequences, GPRA-A and -B, encoded a full-length G protein-coupled receptor with seven transmembrane regions. Using various techniques, we showed that GPRA is expressed in multiple mucosal surfaces including epithelial cells throughout the respiratory tract. GPRA-A has additional expression in respiratory smooth muscle cells. However, in bronchial biopsies with unknown haplotypes, GPRA-B was upregulated in airways of all patient samples in contrast to the lack of expression in controls.

Further support for GPRA as a common mediator of inflammation was obtained from a mouse model of ovalbumin-induced inflammation, where metacholine-induced airway hyperresponsiveness correlated with elevated GPRA mRNA levels in the lung and increased GPRA immunostaining in pulmonary macrophages. Expression analyses revealed that a novel GPRA agonist, Neuropeptide S (NPS), co-localizes with GPRA in human bronchial and colon epithelia, and in inflammatory cells. In a mouse macrophage cell line, NPS stimulated phagocytosis of *Esterichia coli* bacteria indicating a role for GPRA in removal of inhaled allergens.

The suggested GPRA functions prompted us to study whether *GPRA* haplotypes associate with respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD) in infants sharing clinical symptoms with asthma. According to the results, near-term RDS and asthma may also share the same susceptibility and protective *GPRA* haplotypes. As in asthma, GPRA-B isoform expression was induced in bronchial smooth muscle cells in RDS and BPD suggesting a role for GPRA in bronchial hyperresponsiveness.

In conclusion, the results of the present study suggest that the dysregulation of the GPRA/NPS pathway may not only be limited to the individuals carrying the risk variants of the gene but is also involved in the regulation of immune functions of asthma.

B. INTRODUCTION

Asthma is currently defined as a chronic inflammatory disease of the airways. The inflammation causes recurrent episodes of wheezing, breathlessness and cough in susceptible individuals. Often the symptoms are associated with variable airflow limitation that is at least partly reversible and includes increased airway responsiveness to various stimuli (the National Heart, Lung and Blood Institute in the United States / World Health Organization 2002).

Asthma is a worldwide disease with variable expression between countries and different populations within a country. The prevalence of asthma has been increasing worldwide over the last four decades of the 20th century. Just in recent years, evidence is emerging that this trend has flattened or even fallen in some countries and populations (von Hertzen and Haahtela 2005).

The current asthma therapies are not cures. The symptoms return soon after the treatment has been stopped even after long-term therapy. The mechanisms involved in the persistence of inflammation are poorly understood. It is still unknown, why some patients suffer from severe life-threatening disease, whereas the management of the disease is easier with others. Thus, there is a constant demand for knowledge and new therapies for asthma.

Although environmental factors such as life style and allergen exposure are undoubtedly risk factors for asthma, both familial and twin studies support a definite role for inherited features in the disease mechanism (Harris et al.1997, Panhuysen et al.1998, Koppelman et al.1999, Räsänen et al. 2000). Traditionally, genome-wide screening and candidate gene approach have been the two strategies in mapping the susceptibility alleles for asthma-related traits. In the former, markers evenly distributed across the whole genome are used to identify novel loci involved in the genetic susceptibility of the disease in an unbiased manner. In the latter, knowledge of genome-wide scans and molecules involved in the pathogenesis of asthma are utilized to study appropriate candidate gene regions. Only recently, several groups have progressed from broad linkage regions to identification of positional candidate genes for asthma. These approaches have exposed totally new avenues for asthma research.

The basis of this work was the identification of a genomic region on chromosome 7p14-p15 that strongly associated with asthma and high serum total immunoglobulin E (IgE) in a founder population recruited from Kainuu province of Finland (Laitinen et al. 2001). Further finemapping of the linkage region exposed a 133 kb segment, which could be divided into three susceptibility and four neutral/protective haplotypes (Laitinen et al. 2004). Utilization of computer algorithms for gene prediction over the disease-associated region revealed two previously uncharacterized genes, *GPRA* (G Protein coupled Receptor for Asthma susceptibility) and *AAA1* (Asthma Associated Alternatively spliced gene 1), with unknown biological functions. In 2005, two independent reports replicated the genetic findings in large European cohorts of up to 5000 study subjects (Melen et al. 2005, Kormann et al. 2005). Most recently, a single nonsynonymous polymorphism in the *GPRA* gene was associated with increased susceptibility to methacholine-induced bronchial hyperresponsiveness in Chinese population (Feng et al. 2006)

suggesting *GPRA* to become the prime candidate for the linkage observed within the 7p14.3 region.

Other groups were studying GPRA functions in parallel with the work carried out in our group. Vassilatis and co-workers identified novel G protein-coupled receptors (GPCRs) including GPRA by sequence pattern mining in a systematic screening approach to construct a phylogenetic tree for GPCRs (Vassilatis et al. 2003). Gupte and coworkers utilized a chimeric receptor approach where the intracellular signaling parts of the vasopressin V1a receptor were joined to the C-terminal portion of GPRA (Gupte et al. 2004). The authors showed activation of the chimeric receptor by arginine vasopressin and suggested that GPRA has dual signalling properties by coupling to both Gq and Gs pathways (Gupte et al. 2004). While the authors where processing their manuscript, a patent application reported identification of a linear 20-residue proteolytic fragment peptide that acted as an endogenous ligand for GPRA (Mori et. al 2003). These findings paved the way for functional studies utilizing the novel GPRA agonist named Neuropeptide S (NPS) (Xu et al. 2004). The main objective of this study was to clarify the role of GPRA in the pathogenesis of asthma to reveal novel disease mechanisms, and possibly, to find novel treatment opportunities for the disease.

C. REVIEW OF THE LITERATURE

1. CLINICAL FEATURES OF ASTHMA

1.1 Definition

In 2002, the National Heart, Lung and Blood Institute in the United States published the following definition of asthma: "*Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial responsiveness to a variety of stimuli.*" The symptoms can be induced e.g. by viral infections in childhood, exercise, or inhalation of cold air and allergens. The typical histological alterations in the airway wall include basement membrane thickening, goblet cell hyperplasia, as well as epithelial damage and SMC hypertrophy. These changes are often termed "airway remodelling" (Davies et al. 2003). However, none of the single features or group of features can be defined as common to all asthmatics. Both environmental and genetic factors attribute to the progress of the disease.

The clinical diagnosis of asthma is accomplished by demonstrating reversible airflow obstruction on spirometry. This can be carried out using sequential measurements of peak expiratory flow, forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), and FEV_1/FVC before and after inhalation of a short-acting bronchodilator. Clinically significant reversibility is defined as a significant increase of more than 12 % or 200 ml in FEV_1 after inhalation of a short-acting bronchodilator or treatment with corticosteroids (the National Heart, Lung and Blood Institute in the United States / World Health Organization 2002).

1.2 Prevalence

The lack of a generally accepted asthma definition for epidemiological studies complicates the comparison of studies on time trends in asthma prevalence. According to the estimations, asthma affects up to 20% of children and 5-12% of adults in industrialized countries (ISAAC Steering Committee 1998). The prevalence of asthma has been increasing worldwide over the last four decades of the 20th century, although evidence is emerging that in recent years this trend has flattened or fallen in some countries and populations (Flemming et al. 2000, Verlato et al. 2003, Braun-Fahrländer et al. 2004, von Hertzen and Haahtela 2005). However, a nationwide study on time trends in prevalence of asthma and allergy in Finnish young men at call-up for military service during the years 1966-2003 found no evidence of a reduction in the prevalence of either asthma or allergic rhinitis (Latvala et al. 2005). Asthma has become milder or more controlled

disease during the past 15 years, but still remains the most common chronic disease among children. Fortunately, the prevalence of symptoms in asthmatic children decreases towards adulthood.

1.3 Risk factors

1.3.1 Atopy

The term "atopy" is commonly defined as genetic predisposition for immunoglobulin E (IgE) - mediated responses such as skin reactions to minute amounts of common allergens (Johansson et al. 2004). Atopy is the strongest identifiable predisposing factor to the developing asthma and more than 80% of asthmatics have an atopic background. However, also non-atopic asthma exists. Strategies that abolish allergen-specific IgE responses (Barnes 1996, Creticos et al. 1996, Adkinson et al. 1997) or that remove total serum IgE (Holgate et al. 2004) are not of complete efficacy for asthma treatment. Although most of the children with asthma are atopic, approximately 10% of patients with childhood asthma have normal levels of total IgE and do not have specific IgE responses to common allergens (Romagnani 2004). Typically, these non-atopic patients display variable but incomplete reversibility of airway obstruction, and are clinically difficult to distinguish from atopic patients (McConnell and Holgate 2000). The two types of asthma probably represent different conditions with distinct disease mechanisms and genetic backgrounds.

The common syndrome of atopic diseases also includes atopic dermatitis (AD) and hay fever (allergic rhinitis), which are risk factors for asthma (Spergel 2005). AD, also called eczema, is a chronically relapsing inflammatory skin disease characterized by dryness of the skin and itching, which results in scratching and excoriations. In a German atopy study containing 1300 children, patients with AD showed increased risk for asthma at the age of seven, whereas patients with AD and no wheezing during the first 3 years did not. Thus, the authors proposed that AD may not develop into asthma, but is part of a syndrome consisting of both diseases (Lau et al. 2002). In a questionary consisting of over 90000 individuals, patients with rhinitis had an increased risk for asthma and lower airway reactivity when compared with patients without rhinitis (Leynaert et al. 2004). In contrast to asthma, allergic rhinitis appears to be associated primarily with skin test reactions to common aeroallergens, independent of the serum IgE level. It is defined as an epithelial inflammation of the nose, and characterized by nasal congestion, rhinorrhoea, sneezing and itching.

1.3.2 Genetics

Both familial and twin studies support a role for inherited features in the disease mechanisms of asthma (Harris et al.1997, Panhuysen et al.1998, Koppelman et al. 1999, Räsänen et al. 2000). First, asthma and atopic diseases cluster in families. The risk that a first-degree family member of a patient with asthma will develop asthma has been calculated to be less than two to almost six

times higher than the risk for individuals in the general population. Because the presence of aggregation among related individuals may indicate either shared genes or a common household environment, the genetic influence on asthma has also been sorted out by using twin studies and segregation analyses. Monozygotic twins have identical genetic make-up whereas dizygotic twins share approximately 50% of their genes. If genes were influenced by a particular trait, monozygotic twins should resemble each other to a greater extent than dizygotic twins because of the greater genetic similarity. Regarding to asthma, the first large twin study was published in 1971 on a Swedish population of 6996 twin pairs (Edfors-Lubs et al. 1971). The results showed that the concordance for self-reported asthma of monozytic twins and dizygotic was 19.0% and 4.8%, respectively, illustrating the genetic influence. In the Finnish twin cohort, the heritability of asthma was estimated to be 35.6% (Nieminen et al. 1991). Among the younger twin cohort, the genetic effect accounted for 79% of the variance in the development of asthma (Laitinen et al. 1998). These studies provide strong evidence for hereditary basis in the disease mechanisms, although the genetic susceptibility for asthma is unlikely to explain the observed increase in the prevalence of asthma. Environmental influences in asthma are established with the finding that occasionally in monozygotic twins only one of the twins is affected by the disease. Genetic studies have also demonstrated that atopy and bronchial hyperresponsiveness (BHR) have different patterns of inheritance. Whereas the ability to produce IgE is regulated at genetic level, the specificity of the IgE response may mainly be affected by the environment.

1.3.3 Environmental factors

Asthma is a worldwide disease with variable expression between countries and between different populations in a country. Its prevalence is generally higher in the western countries than in the developing countries indicating that western lifestyle with indoor environment, diet, air pollution and psychological stress might play a role in the onset of the disease. This has given rise to the widely debated theory called “hygiene hypothesis” stating that exposure to microbes in childhood protects against asthma. The four main environmental candidates for the elevated asthma prevalence in the western countries are changes in exposure to infectious diseases in early childhood, environmental pollution, allergen levels, and dietary changes (Arruda et al. 2005). The length and type of exposure to infectious agents as well as the time when it happens have been shown either to promote or to suppress an inflammatory process and influence on a subsequent history of asthma. Childhood on a traditional farm seems to confer protection against the development of asthma and atopy suggesting that continuous inhalation of lipopolysaccharide (LPS) might somehow dampen the harmful immune responses (von Ehrenstein et al. 2000, Riedler et al. 2001, Braun-Fahrländer et al. 2002). Exposure to environmental pollution could worsen the expression of atopy and asthma, but the best evidence, however, shows the opposite. For example, children in the cities of Halle and Leipzich in the former East Germany had lower prevalence of atopy and asthma when compared with ethnically matched population in Munich from the western part of the country with cleaner air. In the former population, children had,

however, a higher overall prevalence of respiratory diseases than in the latter. Nevertheless, those diseases were not of allergic origin (von Mutius et al. 1994). Five years after reunification, allergy rates had risen up in East Germany and the allergic sensitization rates had equalized to the same extent to the western part of the country (Weiland et al. 1999). A similar west-east trend in childhood allergic sensitization, hay fever, and eczema was observed in a recent study reporting higher rates of allergy among Finnish children in North-Karelia when compared to their adjacent Russian counterparts (von Hertzen et al. 2006). As a hint of the influence of the western nutrition, there is even a study suggesting that frequent consumption of hamburgers shows a dose-dependent association with asthma symptoms (Wickens et al. 2005). Thus, these findings may indicate that locally operating factors such as pollutant exposure, diet, and respiratory virus infections may indeed predispose individuals to asthma.

1.3.4 Neonatal characteristics

Epidemiological studies have repeatedly reported an increased risk of asthma or wheezing disease among preterm children with respiratory distress syndrome (RDS) or RDS and subsequent bronchopulmonary dysplasia (BPD) (Schaubel et al. 1996, Evans et al. 1998, Smith 2003). In a study utilizing data from a provincial health organization to evaluate the effect of birth characteristics on asthma incidence and hospitalization for asthma during the age of 0-4, several neonatal characteristics were important determinants for the risk of physician-diagnosed preschool asthma (Schaubel et al. 1996). Significant association with preschool asthma included male gender, low birthweight and prematurity. RDS in the presence or absence of BPD showed associations both with asthma incidence and hospitalization due to asthma. Other perinatal factors that associate with the development of asthma and atopic diseases include low birth weight, prenatal exposure to tobacco smoke, severe respiratory infection in the first year of life and lack of breast feeding (Young et al. 1991, Evans et al. 1998, Friedman and Zeiger 2005).

1.4 Pathogenesis

According to the current consensus view high IgE-levels derived as a result of cluster of differentiation (CD) 4+ T helper type 2 (Th2) cell responses account for the pathogenesis of atopic diseases. Th2 cells promote B-cell derived IgE production (Kay 2001). Allergen-specific IgE binds to its high-affinity Fc receptor (FcεRI) on mast cells and basophils and low affinity receptors (FcεRII or CD23) on T -lymphocytes, eosinophils, and antigen presenting cells, such as B-cells, monocytes and dendritic cells. The immediate symptoms of asthma are triggered, when allergens penetrated through the bronchial epithelium induce crosslinking of FcεRI. Subsequent mast-cell degranulation releases pro-inflammatory molecules and potent bronchoconstrictors such as histamine, cysteinyl leukotriene (LT) C_4 and prostaglandin (PG) D2 (Cookson 2004).

The development of allergic asthma can be separated into three phases (Elsner et al. 2004).

1) In the initiation phase, genetic predisposition to atopy predominates and the development of IgE antibodies against environmental antigens occurs. According to the hygiene hypothesis, antigen presentation in susceptible individuals results in polarized T-cell differentiation towards a Th2 instead of an opposite Th1 phenotype (Wills-Karp et al. 2001, Yazdanbakhsh et al. 2002). The Th2 cells secrete preferably interleukins (ILs) 4, 5, 9 and 13, and the Th1 cells interferon-γ and IL-2. The effective IgE production by B cells depends on support by Th2 cells (Kay 2001).
2) In the propagation phase of allergic asthma, Th2-polarized T lymphocytes and eosinophils infiltrate the airways, leading to a chronic inflammatory state. IL-4 is essential for T-cell survival and expansion, IL-5 for eosinophil maturation and IL-13 for mucus production and SMC contraction. Increasing body of evidence also shows the importance of chemokines and their receptors in the mechanisms of recruiting eosinophils into the lung and maintaining local Th2-cell dominance (section 3.3.2).
3) In the final effector phase of the disease, the inflammation leads to edema of the lung tissue along with complement activation, and cell extravasation (Humbles et al. 2000). The production of mediators of chronic inflammation leads to airway hyperresponsiveness (AHR), mucus hypersecretion and structural changes of airway architecture. Loss of bronchodilators such as PGE2 and nitric oxide secreted by epithelium also contribute to AHR in allergic asthma. Eosinophils are able to damage the epithelium due to the release of reactive oxygen species and their basic proteins such as eosinophil cationic protein and major basic protein (Andreadis et al. 2003). These compounds are also known to cause epithelial shedding, which exposes sensory nerve endings to the airway lumen (Gleich 1988). Afferent sensory nerve endings are also involved in central reflex bronchoconstriction upon stimulation by inflammatory mediators such as histamine, adenosine and endothelin-1 (Coleridge et al. 1989, Riccio et al. 1995). Eosinophils also secrete cytokines such as transforming growth factor-β, epidermal growth factor and IL-13. Both the toxic compounds and cytokines promote smooth muscle thickening, AHR and mucus production in allergic asthma.

2. FINDING GENES FOR ASTHMA SUSCEPTIBILITY

In asthma, the susceptibility alleles have relatively high population frequencies, but not everyone with the susceptibility alleles is affected by the disease (reduced penetrance). Because clinically similar phenotype may also result from a different set of genes or environmental exposure, the risk effects are expected to be modest as a maximum (Lander and Kruglyak 1995). In most genetic studies, self-reported questionnaires in addition to reported doctor's diagnosis of asthma,

bronchial hyperresponsiveness, or combinations of these parameters are used for definition of asthma. Because of the lack of a clearly defined asthma phenotype, additional quantitative traits (intermediate phenotypes) such as serum total and allergen specific immunoglobulin E levels, blood eosinophil count and bronchial peak flow variability are commonly used as surrogate markers (Martinez et al. 1997). Recent studies have shown that global microarray gene-expression approaches might allow novel asthma candidate genes to be identified. However, the hypothesis-independent genome-wide search and the hypothesis-dependent candidate-gene approach have traditionally been, and still exist, as the two basic methods in the genetic asthma research (Lander and Schork 1994). Regarding the complex and heterogeneous etiopathogenesis of asthma, it is not surprising that numerous genes have been reported. Because many genes with individually small effects are likely to contribute to the risk, replication of the linkage results and association studies has not been trouble-free. Utilization of larger sample numbers in the study cohorts as well as consideration of multiple gene variants and haplotypes in the latest studies might have reduced the possibility of false association results. The development and utilization of more advanced software platforms for association analyses will be useful in gathering novel data concerning the interaction between the susceptibility genes.

2.1 Genome-wide linkage studies

In genome wide scans, markers evenly distributed across the genome are genotyped to implicate linkage to chromosomal segments of 10–20 megabases in an unbiased manner. Microsatellites, also known as short tandem repeats (Weber and May 1989, Litt and Luty 1989), have traditionally been utilized as markers. However, the use of single nucleotide polymorphisms (SNPs) has gained popularity along with the HapMap project and the development of commercial microarrays, “SNPchips” (Kruglyak 1997). SNPs consist of biallelic base-pair substitutions occuring randomly in the genome, and are the most common forms of genetic polymorphisms. Launched originally in October 2002, the HapMap project has now a goal of increasing the density of SNPs across the genome from the average of 1/3,000 bases to about 1/600 bases.

The first genome-wide screen for asthma susceptibility loci was published in 1996 (Daniels et al. 1996). Since then, at least 17 additional genome-wide surveys have been carried out in a variety of populations and suggested more than 30 different genomic regions for linkage to asthma related traits (Ferreira et al. 2005) (Table 1). However, many studies lack proper replication of the initial findings. Although small sample populations may explain some of the false positive results, the study designs in most genome-wide scans are more likely to lead into false negative findings because of the relatively wide gaps between the markers. Nevertheless, there are several regions that have consistently shown a significant linkage in more than a few studies and are likely to represent true asthma or atopy susceptibility loci (Wills-Karp and Ewart 2004).

Table 1. Summary of results from genome-wide scans of asthma or related traits. Modified from Wills Karp and Ewart 2004 and Ferreira et.al 2005. The most significant primary linkage regions are 2q14-q32 (containing e.g. *cytotoxic T-lymphocyte associated protein 4* and *IL-1 receptor antagonist*), 5q31-q33 (Th2 type cytokine gene cluster including *IL-4*, *IL-13*, *CD14* and *β-adrenergic receptor* (*β-AR)*); 6p24-p21 (*human leukocyte antigen DRB1* (*HLA-DRB1)*, *tumor necrosis factor α*); 7p15-p14; 11q13-q21 (near the B chain of the high affinity receptor for IgE); 12q21-q24 (*stem cell factor*, *interferon-γ*, *signal transducer and activator of transcription-6*); 13q12-q14; and 20p13.

Population	**Study design: Number of families (study subjects; markers)**	**Phenotypes**	**Chromosomal regions reported**	**References**
Australian, British*	80 (364; 296) 77	Asthma, atopy, total IgE, SPT, blood eosinophils, BHR	4q‡, 6‡, 7, 11q‡, 13q‡, 16‡	Daniels et al. 1996
African-American, Caucasian, Hispanic	43–115 45–138 18–34 (1931; 323)	Asthma, atopy (SPT), total IgE, Der-p-specific IgE, asthma and environmental tobacco smoke exposure, Der p and Der f mite reactivity	1p32, 1q, 2q21-23‡, 2q33‡, 3, 5q23-31‡, 5p15, 6p21.3-23‡, 8p23-21, 8p32, 9q,11p15, 11q21‡, 12q14-24.2‡, 13q21.3 qter‡, 14q11.2-13, 14q32, 15q13, 17p11.1-q11.2, 18, 19q13‡, 20p, 21q21	Xu et al. 2001a, Mathias et al. 2001 Blumenthal et al. 2004*a*, *b*
German	97 (415, 156 sib pairs; 351)	Asthma, total IgE, specific IgE	2‡, 6‡, 9, 12‡	Wjst et al. 1999
Hutterites (a founder population of European descent)	1 (361-693; 563) 1 (292–324; 563)*	Asthma, BHR, SPT	1, 3p24.2-22, 5q23-31‡, 6‡, 12q15-24.1‡,16‡, 19q13‡, 21q21	Ober et al. 1998, 1999, 2000
Japanese	47 (197, 65 affected sib pairs; 398)	Mite-sensitive atopic asthma	4q35‡, 6p22-21.3‡, 5q31-33‡, 12q21-23‡, 13q11‡, 13q14‡	Yokouchi et al. 2000
Japanese	48 (188, 67 affected sib pairs; 400)	Total IgE, orchard grass -specific IgE, orchard grass -sensitive allergic rhinitis	1p36.2, 3p24.1, 4p16.1‡, 4q13.3‡, 5q33.1‡, 9q34.3, 11q14.3‡, 12p13.1‡, 12q24.2‡, 16p12.3‡	Yokouchi et al. 2002
French	107 (493; 254)	Asthma, total IgE, BHR, SPT, blood eosinophils, age of onset	1p31, 7q, 11p13, 11q13‡, 12q24‡, 13q31‡, 17q12-21, 19q13‡	Dizier et al. 2000
Finnish French-Canadian*	86 (443; 312) 22	Asthma, total IgE, asthma and high IgE	7p14-15§, 4q	Laitinen et al. 2001

Chinese	533 (2551; 422)	Total IgE, specific IgE, FEV_1, FVC, BHR, blood eosinophils, SPT	1, 2‡§, 4‡, 10, 16‡, 19‡, 22	Xu et al. 2001*b*
Dutch	200 (1174; 366)	Total IgE, Der-p and aeroallergen -specific IgE, SPT, SPT to house dust mite, blood eosinophils	2q‡, 6p‡, 7q, 11q‡, 13q‡, 17q, 22q	Koppelman et al. 2002
Danish	48 (206; 446)	Atopy, allergic asthma, total IgE	1p36, 3q21-22, 5q31‡, 6p24-22‡	Haagerup et al. 2002
Caucasian	460 (920; 387)	Asthma, asthma and BHR, asthma and total or specific IgE	20p13§	Van Eerdewegh et al. 2002
Icelandic	175 (1134; 976)	Asthma	14q24§	Hakonarson et al. 2002
French	295 (1,317; 396)	Asthma, total IgE, SPT, SPTQ, specific-IgE), eosinophils, BHR, FEV_1	12p13, 12q24	Bouzigon et al. 2004
Australian	539 (2,360; 396)	Eosinophil count	2q33, 8q24	Evans et al. 2004
European	82 (366; 603)	Asthma, mite-sensitive atopic asthma	2p12, 3q21, 16q21	Kurz et al. 2005
Australian	202 (591)	SPT to house dust mite, atopy BHR, FEV_1, FVC, asthma	20q13, 12q24	Ferreira et al. 2005
Caucasian	364 (1551; 396)	Asthma, atopic asthma, BHR	2p, 4p	Pillai et al. 2006

*Replication study
‡Linkages identified in four or more studies
§Chromosomal regions providing significant evidence for linkage (many chromosomal regions reported in the literature did not provide significant evidence for linkage).
BHR, bronchial hyperresponsiveness to methacholine challenge; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; IgE, immunoglobulin E; SPT, skin prick test. SPT (positive skin prick test response to at least one of 11 allergens), SPTQ score being the number of positive skin test responses to 11 allergens and % predicted FEV.

2.2 Association studies

The hypothesis-dependent approach selects candidate genes that might have relevance in the disease process (biological candidates) or of candidate-gene regions previously linked to the trait of interest (positional candidates). The study designs can be either family- or population-based (Risch 2000, Cardon et al. 2001). The population-based case-control studies are utilized in studying the probabilities whether a specific genetic variant is overrepresented (or underrepresented) in affected cases in comparison to non-affected controls. Haplotypes are defined as a linear set of genetic markers on one chromosome that are inherited together

(haplotype block) (Gabriel et al. 2002). By obtaining knowledge from several polymorphisms, the misleading results often received by genotyping single polymorphisms can be avoided. However, the controls and cases have to be carefully matched for age, sex and origin in order to reduce genetic heterogeneity. These problems can be overcome with family-based settings, where transmission disequilibrium tests (TDTs) are utilized in comparing the transmissions and non-transmissions of the marker allele from heterozygous parents to the affected offspring (Spielman and Ewens 1996).

The current tools for biocomputing allow researchers to relatively easily dissect the disease-associated polymorphisms to exceed the statistical treshold. However, a possibility of false positive results remains with each test performed. This can be corrected for multiple testing, where the probability of false positive results is simulated by repeated analysis of randomly selected samples within the study population (permutation test).

Regarding asthma, more than 150 genetic linkage studies have suggested a variety of candidate genes in 500 loci (Wills-Karp and Ewart 2004). A recent review reported 118 genes associated with an asthma- or atopy-related phenotype, 54 genes that were replicated in 2-5 independent samples, 15 genes in 6-10 independent samples, and 10 genes in >10 independent samples, reported in a total of 492 articles (Ober and Hoffjan 2006). Genome-wide screening studies have provided novel candidate loci with potential susceptibility genes, which often show important immune-responses. In particular, variation in ten genes has been associated with asthma-related phenotypes in ten or more studies. These genes are *IL-4* and *IL-4RA*, *IL13*, *β-AR*, *tumor necrosis factor*, *HLA-DRB1* and the *B-subunit of the high-affinity IgE receptor*, as well as *CD14*, *HLA-DQB1*, and *a disintegrin and a metalloproteinase 33* (*ADAM33)* genes.

2.3 Positional cloning

In positional cloning, the susceptibility loci revealed after genome-wide searches are narrowed down by genetic association analysis using genetic markers at higher density in order to identify the genes responsible for the linkage. Finally, expression pattern analysis and further functional characterization of the gene and its variants are warranted to indicate proper causality. Only recently, a few groups have progressed from broad linkages to identification of the susceptibility gene (Kere and Laitinen 2004). This has led to the discovery of novel signaling pathways transmitted by susceptibility genes such as *ADAM33* and *GPRA*.

2.3.1 ADAM33

ADAM33 was the first asthma susceptibility gene identified by positional cloning. In the original report published in 2002, linkage to asthma was studied in 362 and 98 Caucasian families with asthmatic sibling pairs in the United Kingdom and United States, respectively (Van Eerdewegh et al. 2002). Followed by a microsatellite marker genome scan, a linkage region of about 2.5 megabases containing 40 genes was discovered on chromosome 20p13. Case-control studies

carried out with 135 SNPs showed that 14 SNPs localized to the *ADAM33* gene region. TDT and haplotype analyses of five *ADAM33* SNPs supported an association with asthma and BHR.

ADAM33 belongs to a family of zinc-dependent membrane-anchored metalloproteinases that also have effects on cell adhesion and intracellular signalling. It is selectively expressed in mesenchymal cells (airway SMC and fibroblasts) (Van Eerdewegh et al. 2002), but not in the immune or inflammatory cells (Umland 2003, Haitchi 2005). Thus, it could play a role in predisposing to the reduced lung function characteristic of asthma, possibly by influencing airway wall remodelling.

The over ten replication studies of *ADAM33* and asthma have yielded somewhat conflicting results, although the results have been confirmed in African American, Hispanic and US Caucasian outbred populations, as well as in Dutch (Howard et al. 2003), German (Werner et al. 2004), Korean (Lee et al. 2004) and Japanese (Noguchi et al. 2006) populations. However, also negative association studies exist (Lind et al. 2003). A recent meta-analysis combining the results of positive and negative studies from eight populations yielded a maximum odds ratio of 1.46 (p=0.0001) for a common *ADAM33* SNP called ST+7 (risk allele frequency 84.9% in asthmatics and 79.1% in controls) (Blakey et al. 2005). In this method, the chromosomes are divided into bins of defined length, and categorized according to their linkage scores (Wise et al. 1999). As observed in many association studies, the risk effects are smaller than in the original report (the odds ratio in the most significantly associated SNP was 1.95 in the original study).

2.3.2 DPP10

Dipeptidyl peptidase 10 (*DPP10*) was discovered by refining the linkage peak on chromosome 2q14-q32 (Allen et al. 2003). An association study revealed a 460-kb genomic region with high linkage disequilibrium. The authors located the first, alternatively spliced exons belonging to a large gene spanning over 1 Mb of genomic DNA and completed the gene sequence with three rounds of rapid amplification of cDNA ends (RACE) -PCR. The gene, *DPP10* (also known as *DRP3* and *DPPY*), encoded a transmembrane protein with a small intracellular amino terminus and a large extracellular domain (Engel et al. 2003, Hiramatsu et al. 2003, Ludwig et al. 2003, Rasmussen et al. 2003). DPP10 was suggested to act as a peptidase on the two terminal peptides of cytokines and chemokines such as IL-2, RANTES and eotaxin (Allen et al 2003), although the enzymatic region has been previously presumed non-functional (Wada et al. 1992, Kin et al. 2001, Qi et al. 2003). Because there were no coding polymorphisms in the *DPP10* gene, the influence of the associated locus was proposed to derive from indirect actions of the adjacent CdxA promoter on DPP-10 expression. Initial functional evaluation of this hypothesis demonstrated that the CdxA promoter was capable of differentially binding to nuclear protein extracts in a manner dependent on the present allele (Allen et al. 2003).

DPP10 is prominently expressed in the brain and additionally in pancreas, adrenal glands and trachea (Allen et al. 2003, Qi et al. 2003). Recently, DPP10 has been found to modulate the properties of Kv4.2 channels that are responsible for rapidly inactivating outward K+ current in

many neuroendocrine cells (Zagha et al. 2005, Ren et al. 2005). This suggests that DPP10 may have additional functions in regulating airway smooth muscle tone.

Consistent with the original report, independent mouse genome screens have linked AHR in mice to a region homologous to the location of *DPP10* in humans (De Sanctis et al. 1995, Ewart et al. 2000), but thus far, there are no replication studies among humans.

2.3.3 PHF11

One of the most replicated segments in asthma genetics is the chromosomal region 13q14 (Daniels et al. 1996, Hizawa et al. 1998, Kimura et al. 1999 and Beyer et al. 2000). The gene named *plant homeodomain finger protein 11* (*PHF11* alias renal carcinoma autoantigen) was identified in a similar approach to *DPP10*. In the linkage region, Zhang and co-workers assessed linkage disequilibrium patterns around a single significantly associated marker using SNPs between brief gaps (Zhang et al. 2003). Concurrently, they studied the gene content of the segment and found strongest associations of SNP markers to a 100-kb high linkage disequilibrium interval containing the gene for *PHF11*. The gene product contains two zinc fingers suggesting involvement in chromatin remodelling or transcriptional regulation. The close proximity of the *PHF11* gene to a protein complex containing a histone methyl transferase *SETDB2* suggests that the two genes might be co-ordinately expressed or that a combined product might be expressed. Both PHF11 and SETDB2 proteins are expressed in B lymphocytes. PHF11 contains several splice variants, which were overexpressed in activated peripheral blood lymphocytes (Zhang et al. 2003). The interactions and genetic mechanisms underlining the associations are complex. Thus far, there are no replication studies for asthma, but two polymorphisms in the *PHF11* gene were associated with childhood atopic dermatitis in Australian population (Jang et al. 2005).

2.3.4 CYFIP2

Noguchi and coworkers constructed a saturation map using 27 microsatellite markers around the 9.4-Mb linkage region that had previously shown linkage to atopic asthma (Yokuchi et al. 2000) and other asthma-related phenotypes on chromosome 5q33 (Ober et al. 1998). The authors performed mutation screening and association analyses of 26 genes that existed between the markers (Noguchi et al. 2005). Transmission disequilibrium test analysis of altogether 105 polymorphisms in 155 Japanese families with asthma revealed strong association between asthma and polymorphisms in *cytoplasmic fragile X mental retardation protein -interacting protein 2* gene (*CYFIP2)*. Six polymorphisms in *CYFIP2* were in complete linkage disequilibrium and associated significantly with the development of asthma (odds ratio 5.9). In real-time quantitative PCR analysis, subjects homozygous for the haplotype overrepresented in asthmatics showed significantly increased levels of *CYFIP2* expression in lymphocytes when compared with the subjects heterozygous for the haplotype.

Interestingly, CYFIP2 protein expression was increased in CD4+ and CD8+ T cells from multiple sclerosis (MS) patients when compared with the cells from healthy controls or patients with inflammatory bowel disorder (IBD) (Mayne et al. 2004). Several findings suggested that overexpression of CYFIP2 facilitates increased T cell adhesion properties.

2.3.5 HLA-G

Linkage of asthma and related phenotypes to chromosome 6p21 has been reported in seven genome scans making it the most replicated susceptibility loci for asthma (Table 1). Nicolae and coworkers conducted a genome-wide screen of families who participated in the Collaborative Study on the Genetics of Asthma (CSGA 1997, Xu et al. 2001a, Nicolae et al. 2005). They focused on 35 white families and 46 white child-parent trios with best associations with asthma.

The linkage region contained 20 genes and at least 30 pseudogenes spanning 1 Mb of genomic DNA. Additional 59 polymorphisms between 10–20-kb intervals in 19 genes, two pseudogenes, and the intergenic regions flanking the *HLA-A* and *HLA-G* loci were genotyped. Five linkage disequilibrium blocks were identified and variation in the *HLA-G* gene accounted for most of the linkage as confirmed by TDT. The results were replicated in two distinct populations, although the gene variants differed between populations. In both Hutterites (a founder population of European descent) and Dutch families, significant association of *HLA-G* with BHR was observed (Ober et al. 2000, Koppelman et al. 2002).

HLA-G undergoes alternative splicing events providing either membrane-bound or soluble proteins. In addition to its antigen presenting properties, HLA-G may have immunosuppressive properties e.g. in acute and chronic organ rejection (Lila et al. 2000). HLA-G is expressed in pulmonary macrophages and dendritic cells (Pangault et al. 2002) and its expression has also been linked to ulcerative colitis (Torres et al. 2004), psoriasis (Aractingi et al. 2001) and MS (Mitsdoerffer et al. 2005). HLA-G has unique tissue expression pattern in fetal cells at the maternal–fetal interface indicating that it may be involved in the biological mechanisms that protect the fetus during pregnancy (Hunt et al. 2005). In peripheral blood monocytes, HLA-G is expressed as membrane-bound, but is secreted as soluble molecules upon IL-10 activation. Regarding to asthma, LPS stimulation induced the secretion of IL-10 and soluble HLA-G1/HLA-G5 molecules in healthy subjects, whereas the levels of IL-10 and detectable soluble HLA-G molecules were reduced in asthma patients (Rizzo et al. 2005). Thus, the loss of immunosuppressive effects of HLA-G could contribute to the persistence of airway inflammation in asthma.

2.3.5 GPRA

Genome-wide scan in 86 pedigrees recruited from a founder population in Kainuu province revealed one genomic region that showed significant linkage to asthma-related traits on chromosome 7p (Laitinen et al. 2001). Further evaluation of the statistical significance of the finding was performed with simulations. The permutation tests showed that linkage peak

exceeded the genome-wide significance level and was highly unlikely a false positive finding. The results were then further confirmed in two independent data-sets: French-Canadians and North-Karelians. Interestingly, the 7p14 region had exceeded the threshold levels in an Australian sib study to detect linkage to allergic asthma (Daniels et al. 1996). In an attempt to identify the effective gene, an association study for a potential candidate gene encoding the γ-chain of the heterodimeric γ/δ T cell receptor on the linkage region showed, however, no evidence of associated polymorphisms (Polvi et al. 2002).

The further finemapping of the associated region led to the discovery of two novel genes: *GPRA* and *AAA1*. As the genes were identified in the first publication of this thesis, the positional cloning and the characterization of the genes will be described in the results section in more detail. Similarly, the results of the replication studies will be enlightened in the discussion part.

Identification of a novel GPCR rapidly received a lot of attention. Research carried out in other groups led to the discovery of a novel linear 20 residue peptide that activated GPRA via both Gαq and Gαs pathways resulting in intracellular Ca^{2+} mobilization as well as protein kinase C and A activation (Mori et al. 2003, Gupte et al. 2004, Xu et al. 2004). The polypeptide might derive from an uncharacterized precursor, mapping in humans to chromosome 10q26, which is phylogenetically conserved across mammals. The precursor protein contains the typical structural features of a neuropeptide precursor. A hydrophobic signal peptide immediately follows the initiation of translation (AUG), and the immature peptide is preceded by a pair of basic amino acids (Lys-Arg) that are presumed processing sites for proteolytic cleavage. Because of the amino terminal serine, this molecule and its cognate receptor were termed neuropeptide S (NPS) and neuropeptide S receptor (NPSR), respectively (Xu et al. 2004). The primary structure of NPS is highly conserved among vertebrates, but the gene is absent in fish (zebrafish or fugu), amphibian, and reptile DNA sequences indicating that this transmitter peptide may have occurred quite late during vertebrate evolution. The residues 1-10 of human NPS are sufficient for mimicking the effect of the complete peptide (Roth et al. 2006). The peptide may contain several domains: The domain containing the residues Phe2, Arg3, and Asn4 crucial for biological activity is separated by a hinge region Val6-Gly7 from the sequence Thr8-Gly9-Met10 required for receptor activation (Roth et al. 2006).

3. G PROTEIN-COUPLED RECEPTORS IN ASTHMA

A variety of important effector molecules in asthma mediate their effects on target cells through GPCRs, which contribute to both the development and the maintenance of allergic processes. In airway smooth muscle, signaling through GPCRs mediates numerous functions including contraction, growth and functions that orchestrate airway inflammation and promote remodeling of the airway wall. Mediators of allergic inflammation that trigger cyclic adenosine monophosphate (cAMP) -associated GPCRs might play a role in peripheral tolerance

mechanisms against allergens. Current strategies for drug development and allergen-specific immunotherapy exploit these observations.

GPCRs are the most prominent targets for drug discovery and according to estimations, up to half of all modern drugs are targeted at GPCRs (Gudermann et al. 1995, Flower 1999). In addition, several ligands for GPCRs are found in the top-100 selling pharmaceutical products. 200-500 orphan GPCRs may still exist in the human genome providing many opportunities for drug discovery (Howard et al. 2001). Pharmacological agents that block or stimulate GPCR action are commonly utilized in the treatment of allergic diseases.

3.1 The GPCR superfamily: classification

The superfamily of GPCRs is evolutionary highly conserved and one of the largest groups of proteins in animals (Lander et al. 2001, Venter et al. 2001). The structural characteristics of GPCRs show a topology of seven transmembrane (TM) α-helices connected by three intracellular loops and three extracellular loops (Baldwin et al. 1993). The human genome contains more than 800 genes for GPCRs, which may represent up to 2-3 % of all protein coding genes (Fredriksson et al. 2003). GPCRs play important roles both in the major peripheral organ systems and in the central nervous system. The receptors can be classified by several ways based e.g. on their amino acid similarities (Vassilatis et al. 2003), ligand binding sites, receptor function and ligand structure (Bockaert and Pin 1999) as well as homology in the human genome (Fredriksson et al. 2003).

According to the phylogenetic analysis of GPCRs in the human genome, the receptors can be divided into five main families, Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2 and Secretin (Fredriksson et al. 2003). Rhodopsin, Secretin and Glutamate families correspond to A, B and C clans in the traditional A-F classification of GPCRs, respectively, where the subclans are defined using roman number nomenclature (Attwood and Findley 1994, Kolakowski 1994). The A-F classification covers all GPCRs in both vertebrates and invertebrates, and some families of the system do not exist in humans.

The Rhodopsin family is by far the largest of the five human GPCR families and most of the current drug targets among GPCRs are found within this family, which contains 15, 241, 24, 24 and 15 non-olfactory receptors, respectively (Attwood and Findley 1994). The rhodopsin family has several characteristics such as the Asn-Ser-x-x-Asn-Pro-x-x-Tyr motif in TMVII, the Asp-Arg-Tyr (DRY) motif or the Asp(Glu)-Arg-Tyr(Phe) sequence at the border between TMIII and the second intracellular loop. The ligands for most of the rhodopsin family receptors bind within a cavity between the TM regions (Baldwin et al. 1993) except for the glycoprotein binding receptors such as luteinizing hormone, follicle stimulating hormone and thyroid-stimulating hormone receptors, where the ligand-binding domain is in the amino terminus. In addition to hormones, rhodopsin family GPCRs can be activated by biogenic amines, peptides, nucleosides and nucleotides as well as lipids and eicosanoids. The large group of olfactory receptors belongs to this family but two thirds of the 900 genes in the subgroup represent pseudogenes in humans.

Most of the olfactory receptors are orphans with unknown ligands (Lane et al. 2002). The rhodopsin family can be further subdivided into four evolutionary collectively inherited receptor groups (Fredriksson et al. 2003). The receptors with bold font are presented in more detail in further chapters.

I) The α-group of rhodopsin family receptors consists of five main branches including the prostaglandin, amine, opsin, melatonin, and MECA receptor clusters (including receptors for melanocortin, endothelial differentiation, cannabinoids and adenosine). Especially, the prostaglandin and amine receptor clusters are of importance in the disease mechanisms of asthma. The former includes receptors for eicosanoids such as various **prostaglandins and thromboxane A2**, whereas the latter includes receptors for serotonin, dopamine, and histamine as well as muscarinic and **adrenergic** receptors. All the known ligands for the amine receptor cluster are structurally related small amine molecules with a single aromatic ring.

II) The β-group includes several neuropeptide receptors such as the neuropeptides FF and Y receptors, but also endothelin-related receptors, arginine-vasopressin receptors and oxytocin receptor. This group of receptors has no branches and it consists of receptors with peptides as ligands. GPRA belongs to this group of receptors.

III) The γ-group has three main branches: the SOG receptor cluster (including somatostatin and opioid receptors), melanin-concentrating hormone (MCH) receptor cluster, and the **chemokine** receptor cluster. In addition to the classical chemokines, known ligands of the last cluster include **complement** component 5a.

IV) The δ-group has four main branches: MAS1-related receptor cluster, glycoprotein receptor cluster, purin receptor cluster, and olfactory receptor cluster. The known ligands for the purin receptor cluster include extracellular nucleotides, leukotrienes and thrombin. The nucleotide-binding and related receptors have the most diffuse topology within this group. These receptors include the formyl peptide binding receptors, thrombin receptors and **cysteinyl leukotriene** receptors (CysLTRs).

3.2 G protein-coupled signal transduction

GPCRs primarily elicit responses to external stimuli by coupling to heterotrimeric G proteins (guanine nucleotide-binding proteins), which transduce signals to various intracellular effectors (Figure 1). G proteins are composed of a guanine nucleotide-binding α subunit and a high-affinity dimer consisting of β and γ subunits. In the inactive state, the G protein exists as a trimer of the guanosine diphosphate (GDP) -bound α-subunit and the βγ -complex associated together. Stimulation (e.g. ligand binding) induces a conformational change in the receptor, which activates the heterotrimeric G protein to exchange GDP to guanosine triphosphate (GTP). This in turn leads to the dissociation of the α-subunit from the βγ -complex, which modulate the

downstream effectors of the signaling cascades. The α-subunit possesses very low intrinsic GTPase activity, which hydrolyses GTP back to GDP and Pi terminating G protein activation. The inactive α-subunit is able to reassociate with the βγ dimer (Neves et al. 2002).

Gα subunits are classified into four subfamilies based on their amino acid sequences and function: the Gi, Gq, Gs and G12 subfamilies (Neves et al. 2002). The type of the Gα subunit determines the specific signaling pathways that are turned on upon receptor activation. Briefly, G_s proteins activate adenylyl cyclase to stimulate the production of cAMP; G_q proteins activate phospholipase Cβ to produce diacylglycerol and inositol 1,4,5-trisphosphate, which facilitates the controlled release of Ca^{2+} from intracellular stores (Smrcka et al. 1991, Taylor et al. 1991). G_i proteins inhibit adenylyl cyclase, regulate ion channels, and activate some isoforms of phospholipase Cβ via the Gβγ subunits. G_{12} proteins activate, for example, the Rho guanine nucleotide exchange factor p115 Rho GEF, which controls cell motility (Hart et al. 1998).

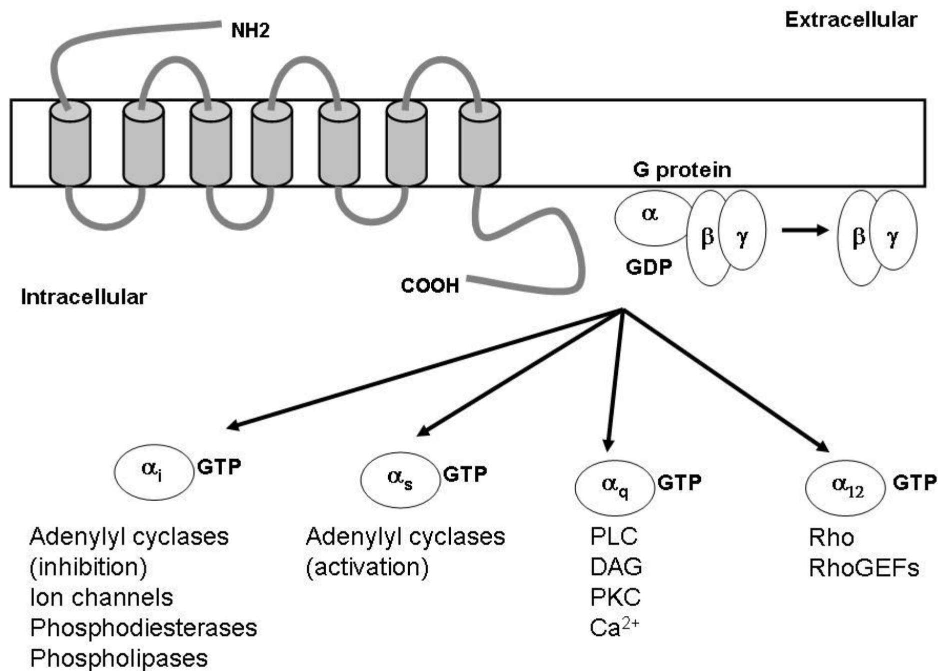

Figure 1. G protein-coupled signaling pathways. Abbreviations: cAMP, cyclic adenosine monophoshate; DAG, diacyl glycerol; GDP/GTP, guanosine di/triphosphate; PLC, phopholipase C; PKC, protein kinase C; Rho GEF, Rho guanine nucleotide exchange factor

In addition, the downstream effector molecules stimulated via G protein activation may include guanylyl cyclases, phosphodiesterases, phospholipase A2, and phosphoinositide 3-kinases, which activate or inhibit second messengers cyclic guanosine monophosphate, phosphatidyl inositol (3,4,5)-triphosphate, arachidonic acid and phosphatidic acid, respectively (Marinissen and Gutkind 2001, Kozasa 2001, Offermanns 2003).

3.3. GPCRs associated with asthma susceptibility

Genetic variations in several GPCRs associate with asthma susceptibility and some of them are reviewed below. Although β_2-adrenoreceptors (β-ARs) may not be causally involved in the pathophysiology of asthma, they are important therapeutic targets for symptoms of asthma. The positional cloning of *DPP10* (section 2.3.2) and the association of chemokines and their receptors with asthma emphasize the role of adaptive immunity and leukocyte activation in the disease mechanisms. On the other hand, the importance of crosstalk between innate and adaptive immunity is underlined with genetic studies demonstrating associations with the complement system and asthma. In addition, recent experimental and genetic studies have highlighted the role of effector molecules such as arachidonic acid metabolites (prostaglandins, thromboxanes and leukotrienes) and their GPCRs in the pathogenesis of allergic inflammation and asthma. The complex etiology of aspirin-induced asthma observed in up to 10 % of asthmatic patients points towards an abnormality of arachidonic acid metabolism (Figure 2) and overexpression of cysteinyl leukotrienes.

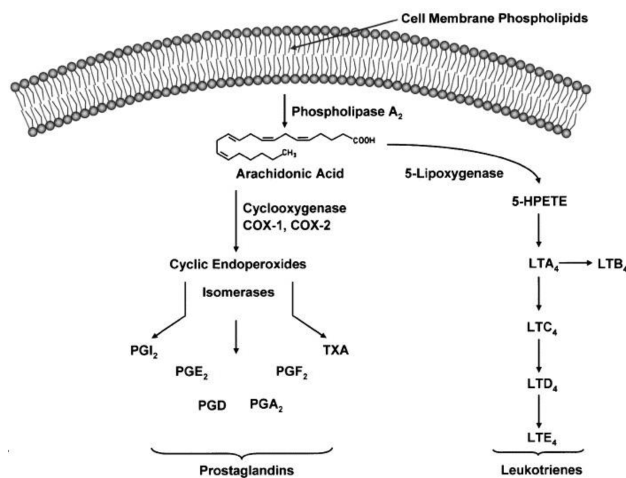

Figure 2. Prostaglandin H2 is the precursor of many prostaglandins and thromboxanes, which are formed by the phospholipase A pathway in a process involving free arachidonic acid release. The cyclooxygenase (COX) enzymes are needed for the further synthesis of prostaglandins and thromboxane A_2. Leukotrienes are synthesized by a distinct lipoxygenase pathway, which cannot be inhibited by aspirin. LTC_4 is the parent compound of the cysteinyl leukotrienes (LTs) including LTD_4 and LTE_4. Arachidonic acid metabolites participate both into early and late phases in allergic responses. Modified from Cabral et al. 2005.

3.3.1 Prostaglandin, leukotriene and thromboxane receptors

Prostaglandin (PG) D2 mediates its effects via two receptors, D prostanoid (DP) 1 and 2, which differ in their ligand binding affinities and expression patterns. DP1, although not expressed by T cells, mediates PGD2-directed T-cell trafficking through the induction of chemokine expression (Hirai et al. 2001). In mice lacking the prostanoid DP1 receptor (DP1-/- mice), the concentrations of the Th2 cytokines and the extent of lymphocyte accumulation in the lung of OVA-challenged DP1-/- mice were reduced when compared with the wild-type controls. Furthermore, DP1-/- mice showed only marginal infiltration of eosinophils into the lung and failed to develop BHR upon OVA challenge (Matsuoka et al. 2000). DP2 receptor was originally termed as chemoattractant receptor-homologous molecule expressed on Th2 (CRTH2) cells and it is a member of the chemoattractant receptor subgroup (Abe et al. 1999, Nagata et al. 1999).

Previous linkage studies have shown that the human genome region 14q22.1 associates with asthma. An intronic SNP in the *DP1* gene is preferentially transmitted to asthmatic children (Noguchi et al. 2002). In 2004, Oguma and co-authors identified four novel and two previously reported SNPs on 14q22.1 in *DP1* and its vicinity, which significantly associated with asthma in both white and black populations (Oguma et al. 2004). The polymorphisms in the promoter region of the gene were underrepresented in asthmatic patients and led to a reduced mRNA transcription and lower levels of receptor protein. In asthma, PGD2 production is activated in mast cells and monocytes/macrophages. PGD2-mediated events include vasodilatation and increased capillary permeability, leading to oedema formation, increased mucus production, bronchoconstriction and eosinophil, basophil and Th2 cell recruitment (Hirai et al. 2001). Thus, the reduced receptor levels in control population lead to an overall asthma-protective effect.

Similar associations have been detected with CysLTRs. The cysteinyl leukotrienes are peptide-conjugated bioactive lipids that are prominent products of immunologically activated eosinophils, basophils, mast cells and macrophages recognized as potent mediators of bronchoconstriction (Dahlén et al. 1980, Kanaoka and Boyce 2004). In addition, they are potent inflammatory mediators that initiate and propagate a diverse array of biologic responses such as increase microvascular permeability, stimulate mucus secretion, decrease mucociliary clearance and recruit eosinophils into airways (Holgate et al. 2003). These effects are mediated through activation of at least two receptors, $CysLTR_1$ and $CysLTR_2$ expressed on bronchial smooth muscle (Lynch 1999 and Heise 2000), pulmonary interstitial macrophages, mast cells (Mellor et al. 2001) and peripheral blood leukocytes (Figueroa et al. 2001). For CysLTR1, the order of ligand potency is ranked as $LTD_4 > LTC_4 > LTE_4$. The CysLTR1-specific antagonists, such as montelukast, zafirlukast and pranlukast, are currently used to control bronchoconstriction and inflammation in asthmatic patients. However, the individual responses are highly variable. In fact, approximately 30% of the asthmatics do not respond to the treatment (Bleecker et al. 2000, Asano et al. 2002), although montelukast significantly reduced the airway remodelling events in a mouse model of asthma (Jones et al. 1995, Henderson et al. 2002). For CysLTR2, the rank order of potency has been reported as $LTC_4 = LTD_4 > LTE_4$ (Heise et al. 2000, Nothacker et al. 2000, Takasaki et al. 2000). *CysLTR2* maps to 13q14 near a linkage region for atopic asthma. In a recent study, a coding polymorphism in the *CysLTR2* was associated with asthma in two family-based collections from Denmark and the United States. The polymorphism resulted in reduced receptor affinity to LTD_4 providing an asthma-protective effect (Pillai et al. 2004).

Thromboxane A_2 (TXA_2) actions are mediated through a G protein-coupled TBXA2R receptor (Coleman 1994). The two isoforms, named TPα and TPβ, share the first 328 amino acids but have differences in the length of their carboxy-terminal tails. *TBXA2R* is located on chromosome 19p13.3 and is composed of 343 amino acids. Significantly, a synonymous polymorphism in the *TBXA2R* gene has been found to associate with asthma related traits in Japanese, Chinese and Korean populations (Unoki et al. 2000, Leung et al. 2002, Shin et al. 2003, Hong et al. 2005, Kim et al. 2005). The polymorphism may regulate *TBXA2R* gene expression at

transcriptional or translational level, but the mechanisms are yet unclear. In addition to inducing platelet aggregation, TXA_2 has potent bronchoconstricting activity (Nagai et al. 1993, Francis et al. 1991). Therefore TXA_2 and its receptors play a role in AHR and are plausible candidate genes in the pathogenesis of asthma (Fujimura et al. 1991, Nagai et al. 1993).

3.3.2 Chemokine receptors

Chemokines are divided into four subclasses distinguished on the basis of the arrangement of the amino terminal cysteine residues (Zlotnik and Yoshie 2000): CXC chemokines (CXC ligands, CXCL), CC chemokines (CCL), C chemokines (XCL), and CXXXC chemokines (CX3CL). Chemokines mediate their effect via GPCRs. Up to now, at least 10 CC chemokine receptors (CCRs) and six CXC chemokine receptors (CXCRs) comply with the criteria of the chemokine nomenclature (Elsner et al. 2004) (Table 2).

Of the various chemokine receptors, especially CCR3, also known as the eotaxin receptor appears to play a major role in allergic diseases (Bisset and Schmid-Grendelmeier 2005). The receptor has several ligands as chemoattractants (Table 2) and polymorphisms in the *CCL11* (Eotaxin-1) and *CCL24* (Eotaxin-2) genes associate with asthma-related traits (Shin et al. 2003, Chang et al. 2005). CCR3 is expressed constitutively on the surface of eosinophils, where the receptor is the dominant chemokine receptor (Ponath et al. 1996). In CCR3-deficient mice, eosinophils are unable to extravasate through the vessel wall (Humbles et al. 2002). Also other cell types including Th2 cells express CCR3 constitutively or upon cytokine activation (Sallusto et al. 1997, Uguccioni et al. 1997, Ochi et al. 1999). In mast cells, CCR3 can be stored in secretory granules and is mobilized towards the cell surface following activation mediated by FcεRI for IgE (Price et al. 2003). The binding of CCL11 ligand to the newly exposed CCR3 is able to provide a second signal, leading to amplification of Fc-receptor for IgE-dependent IL-13 production.

Interestingly, CCL11 acts as an antagonist to CXCR3, the gene of which is located on chromosome Xq13 and associates with asthma and atopy especially in atopic male subjects (Cheong et al. 2005). CCL11 is also another ligand for CCR5. A 32-bp deletion in the *CCR5* gene, initially linked with low human immunodeficiency virus 1 prevalence (Samson 1996), associates with reduced prevalence of asthma in several studies (Hall et al. 1999, Mitchell et al. 2000, McGinnis et al. 2002), especially during childhood (Srivastava et al. 2003). The *CCR5* gene with the deletion is in close proximity on the 3p21 chromosome to *CCR1*, *CCR2*, and *CCR3*. Thus, the linkage disequilibrium does not exclude an association between these other genes and asthma. Indeed, a silent polymorphism in the *CCR3* gene showed significant association with asthma in Japanese but not in British population (Fukunaga et al. 2001). However, no replication studies exist so far.

Table 2. CC and CXC chemokine receptors: Cellular distribution and ligands. Modified from Elsner and coauthors 2004.

Receptor	Cellular distribution	Ligand
CCR1	Monocyte, macrophage, dendritic cell, T cell, B cell, eosinophil, basophil, neutrophil	CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3),CCL8 (MCP-2), CCL14 (HCC-1), CCL15 (HCC-2), CCL16 (HCC-4), CCL23 (MPIF-1)
CCR2	Monocyte, macrophage, immature dendritic cell, natural killer cell, basophil	CCL2 (MCP-1), CCL7 (MCP-3), CCL8 (MCP-2), CCL12, CCL13 (MCP-4), CCL16 (HCC-4) *Antagonistic:* CCL11 (Eotaxin), CCL26 (Eotaxin-3)
CCR3	Eosinophil, basophil, mast cell, T cell (Th_2), keratinocyte	CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL11 (Eotaxin), CCL13 (MCP-4), CCL14 (HCC-1), CCL15 (HCC-2), CCL24 (Eotaxin-2), CCL26 (Eotaxin-3), CCL28 (MEC) *Antagonistic:* CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC), CCL18 (MIP-4/Ckβ7/PARC)
CCR4	T cells (Th_2), basophil, immature dendritic cell	CCL17 (TARC), CCL22 (MDC)
CCR5	Monocyte, macrophage, dendritic cell, natural killer cell, T cell (Th_1), thymocyte	CCL3 (MIP-1α), CCL4 (MIP-1ß), CCL5 (RANTES), CCL8 (MCP-2), CCL11 (Eotaxin), CCL14, CCL16 (HCC-4), CCL26 (Eotaxin-3)
CCR6	Monocyte, macrophage, dendritic cell, T cell, B cell	CCL20 (MIP-3α)
CCR7	Dendritic cell, T cell, B cell	CCL19 (MIP-3β), CCL21 (SLC)
CCR8	Monocyte, T cell, B cell, neutrophil, thymocyte	CCL1 (I-309), CCL4 (MIP-1β), CCL17 (TARC)
CCR9	T cell, thymocyte	CCL25 (TECK)
CCR10	T cell, melanocyte, Langerhans cell	CCL27 (CTACK), CCL28 (MEC)
CXCR1	Neutrophil, T cell, monocyte, macrophage, dendritic cell	CXCL1 (GRO-α), CXCL6 (GCP-2), CXCL8 (IL-8)
CXCR2	Neutrophil, T cell, monocyte, macrophage, dendritic cell	CXCL1 (GRO-α), CXCL2 (GRO-β), CXCL3 (GRO-γ), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (NAP-2), CXCL8 (IL-8)
CXCR3	Neutrophil, T cell (Th_1), B cell, dendritic cell	CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC) *Antagonistic:* CCL11 (Eotaxin)
CXCR4	Neutrophil, T cell, B cell, monocyte, macrophage, dendritic cell, eosinophil, natural killer cell (CD16)	CXCL12 (SDF-1α, -β)
CXCR5	T cell, B cell, monocyte, macrophage	CXCL13 (BCA-1)
CXCR6	T cell, dendritic cell	CXCL16

MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T-cell expressed and secreted; MCP, monocyte chemotactic protein; HCC, hemofiltrate CC chemokine; MPIF, myeloid progenitor inhibitory factor; MEC, mucosae-associated epithelial chemokine; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine; SLC, secondary lymphoid-tissue chemokine; TECK, thymus-expressed chemokine; GCP-2, granulocyte chemotactic protein; NAP, neutrophil activating protein; ENA-78, epithelial neutrophil activating protein; GRO, growth-related oncogene; IP-10, interferon-γ-inducible protein; MIG, monokine induced by INF-γ; I-TAC, IFN-inducible T cell alpha chemoattractant; SDF, stromal cell-derived factor; BCA, B cell-attracting chemokine; PARC, pulmonary and activation-regulated chemokine; Ckβ7, chemokine β7; CCL14 is HCC-1.

The paradigm that Th1 cells preferentially express CXCR3 and CCR5 and Th2 cells preferentially express CCR3, CCR4, and CCR8 has been well established in the settings of polarized cell lines *in vitro*, although the situation appears more complicated *in vivo* (D'Ambrosio et al. 1998, Sallusto et al. 1998). After extensive studies, small molecular inhibitors for CCR2, CCR3, CCR4 and CCR8 are in the development for asthma. Because these chemokine receptors are expressed on inflammatory cells in the circulation that respond to chemokines released locally in the airways, the inhibitors are more likely to be useful as oral, rather than inhaled drugs (Barnes 2004).

3.3.3 Complement receptors

The complement system consists of approximately 20 circulating serum proteins. Activation of the system links the innate and adaptive immunity through generating two protein anaphylatoxins C3a and C5a, which mediate their effects on smooth muscle contraction, vascular permeability and leukocyte chemotaxis through specific C3a and C5a receptors (Gerard and Gerard 1991, Ames et al. 1996). In addition to C5aR, C5a binds with high affinity to another receptor C5L2, which is not G protein-coupled (Ohno et al. 2000). Activation products of C3a and C5a play a major role in phagocytosis and inflammation, but also in adaptive immunity. Foreign particles taken up by the complement receptors and processed in antigen-presenting cells are presented to T cells via major histocompatibility complex class II molecules. Thus, the complement receptors play a dual regulatory role in C3a and C5a signaling by protecting against Th2-mediated immune responses at the beginning of the immune responses and a proinflammatory role once the responses are established.

C3aR-deficient mice displayed enhanced susceptibility to LPS-challenge with significantly enhanced mortality (Kildsgaard et al. 2000) and near complete protection from the development of hyperresponsiveness to methacholine in the mouse model of asthma (Humbles et al. 2000). C5aR expression was originally described on myeloid cells (Chenoweth and Hugli 1978, Gerard and Gerard 1991), but it is also expressed in endothelial and epithelial cells of many organs, especially in the lung and liver (Haviland et al. 1995). A natural deletion in the coding sequence of the *C5* gene lead to C5-deficiency and increased susceptibility for allergen-induced AHR in a mouse model of asthma (Karp et al. 2000).

Interestingly, several genome-wide scans for asthma susceptibility loci have intervals that include *C5* (chromosome 9q32-q34) and *C5aR* (chromosome 19q13.3-q13.4) as well as *C3* (chromosome 19p13.2-p13.3) (CSGA 1997, Ober et al. 1998, Wjst et al. 1999, Blumenthal et al. 2004b). In 2004, Hasegawa and co-authors identified a high-risk haplotype of the *C3* gene for childhood and adult bronchial asthma and a low-risk haplotype for adult bronchial asthma in Japanese population (Hasegawa et al. 2004). They also found that severity of childhood bronchial asthma was associated with SNPs in the *C3aR1* gene and a *C5* haplotype was protective against childhood and adult asthma. Variants of the *C3* gene were also associated with asthma and related phenotypes in African Caribbean families (Barnes et al. 2006).

3.3.4 β2-adrenergic receptors

The human β-ARs are classified into β_1, β_2, and β_3 subgroups, which serve as receptors for the catecholamines epinephrine (adrenaline) and norepinephrine (noradrenaline). β_2-ARs are widely distributed throughout the human bronchial tree, and particularly in the airway smooth muscle (Barnes et al. 1982). In addition, inflammatory cells such as mast cells, monocytes, eosinophils, T -lymphocytes, and neutrophils carry β_2-ARs (Johnson et al. 2002). Intracellular signaling after β_2-AR activation is affected through Gs protein coupled adenylyl cyclase. cAMP induces airway relaxation via mediating phosphorylation of bronchial smooth muscle regulatory proteins, attenuation of cellular Ca^{2+} concentrations and inhibition of mediator release from mast cells. Short-acting, inhaled, selective β_2-agonists, which activate these cytosolic effects, were introduced over thirty years ago. To date, they still are among the most prescribed drugs worldwide and remain the mainstay bronchodilators for asthma. Combinations of long-acting β_2-agonists and corticosteroids appear to have additive and/or synergistic effects in inhibiting inflammatory mediator release and the migration and proliferation of airway SMCs.

The relationship between β-AR haplotype and acute and chronic pharmacodynamic (bronchodilator) response is complex and widely studied (Taylor et al. 2005). There are two common polymorphisms in the *β_2-AR* gene: Arg/Gly16 and Gln/Glu27. *In vitro* studies indicate that the *Gly16* allele enhances agonist-induced downregulation of the receptor, whereas the *Glu27* allele enhances resistance to downregulation (Green et al. 1994, Hall et al. 1995, Hall 1999, D'amato et al. 1998). It is plausible that these differences in receptor regulation influence the reactivity of airway smooth muscle in response to airway inflammation and thereby alter the risk of asthma. However, epidemiologic studies have yielded conflicting results. A recent systemic meta-analysis study found no evidence for the association of these *β_2-AR* polymorphisms in adults (Thakkinstian et al. 2005). Instead, a recessive protective effect of *Gly16* for children was suggested. According to the haplotype analysis, there may be an interaction between the two sites with a lower risk of asthma associated with the Glu27 allele (compared with Gln27). The risk may be modified by the allele at position 16 (Thakkinstian et al. 2005).

D. AIMS

Positional cloning of *GPRA* as an asthma susceptibility gene was the starting point of this study. The biochemical properties of GPRA make it an excellent drug target candidate as an early susceptibility factor in the pathogenesis of asthma and allergic diseases, but its role and functions need to be understood in detail for any such applications. The identification of AAA1 in the disease-associated region required its characterization in parallel with GPRA.

The specific aims of the study are

1) To clarify the role of AAA1 in the pathogenesis of asthma.
2) To characterize the expression of GPRA in normal human tissues and during inflammation in asthma.
3) To characterize the expression and function of GPRA utilizing a murine model of asthma.
4) To investigate whether GPRA associates with respiratory distress syndrome.

E. MATERIALS AND METHODS

1. Patients and samples

Asthma family collection (I)

The patient recruitment and verification of diagnoses of subjects from Kainuu (a total of 254 families and 1015 study subjects), North-Karelia and Quebec were carried out as described (Kauppi et al. 1998, Laitinen et al. 1997, Laitinen et al. 2001). Microsatellite genotyping was performed in 103 trios (a total of 853 study subjects) and in all of the 86 pedigrees included in the original genome scan (Laitinen et al. 2001). SNP genotyping was carried out in the subset of families (106 trios, 361 study subjects) informative in the haplotype pattern mining (HPM) analysis (Toivonen et al. 2000) for high serum IgE level. The relative risks for asthma and serum IgE levels were computed both for the Kainuu and French- Canadian data sets among the founders of the families (n=499 and n=402, respectively) to avoid weighing of any chromosome segregating in the families.

Total serum IgE level was determined by Diagnostics CAP FEIA (Kabi Pharmacia) in one batch for all the participants. Individuals from Kainuu and North-Karelia were divided into two groups based on their total serum IgE level: high IgE responders (IgE $>$ 100kU/L) and low IgE responders (IgE $\leq$ 100 kU/L).

Diagnosis of RDS (IV)

The study population for association analysis of *GPRA* and RDS consisted of 521 Finnish infants born during the years 1996–2001 in the Oulu University Hospital. The clinical diagnosis of RDS in the newborn infants was made by the presence of both clinical and X-ray definition. They include requirement of supplemental oxygen and continuous distending airway pressures for at least 48 hours after birth unless treated with exogenous surfactant in established respiratory failure, diffuse reticulogranular pattern with air bronchograms and ground glass appearance of lung fields in the chest X-rays. Infants receiving surfactant therapy prior to radiography were excluded. Neither pneumonia nor transient tachypnoea was diagnosed as RDS. BPD was defined as requirement of supplemental oxygen at postmenstrual age of 36 weeks for those born before 32 weeks of gestation (Jobe and Bancalari 2001).

Human sputum samples (III)

2 atopic and 6 non-atopic patients with asthma diagnosed according to American Thoracic Society guidelines (ATS 1995) and 14 non-asthmatic subjects (6 atopic and 8 non-atopic) were recruited for the study. Four subjects used inhalation steroid (beclomethasone) 800 µg per day and one subject 1600 µg per day. The study was approved by the Ethics committee, Department of Medicine, Helsinki University Hospital. Skin prick tests were made using 10 common inhalant allergens and positive (histamine dihydrochloride, 10 mg/ml) and negative (solvent) control

solutions. A subject was classified as atopic if any allergen induced a red spot of three millimeters or more in diameter and control solutions gave expected results (Dreborg 1993).

Sputum was induced by inhalation of nebulized 5 % hypertonic saline via an ultrasonic nebulizer (Rytilä et al. 2000). All subjects were pre-treated with 0.5 mg terbutaline (Bricanyl Turbuhaler®) and peak expiratory flow was measured before the pre-treatment and after induction.

Sputum processing was carried out as previously described (Rytilä et al. 2000). Cytospins with a minimum of 300 nonsquamous cells were prepared and stained using the May-Grünwald-Giemsa method or with the GPRA specific antibodies. All analyses were performed blind to the clinical characteristics or treatment. The results are expressed as a percentage of individual cells in relation to total nonsquamous cell count.

Peripheral blood mononuclear cells and eosinophils (III)

Peripheral blood cells were isolated from healthy volunteers or buffy coat donors by Ficoll density gradient centrifugations (Biochrom KG). From the granulocyte fraction, erythrocytes were first lysed and eosinophils were further separated by depletion of neutrophils using MACS CD16 Microbeads (Miltenyi Biotec). Eosinophils were cultured for 24 h with 25 ng/ml granulocyte-macrophage colony stimulating factor in complete RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium puryvate, 1% MEM non-essential amino acids and vitamins, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Life Technologies) and 10% fetal bovine serum (Sera-Lab). The purity of the eosinophil fraction was as high as 99 % as detected by microscopic evaluation of cytospin preparations. Peripheral blood mononuclear cells (PBMC) were cultured in complete RPMI 1640 over night and stimulated then for 6, 16, 28 or 48 h either with 10 ng/ml LPS or plate-bound anti-CD3 (1 µg/ml, clone OKT3, ATCC) and anti-CD28 (2 µg/ml, PeliCluster clone 15E8, Sanguin). CD4+ T cells were isolated from PBMCs using Dynal CD4 Positive Isolation Kit (Dynal) and rested o/n in serum free AIM-V medium (Life Technologies, Basel, Switzerland). Monocytes were isolated by incubating freshly separated PBMC's in complete RPMI 1640 medium in cell culture bottle for 10-20 minutes. Unattached cells were washed out and the adherent monocytes were trypsinized, washed and rested o/n in complete RPMI 1640.

Experimental mouse models (I, III)

BALB/c female mice, age 6–8 weeks, free of specific pathogens, were obtained from M&B, Ry. The mice were housed under pathogen-free conditions and maintained on OVA-free diet. All experiments conducted were approved by the Animal Experimental Committee of the State Provincial Office of Southern Finland.

Two models were applied. In the first model with OVA-sensitization and challenge, mice were sensitized intraperitoneally on days 1 and 10 with 20 µg grade V OVA (Sigma-Aldrich) adsorbed to 2 mg of alum adjuvant (Pierce Biotechnology Inc.) diluted in saline (III). In the

second model used for GPRA mRNA expression studies, mice were sensitized and challenged with OVA as above in combination with 7 intranasal applications of *Stachybotrys chartarum* mold (NRRL 6084) over a period of 22 days (I). In both models, unsensitized and naïve control mice received 200 µg of saline-diluted alum intraperitoneally. Challenge of the unsensitized control and OVA-sensitized mice consisted of a 40-minute exposure to 1% (wt/vol) OVA in saline on days 20, 21, and 22. All mice were exposed to aerosol in an exposure chamber connected to the outlet of a six-jet atomizer that delivered an aerosol of particles with a mean diameter of 0.3 µm (TSI Inc.).

The mice were killed by CO_2 asphyxiation at day 23. The blood was drained from the vena cava. The lungs were lavaged with phosphate-buffered saline (PBS) via the tracheal tube (1 x 800 µl for 10 s). The bronchoalveolar lavage (BAL) sample was cytocentrifuged (Cytospin, Shandon Ltd) onto a slide and the cells were stained with the MayGrünwald–Giemsa (MGG) stain. The cell population on the cytospin slide was counted under light microscope. The left lung was removed for mRNA isolation, quick-frozen and kept at −70°C. For histologic examination, the right lung was perfused with 10% formalin and then excised *en bloc*. The lungs were embedded in paraffin, cut into 5-mm-thick sections, affixed to microscope slides and deparaffinized. The slides were stained with haematoxylin and eosin and examined under light microscope.

Cell cultures (II, III)

COS-1 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (GibcoBRL/Invitrogen) supplemented with 10% fetal bovine serum (Perbio), 1% Penicillin/Streptomycin (GibcoBRL) and 1x non-essential amino acids (GibcoBRL). Human lung epithelial carcinoma cell line NCI-H358 (ATCC) and was cultured in RPMI 1640 medium (GibcoBRL) supplemented with 1 mM sodium puryvate (GibcoBRL), 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), and 1% Penicillin/Streptomycin. BEAS-2B cell line originating from normal human bronchial epithelium was cultured in Basal Medium (Cambrex) supplemented with Bullet Kit (Cambrex). Myoblast cells were isolated from normal human skeletal muscle (kindly provided by Dr. P. Salmikangas) and cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 15% fetal bovine serum (Perbio), 4% Ultroseer G (BioSepra,) and 1% Penicillin/Streptomycin. Murine macrophage cell line, RAW 264.7 (ATCC) was cultured in RPMI 1640-medium supplemented with 10% of heat inactivated fetal bovine serum, 1% of L-glutamine and 1% of Penicillin–Streptomycin antibiotic mixture (all from Gibco/Invitrogen). All cell lines were cultured at 37 ºC in a CO_2 conditioned, humidified incubator.

In order to obtain GPRA-A stable cells, GPRA-A cDNA was cloned into a pQM vector under CMV promoters (produced by Quattromed AS). 293H cells were transfected with Lipofectamine 2000 (Gibco BRL) and clones were cultured under puromycin selection. GPRA-A positive clones were characterized by RT-PCR or real-time PCR, and Western blotting.

Minimum of three different GPRA-A positive and negative clones and parental 293H cell line were used for different studies in which the clones were cultured in 293 SFM II medium (Gibco BRL) with or without 0.8 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO).

2. Association analyses

Genotyping, SNP discovery and sequencing (I, IV)

In order to create a dense map of polymorphic markers spread evenly across the 7p14-p15 linkage region, the publicly available genomic sequences for potentially polymorphic tandem repeats were screened (Polvi et al. 2002). All microsatellite markers and deletion/insertion polymorphisms found in the critical region were genotyped using fluorescently labeled primers in gel electrophoresis on an ABI377 sequencer. SNP genotyping was carried out using two different methods: single base pair extension with the chemistry of Molecular Dynamics on a MegaBASE 1000 sequencer (Molecular Dynamics) or PCR (using 20 ng of genomic DNA as starting material) followed by restriction enzyme digestion. Altogether 8 individuals were resequenced for nonrepeated DNA segments by direct sequencing of PCR products. Purified PCR fragments (Quickstep 2 PCR purification Kit, Edge BioSystems) were sequenced from both directions using ABI Prim3100 (Applied Biosystems) sequencer and dye-terminator chemistry. We assembled forward and reverse sequence reads using the Gap4 program (Staden Package software). All the markers were in Hardy-Weinberg equilibrium and observed Mendel errors were less than 0.1%.

For the association study of *GPRA* with RDS (IV), DNA was extracted from a 3-mm disk punched from a blood spot dried on a filter paper. Whole genome amplification was carried out with the extracted DNA using the improved primer preamplification (I-PEP-L) method and genotyped using the Sequenom platform (Hannelius et al. 2005, Sun et al. 2005). Seven haplotype tagging intronic SNPs in the *GPRA* gene (Melen et al. 2005, Table 5) were genotyped using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) technology. Primers were designed with the SpectroDesigner software (Sequenom GmbH, USA). All samples were analyzed in a sex-specific assay (AMELXY, MWG-Biotech AG) to validate the sample logistics. Three positive and three negative controls were included in each 96 well DNA plate, and all genotyping results were confirmed independently by two persons.

Statistical and computational analyses (I, IV)

In the asthma studies (I), haplotypes were analyzed with the HPM software (Toivonen 2000), whereas the SNPHAP program v.1.2.1 (http://www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt) was utilized in the RDS studies (IV).
HPM is a data mining-based algorithm that searches for shared patterns among haplotypes, scores each marker location for the number of shared patterns, and evaluates empirically the significance of the scores by a permutation test. Its utility for locating genes within haplotype data has been shown (Sevon et al. 2001). Linkage disequilibrium between SNP markers was

calculated and the graphical output (Laitinen et al. 2001) was produced by the Haploblocks software (M. Zucchelli and J. Kere, unpublished).

SNPHAP uses an expectation maximization (EM) algorithm to calculate maximum likelihood estimates of haplotype frequencies from population-based unphased genotype data. SNPHAP lists the individual predicted chromosomewise haplotype assignments and their probabilities. The single-marker data and pairwise linkage disequilibrium (D') values were calculated by Haploview v. 3.2 available at http://www.broad.mit.edu/mpg/haploview/ (Barrett 2005). Haplotype comparisons were performed using a χ^2 test in a 2x2 contingency table (Arcus Quickstat Biomedical v. 1.0).

3. Gene discovery

Exon prediction (I, II)

Exon predictions were performed by the GENSCAN (http://genes.mit.edu/GENSCAN.html) software using genomic clones AC005493.1 and AC005826.1 (690 kb) (Polvi et al. 2002) as one block. Predictions were performed for both unmasked and masked sequence. In the latter, the repetitive sequences were masked using the Repeat Masker Service (http://repeatmasker.genome.washington.edu/).

RACE-PCR (I, II)

To generate 3' and 5' cDNA ends, rapid amplification of cDNA ends (SMART RACE) was performed using human testis cDNA and Human Marathon-Ready Fetal Thymus cDNA (Clontech) according to the manufacturer's protocol for the SMART RACE cDNA Amplification Kit (Clontech). RACE-PCR products were cloned using pGEM-T Easy Vector system (Promega) or TOPO TA Cloning kit (Invitrogen) and plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen). The purified RT-PCR products and the cloned RACE-PCR products were verified by automated sequencing with dye-terminator chemistry (ABI Prism3100, Applied Biosystems, Inc).

Cloning (I)

Nested PCR amplification using two set of primers was used in cloning of the full length cDNAs for *GPRA* splice variants A and B. Primary PCR amplifications were performed in 25 µl volumes using 2.5 µl Human Brain Marathon-Ready cDNA (Clontech) as template, 1x DyNAzyme EXT buffer (containing 1.5 mM $MgCl_2$), 0.2 mM deoxy-nucleotide triphosphates (Finnzymes), 0.52 µM primers, 5% dimethyl sulfoxide and 0.5 U DyNAzyme EXT (Finnzymes) under the following conditions: 94°C for 4 min, 38 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min followed by final extension of 72°C for 10 min. The aliquot of primary PCR product was re-amplified by 30 PCR cycles under the same conditions as above.

PCR products were cloned into pCR 2.1 TOPO -vector using TOPO TA cloning kit (Invitrogen) according to manufacturer's instructions and plasmid DNA were purified using QIAprep Spin Miniprep Kit (Qiagen). The cloned RT-PCR products were verified by automated sequencing with dye-terminator chemistry (MegaBACE 1000, Amersham Biosciences).

In vitro translation (I)

In order to investigate whether AAA1 is translated to any polypeptide, in vitro translation experiments were performed. Capped RNA of AAA1 gene variants I and III were transcribed from DNA constructs with T7 RNA polymerases (mMESSAGE mMASCHINE system, Ambion, USA). Translation was performed with rabbit reticulocyte lysate translation machinery (Riboprobe *in vitro* translation system, Promega) in the presence of S^{35} -labeled methionine (PerkinElmer) in the reaction mixture. The Xenopus elongation factor α (pTRI-Xef) DNA template was used as a positive control for transcription and translation. In negative control, water was used instead of DNA. The translated polypeptides were detected by autoradiography after Tris-Tricine SDS-PAGE.

Statistical and computational analyses (I)

The transmembrane topology and putative N-glycosylation sites of *GPRA* protein were predicted using the PredictProtein (http://cubic.bioc.columbia.edu/predictprotein/submit_def.html#top) and the TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) softwares. In protein homology comparisons, the BlastP (http://www.ncbi.nlm.nih.gov/blast/) and T coffee (http://www.ch.embnet.org/software/TCoffee.html) softwares were used.

4. mRNA expression studies

Northern blot hybridization (I)

Human multiple tissue 8-lane Northern blot (Clontech) were hybridized using α[^{32}P]-dCTP random-labeled 1285-bp cDNA probe (comprising the full-length GPRA). The probes were generated by PCR amplification of human testis cDNA (Clontech; MTN 8-lane blot) and human brain cDNA (Invitrogen; MTN 12-lane blot), respectively. Prehybridizations were performed in ExpressHyb solution (Clontech) for 3 hours at 65°C. Herring sperm DNA (100 µg/ml) was used as the blocking reagent. The membranes were washed with 2 x SSC and 0.05 % SDS at room temperature and then exposed to X-ray film at –20 °C for 3 d (GPRA) and for 1 h (β-actin control).

Sample preparation, RNA extraction and cDNA synthesis (III, IV)

The subjects for mRNA analysis of the nasal scratch samples were treated in the Hospital for Children and Adolescents, Helsinki University Central Hospital, during the years 2004-2005 (IV). GPRA mRNA expression was studied in the nasal respiratory epithelium of 6 term (gestation age 39.3±1.2 weeks, mean ± SD; birth weight 3480±200 g; male female ratio 3/3) and

7 preterm infants (gestation age 26.4 ± 1.5; 980 ± 180; 2/5), and 10 adults (41.4 ± 8 years; 10/0) with no history of asthma or atopy. In the preterm infants the scratch test was performed twice, at the age of 1 - 4 h and 21 - 48 h as described (Helve et al. 2004). In the first sampling, the postnatal age of the subjects varied between 97 ± 22 min in the term and 62-175 min in the preterm subjects. The nasal samples were gathered under direct vision by scraping the nasal epithelium using a Rhino-Probe (Arlington Scientific), dispersed into lysis buffer and stored at –80°C as described (Helve et al. 2004). Subsequently, total RNA was extracted with RNeasy kit (Qiagen) and quantified using RiboGreen RNA Quantitation Kit according to the manufacturer´s instructions (Molecular Probes).

For analysis of human blood cell preparations (III), total RNA from 0.1 - $5x10^6$ cells was lysed followed by storing and RNA extraction as described above. For analysis of murine lung samples, total RNA from lungs was extracted using Trizol Reagent (GIBCO BRL) according to the manufacturer's protocol. The amount of RNA was quantified by spectrophotometry (GeneQuant II/Pharmacia Biotech).

cDNA synthesis was performed with MultiScribe reverse transcriptase according to the manufacturer's instructions (Applied Biosystems) using either random hexamers or oligo dT primers for 60 min at 48 °C, after which the enzyme was inactivated at 95 °C for 5 min.

Real-time quantitative RT-PCR assay (I, III, IV)

The quantitative real-time RT-PCR was performed by AbiPrism 7500 or 7700 Sequence Detection System Sequence Detector System (Applied Biosystems) according to the manufacturer’s instructions. The sequences of the human and mouse GPRA primers and probes were designed by PrimerExpress software (ABI). Quantitative real-time PCR of human blood cell samples (III) was performed applying SybrGreen chemistry (Applied Biosystems), whereas the Taqman assay was utilized in measuring GPRA mRNA expression in nasal scratch samples (IV) and in murine whole lung (I) and RAW cell line 264.7 samples (III). The sequences for the primers and the probes are given in Table 3.

One µl of the cDNA sample was amplified for GPRA detection in a final volume of 25 µl containing 12.5 µl of TaqMan Universal PCR Master Mix and AmplitaqGold polymerase (Applied Biosystems), 300 nM forward and reverse primers and 200 nM probe. After an initial incubation at 95 °C for 10 min to activate the DNA polymerase, 40 cycles were performed for 15 s at 95 °C and for 1 min at 60 °C. Cytokeratin 18 (CK18) was used as an epithelial marker (GPRA:CK18, ng/fmol) in nasal scratch samples (IV). In murine whole lung (I) and RAW cell line 264.7 samples (III), PCR primers and probes for the housekeeping genes β-actin or 18S rRNA were obtained as predeveloped assay reagents and were ordered from Applied Biosystems. In human blood cell samples (III), elongation factor (EF) 1α was amplified as an endogenous reference gene. The relative gene expression differences were calculated with the comparative ΔΔCT method.

Table 3. Primers and probes for real-time RT-PCR. Abbreviations: EF-1, elongation factor 1

Name	Forward	Reverse
GPRA-A	CCTGCAGGGAGCAAAGATCA	AATCTGCATCTCATGCCTCTCA
GPRA-B	CCTCAACGAGAGAACTGGAAG	AGAGCTGTCACCTTGGAAGAG
NPS	CAAAATGATTAGCTCAGTAAAACTCAATC	GGAACTGGATAACACCAAAACACA
EF-1	CTGAACCATCCAGGCCAAAT	GCCGTGTGGCAATCCAAT
GPRA	CAAAGGCAAAAATCAAGGCTATC	AGGTTGAAATTGTCCAAAATGTCA
probe	6-FAM-5' CATCATCATTCTTGCCTTCATCTGCTGTTG-3'-TAMRA.	
Gpra	GGCTCATCTCTAAGGCAAAAATCA	ACGCTCCTTGGTGTCTGGAA
probe	6-FAM-5' CGTCATAATCCTTGCTTTCATCTGCTGCTG-3'-TAMRA	

In situ hybridization (II)

Antisense and sense probes from DNA templates of *AAA1* (Image clones V3: 482785 in PBluescriptR, 3387 bp, V1 232825 in pT7T30-Pac1, 3482 bp) as well as *GPRA* and *NPS* (full-length GPRA-A and NPS cDNA in pCMV-Script vector) were transcripted by T3 or T7 RNA polymerases in the presence of digoxigenin-11-uridine-5´-triphosphate (Dig-11-UTP, Roche) by MAXIscript *in vitro* transcription kit (Ambion) according to the manufacturer´s instructions. The NPS cDNA sequences were amplified by PCR from a human pancreas cDNA sample (Human Multiple Tissue cDNA Panel, BD Biosciences)

Non-radioactive *in situ* hybridization on tissue sections was performed with Ventana Discovery^{TM} device. Briefly, the samples were frozen sections or deparaffinized with heat treatment followed by post-fixation and RiboClear pre-treatment. Samples were protease treated for 18 min and hybridized for 6 h at 65 °C with both antisense and sense probes. Slides were then washed three times with 0.1X SSC (15 mM NaCl, 150 nM Sodium citrate, pH 7.0) at 75 °C followed by the detection step, which includes 20 min incubation with biotinylated anti-DIG antibody (Jackson ImmunoResearch Laboratories) and 2 h incubation with the BCIP/NBT substrate. After color reaction the slides were washed, dehydrated and mounted with Mountex (HistoLab). All reagents for Discovery^{TM} are provided by Ventana Medical Systems except for protease K (Roche), which was used at a concentration of 350 ng/µl.

Characterization of the alternatively spliced GPRA transcripts (I, II)

Poly A^+ RNAs from human lung epithelial carcinoma cell line NCI-H358 (ATCC) were isolated by Dynabeads mRNA DIRECT Kit (Dynal) according to the manufacturer's instructions and subsequently reverse transcribed to cDNA by using SMART RACE cDNA amplification Kit (BD Biosciences). PCR was performed in 20 µl volume using 2.0 µl NCI-H358 cDNA as template,

1X PCR Gold buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Finnzymes, Espoo, Finland); 1 μM of 3'-tgagcaattgataactctgtgggtcctc-5' and 3'-gaatggtggggaaggaaggcgttt-5' or 3'-tgagcaattgataactctgtgggtcctc-5' and 3'-ggccatcctgctgtgacccatttt-5'; and 0.5 U AmpliTaqGold (Applied Biosystems, Foster City, CA) under the following conditions: 94°C for 10 min; 40 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 1 min followed by final extension of 72°C for 10 min. PCR products were cloned into the pCR 2.1 TOPO -vector using TOPO TA cloning kit (Invitrogen) according to manufacturer's instructions and plasmid DNAs were purified using QIAprep Spin Miniprep Kit (Qiagen). The cloned RT-PCR products were verified by automated sequencing with dye-terminator chemistry (MegaBACE 1000, Amersham Biosciences).

5. Protein expression studies

Antibodies and peptides (I- IV)

Four specific antibodies against the amino terminus, the third cytoloop and the two alternative carboxy termini of GPRA were raised by immunizing rabbits with the following peptides: TEGSFDSSGTGQTLDSSPVAKKG (a KKG core containing multiple antigen peptide corresponding to the nucleotides 16-76 of the N-terminus of GPRA), SSYNRGLISK (corresponding to the residues 258-267 of the third cytoloop), CREQRSQDSRMTFRERTER (corresponding to the resudies 341-359 of the variant A) and CPQRENWKGTWPGVPSWALPR (corresponding to the nucleotides 357-377 of the variant B). A TEGSFDSSGTGQTLDSSPVAC-peptide for purification of the amino terminal antibodies as well as GPRA-N multiple antigen peptide and GPRA-B peptide synthesis were purchased from the University of Helsinki and antibody production from the University of Oulu. GPRA-A and cytoloop 3 peptide syntheses and antibody production were purchased from Sigma-Genosys Ltd. GPRA-B peptide was conjugated to KLH carrier with Imject Maleidine Activated mcKLH kit (Pierce) and purified by gel filtration, whereas the conjugation of GPRA-A and GPRA-CL3 peptides to KLH was carried out in Sigma-Genosys Ltd. To further investigate the possible translation of the *AAA1* gene, a polyclonal antibody against the constant region (YVRRNAGRQFSHC) of the gene was produced in rabbits as above (Sigma-Genosys Ltd). A total of 6 immunizations were performed at 2 weeks intervals using a total of 2 mg of KLH-conjugated peptide as immunogen.

The sera were analyzed by enzyme-linked immunosorbent assay using the uncoupled peptides immobilized onto the nitrocellulose filters as antigens. Positive antisera were affinity-purified using Sulfolink (GPRA-A, GPRA-B and GPRA-N) or Ultralink kits (GPRA-CL3) (Pierce) and analyzed by immunoblotting of recombinant fragments expressed with the pGEX 4T-3 GST fusion expression vector (Amersham Biosciences) as glutathione-S-transferase (GST) fusion protein in *Esterichia coli* except for the C-terminal tail of the A isoform which was expressed as a dihydrofolate reductase (DHFR) fusion protein (Qiagen). Blocking experiments using 10 molar excess of free peptide as a competitor were also performed to demonstrate antibody specificity.

The goat polyclonal glyseraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz) and the horseradish peroxidase -conjugated secondary antibodies from Jackson ImmunoResearch Laboratories Inc. Monoclonal anti-myc and anti-HA antibodies were purchased from Berkeley. The non-immune rabbit IgG fraction was obtained from DAKO. NPS polypeptide (SFRNGVGTGMKKTSFQRAKS) was synthesized by MedProbe.

Transient transfections (II)

COS-1 cells were transiently transfected using Fugene6 transfection reagent (Roche) according to the manufacturer's protocol. The empty pCMV vector, beta-gal-pCMV, and myc-tagged luciferace-pCMV vectors (Stratagene) were used as controls. In transfections, 2.5 µg of DNA and 12 µl of Fugene6 (GPRA-C, -D, -E, -F, and -B_{short} vectors) or 2.0 µg of DNA and 8 µl Fugene6 (GPRA-A and -B vectors) were used for 5 x 10^6 cells in 35 mm cell culture dishes.

In order to study the effects of AAA1 open reading frames or the various *GPRA* splice variants on the translocation of the full-length GPRA-A or -B receptors to the plasma membrane, COS-1 cells were cotransfected with myc-tagged HA-tagged AAA1 or GPRA-A or -B with 0.3, 1, 3, and 10-fold amount of pCMV-GPRA-A, -B, -Bshort, -C, -D, -E or -F. The empty pCMV-vector and β-gal vector were used as controls. In all transfections, 2 µg of DNA and 8 µl of Fugene6 per 5 x 10^6 cells were used. The cells from one well of 6-well plate were divided into 16-wells of 96-well plate 24 h after transfection. The cells were analyzed with cell-based Elisa assay or immunofluorescence staining 24 h or 48 h after transfection. In addition, the *AAA1*-transfected cells were analyzed with immunoblotting and RT-PCR.

Cell-based Elisa assay (II)

Transfected cells were fixed with 3.5% paraformaldehyde in PBS for 15-20 min at room temperature. Cells were blocked with TBS (25mM Tris-150 mM NaCl, pH 8.0) containing 2% powered milk and 1% goat normal serum at 37 ºC for 30 min. Cells were then incubated with 1:1000 dilution of anti-myc- antibodies for 1 h at 37 ºC, washed three times with TBS and thereafter incubated with a dilution of 1:2000 of horseradish peroxidase-conjugaded anti-mouse IgG antibodies for 30 min at room temperature. TMB-substrate (Sigma Genosys) was added to cells for 3 to 6 min. The reaction was stopped by adding an equal amount of 1.5 M HCl and absorbance was measured at 450 nm. Half of the cells were permeabilized in order to detect the total expression level of the corresponding construct by adding 0.5% Triton-X-100 in PBS for 10-15 min after fixation. The results were normalized using the absorbance values obtained from pCMV and beta-gal control experiments.

Immunofluorescence stainings (II)

Transfected cells grown on coverslips were fixed in 3.5% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, blocked with PBST (PBS/ 0.01%/ Tween 20) containing 0.5% bovine serum albumin at room temperature for 30 min, and then incubated in PBST/ 0.1% bovine serum albumin with anti-GPRA-A, anti-GPRA-B, or anti-HA antibodies for 1 h at room temperature and then washed three times with PBST. Thereafter, the cells were incubated in PBST with (10 µg/ml) Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 488 goat anti-mouse IgG antibodies (Molecular Probes) for 30 min at room temperature and washed three times with PBST. Samples were visualized by fluorescence microscopy.

Western blot analysis (II, III)

For western blot analysis of human tissues (II), lysates from spleen, skeletal muscle, uterine muscle, colon muscle, kidney, colon epithelium, testes, and prostate were obtained by mechanically homogenizing the frozen tissue samples in 10 mM Tris HCl, 100mM NaCl, 2 % Triton X-100 buffer with with Complete Mini -protease inhibitors (Roche). Human cell lysates from various cell lines were obtained by homogenizing the cell samples in RIPA-buffer (1xPBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with Mini Complete protease inhibitors (Roche). For detection of PBMC samples (III), $1x10^6$ cells were lysed directly into sample buffer. For crude membrane preparations (II), transfected COS-1 cells were harvested in TE buffer (10 mM Tris, 0.1 mM ethylenediaminetetraacetic acid, pH 7.5). The membrane fractions were separated by suspending cell pellets in TE buffer with 0.32 mM sucrose, homogenizing mechanically, and centrifuging for 15 min at 380 g at 4 ºC. Supernatant was further centrifuged for 30 min at 40600 g at 4ºC. The pellet was suspended into sucrose-free TE buffer and centrifuged as above. The amount of protein was measured with the BCA Protein Assay Reagent kit (Pierce).

Fifty µg of the protein lysates or $1x10^6$ cells were run on reducing 12.5 % SDS-PAGE gels and electroblotted to the PVDF membrane according to standard procedures. Nonspecific protein binding was prevented by incubating the membrane with 5 % milk in 0.1 % Tween 20 in TBS (TBST). Antigenic sites were revealed by incubating the membrane with the antibodies or preimmune serum followed by the horseradish peroxidase -conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc.) in 5 % milk in 0.1 % TBST. The bands were revealed by the ECL detection kit (Amersham Biosciences). Negative controls did not show specific reactivity.

In human PBMC samples (III), the relative GPRA-A and -B expression was quantified after equalizing with the relative expression of GAPDH used as an endogenous reference gene. Syngene GeneTools software (Synoptics Ltd.) was used for detecting differences between intensities of the corresponding lanes. The unstimulated cells received a relative band intensity value of 1.0.

Immunohistochemistry (I-IV)

Formalin-fixed, paraffin-embedded specimens of normal adult human bronchus, skin and colon as well as human normal tissue array slides (MaxArray, Zymed Laboratories Inc.) containing 30 different tissues were used for immunohistochemistry. Bronchial biopsies from 10 control subjects and 8 asthmatic patients, who used either sodium cromoglycate or short-acting β-AR -agonist, were also studied. The bronchoscopic examination and biopsy-taking (Laitinen A. et al. 1997) have been approved by the appropriate Ethical Review Board. All the subjects for RDS and BPD studies were treated in the Hospital for Children and Adolescents, Helsinki University Central Hospital, during the years 1992-2000. The tissue samples were obtained from autopsies performed within 2 days of death. Fetuses were aborted in mid-pregnancy because of severe extrapulmonary anomalies. The term control group with macroscopically and microscopically normal lungs consisted of newborns who died of causes other than lung diseases at the age of <24 hours. The RDS group consisted of patients who died at the age of 3-16 days. The infants with severe BPD died at the age of 2.5-10 months. All the study protocols have been approved by the ethics committees of the participating centers, and a signed consent was obtained from the parents of the subjects.

For pre-treatment, slides were deparaffinized by xylene-treatment followed by decreasing alcohol series. The slides were heated in microwave oven in 10 mM citrate buffer, pH 6.0 for 5 min and stained using the enzymatic ABC (Avidin and Biotinylated horse-radish peroxidase macromolecular Complex) method (Vectastain Elite ABC kit, Vector Laboratories). The color reaction was revealed by 3-amino-9-ethylcarbazole (Vector Laboratories). Omission of primary antibody and staining with the nonimmunized sera was used as negative control for parallel sections. Neither of these controls showed any immunoreactivity.

6. Functional studies

GPRA-A overexpressing cell line (II, III)

GPRA activation in parental 293H cells, in three GPRA- positive and two negative clones was determined using the non-radioactive GTP-Eu binding assay (Perkin Elmer) according to manufacturer´s instructions in the absence or presence of 1 µM NPS. For the cell growth assay (II), GPRA-A positive cells were seeded at a concentration of 0.3 x 10^6 cells/ml and cultured in 293 SFM II medium (GibcoBRL) containing 0.8 µg/ml puromycin. At days 2 and 4, cell numbers were counted. For a quantitative cell number assay, GPRA-A positive and negative clones were split into 96-well plates into a concentration of 2 x 10^4 cells per well. 1 µM NPS was added and the relative amount of viable cells was determined after 3 days by using CellTiter 96 One solution Cell Proliferation Assay (Promega, Southampton, UK).

To study NPS-mediated signaling events (III), cell clones were divided into serum-free SFM II medium at a density of 2.5×10^5 cells/ml. The cells were loaded with 4 µM Fluo-3, AM (Molecular Probes) for 15 min at 37 °C followed by a 20 min incubation at room temperature.

After two washes, 50000 cells were seeded into 96-well plates and Ca^{2+} -dependent fluorescence traces (1 data-point/s, exitation at 488 nm) were measured by Fluostar Optima (BMG Labtechnologies) microplate reader. NPS (1 µM) was added prior to measurement at 535 nm.

For cAMP assay (III), the GPRA overexpressing cell clones and 293H parental cells were divided into cell culture medium at a density of 5×10^4 cells/well and incubated for 3 h at 37 °C. Thereafter, increasing concentrations (0.1-10 µM) of NPS were added to the cells for 20 min. The cells were lysed with cAMP detection lysis reagent for 10 min and stored at –80 ºC. Before storing, viability of the cells was confirmed with Trypan blue exclusion method. Total cellular cAMP was detected with cAMP Biotrak Enzyme immunoassay System (Amersham Biosciences) according to the manufacturer`s instructions. The reactions were stopped with 1 M H_2SO_4 and the absorbance values were measured at 450 nm. Duplicate wells were studied and half of the total end volume of 200 µl was used for measurements.

RAW 264.7 mouse macrophage cell line (III)

For the cell attachment assay, microtiter wells (Nunc) were coated overnight with 20 ng/ml of fibronectin, 5 ng/ml of collagen or 100 ng/ml of poly-L-lysine at +37°C. The wells were blocked for 60 min with 0.5% bovine serum albumin in PBS. RAW 264.7 cells were harvested by scraping, washed and resuspensed into RPMI medium at a density of 3.5×10^5 cells/ml. The cells were preincubated in the presence or absence of 1µM NPS for 30 min on ice. Subsequently, 3.5×10^4 cells were seeded onto the wells, and the plates were incubated for 50 min at 37°C. After three washes with PBS, the cells were counted using CyQuant reagent (Molecular Probes) and Victor2 plate reader (Perkin-Elmer) at excitation/emission wavelengths of 485/530 nm.

Chemotactic migration was investigated using 24-well Transwell chambers (Corning) and polystyrene cell culture inserts with 6 µm pore size. Transwell culture chambers were coated overnight with fibronectin (20 ng/ml) at +4°C. Cells were washed once with serum-free RPMI and seeded to cell culture inserts at a density of 10^5 cells/insert in serum-free RPMI. Lower chambers contained 200 ng/ml monocyte chemoattractant protein 1 (R&D Systems) or 1 µM NPS in serum-free RPMI as chemoattractants, while media alone was added to the control chambers. The cells were allowed to migrate for 2 h at + 37ºC. The upper side of the membrane was wiped off and the migrated cells in the lower side were lysed into 400 µl of CyQuant reagent (Molecular Probes). Fluorescence was measured with the Victor2 plate reader as above.

Random cell migration was measured by a ‘wound healing assay’. RAW 264.7 cells were grown to confluency for 14 days on a 24-well plate in RPMI with 10% serum. Cells were washed once with PBS and pipet tips were used to scrape a cell free zone with constant width in the middle of the wells. Cells were washed once more with PBS and 1 µM NPS in serum-free RPMI was added to the wells to induce cell migration, while media alone was added to the control wells. The cells were allowed to migrate for 24 h at +37°C followed by analysis with light microscopy. The experiment was repeated three times using 6 parallel wells.

Phagocytosis activity of cells was measured with Vybrant Phagocytosis Assay Kit (Molecular Probes). Briefly, $1x10^5$ cells were seeded to the 96 well plates and incubated in RPMI for 1 hour in the presence or absence of 1 μM NPS followed by 2-h incubation with the fluorescein-labeled *Escherichia coli*. The fluorescence from noninternalized bacteria was then quenched by the addition of trypan blue. Subsequently, the samples were assayed with a Victor2 microplate reader with filters for detection of fluorescein. Tutftsin (Sigma-Aldrich) at a concentration of 1 μg/ml was used as a positive control. In order to identify the specific signaling pathways involved in the NPS-mediated phagocytosis, the specific inhibitors BAPTA-AM, bisindolylmaleimide I and H-89 (Calbiochem) for intracellular Ca^{2+}, protein kinase C and protein kinase A, respectively, were incubated with the respective suboptimal concentrations of 50 μM, 1 μM and 1 μM in the presence of 1 μM NPS. The inhibitors were diluted into dimethyl sulfoxide.

The results were verified by fluorescence microscopy under a magnification of 6300. Briefly, $4x10^5$ cells were seeded on coverslips and incubated for 1 hour in the presence or absence of 1 μM NPS followed by 2-h incubation with the fluorescein-labeled *Escherichia coli*. The fluorescence was not quenched with trypan blue. After two washes with PBS, the cells were fixed with 3.5% paraformaldehyde in PBS, mounted and visualized by fluorescence microscopy.

Statistical analysis (II, III, IV)

All data are expressed as mean ± standard error of mean (SEM) unless stated otherwise. Student's *t* test and analysis of variance were used for parametric comparisons, whereas Mann-Whitney and Kruskal-Wallis tests were used for nonparametric comparisons.

F. RESULTS

1. POSITIONAL CLONING OF *GPRA* AND *AAA1*

To identify the gene(s) accounting for the observed associations, the linkage region was refined by a hierarchical genotyping design until a 133-kb risk-conferring segment was discovered on chromosome 7p14.3. Briefly, the 20-cM linkage region was narrowed down by genotyping 76 microsatellite markers in families from Kainuu. Haplotype patterns spanning 12 microsatellite markers within 3.5 cM were associated with high serum IgE. At the second round of fine mapping, genotyping of 10 additional microsatellites implicated a 301-kb haplotype pattern. At the next cycle, five microsatellites and 13 SNPs with the highest associations were genotyped to reveal a 47-kb haplotype pattern (10 markers). The boundaries of this critical region were determined by genotyping additional SNPs in 131 trios. In data analysis, strongest associations were observed within a conserved 133-kb pattern.

Two other data sets from Quebec and North Karelia were genotyped to replicate the results in independent case-control cohorts. Most of the SNPs were shared, but some of them differed from the ones discovered in Kainuu population. In the three populations, 13 SNPs across the most conserved 77 kb produced seven alternative haplotypes (with frequencies >2% in the population). Sequencing the 133-kb segment from six additional individuals (including an asthmatic subject from Quebec and a North Karelian with high IgE), each homozygous for a different haplotype, confirmed that each of the haplotypes yielded unique SNP compositions. A phylogenetic analysis confirmed that the three risk haplotypes were evolutionary closely related and distinct from the four non-risk haplotypes in all three populations. In the Finnish population, more than half of the asthma patients carried the three risk haplotypes as opposed to only one third of the healthy subjects. The relative risk effect for high serum IgE was 1.4 among H4/H5 carriers in Kainuu, whereas the homozygous H2 haplotype carriers had a 2.5-fold risk for asthma in the Canadian population.

Utilization of gene expression analyses and computer algorithms for gene prediction over the disease-associated region revealed two previously uncharacterized genes named *GPRA* (*G Protein coupled Receptor for Asthma susceptibility*) and *AAA1* (*Asthma Associated Alternatively spliced gene 1*) with unknown biological functions. *AAA1* is transcribed from the complementary DNA strand to the opposite direction from *GPRA* (Figure 3).

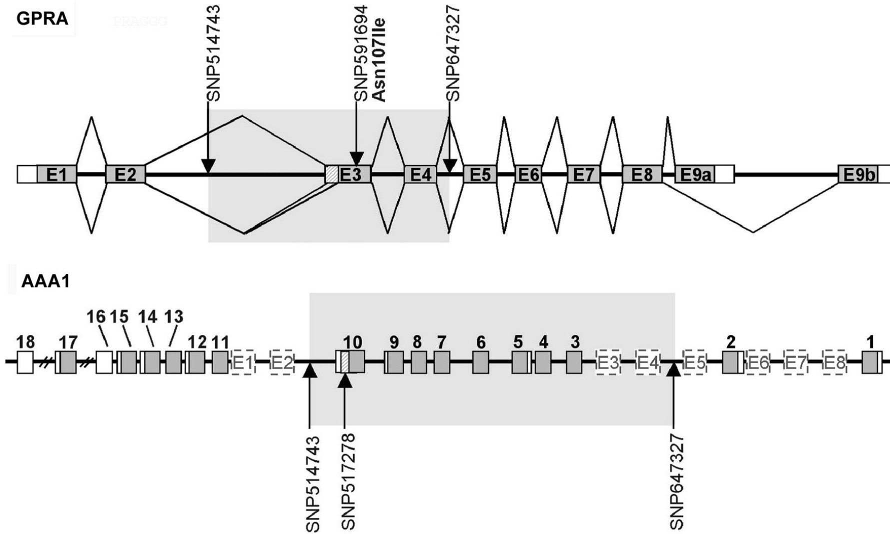

Figure 3. The exonic structures of *GPRA* and *AAA1* genes around the conserved 133-kb haplotype block (gray box). The two genes do not have overlapping exons, but instead are embedded inside each other's genes. There are two alternative 3' exons, 9a and 9b, in the *GPRA* gene, which encode full-length variants of the receptor. The nonsynonymous polymorphism is located in exon 3. In the opposite DNA strand, there is a previously uncharacterized *AAA1* gene, which displays extensive alternative splicing. Eight exons of the *GPRA* gene (E1 to E8) are shown for orientation. Modified from (I).

2. CHARACTERIZATION OF AAA1

2.1 Genomic structure of AAA1 variants (I)

The previously uncharacterized *AAA1* gene is located in the complementary DNA strand transcribed to the opposite direction from *GPRA*. The gene spans over 520 kb of the genomic DNA (contig NT_000380, NT_007819) and is encoded by at least 18 exons (Figure 3). The cDNA sequences of the *AAA1* splice variants were assembled by using RT-PCR with primers designed for predicted exons. Using cDNAs from human lung, kidney, testis and fetal liver as templates, thirteen splice variants termed IA, IB, II, III, IVA, IVB and V-XIII were identified. All variants share exon 6, but use alternative exons to encode the 5´ and 3' ends of the transcript. In most of the variants, the sequence flanking the putative ATG translation initiation site (GCCATGC) contains the –3 purine, but not the +4 guanine residue, of the Kozak consensus sequence surrounding the initiation codon of eukaryotic mRNA.

2.2 Expression pattern of AAA1 mRNA (I)

Northern blot analysis of a multiple tissue expression array showed that AAA1 mRNA is expressed in several human tissues such as testis, brain, placenta, lung, heart, skeletal muscle, kidney, liver, fetal liver and fetal lung. *In situ* hybridization revealed that AAA1 is expressed in the epithelial cells in normal colon and lung tissues.

2.3 AAA1 is a non-coding RNA gene (I)

All predicted AAA1 proteins sequences are small peptides (sizes from 34 aa to 74 aa) with no significant homology to any known modular structures or motifs. There are no AAA1 homologues in the sequence databases of other species, e.g. mouse, rat, drosophila orthologues. All AAA1 isoforms contain the same core sequence (AYVRRNAGRQFSHCNLHAHQFLVRRKQ) flanked by alternative amino- and carboxyterminal tails. To test whether AAA1 encodes for any polypeptides, *in vitro* translation experiments of AAA1 variants I and III in the presence of S^{35} -labeled methionine in the reaction mixture were performed. Reaction products were analyzed by SDS-PAGE and autoradiography. The commercial control DNA construct resulted in the synthesis of a 50-kDa polypeptide as expected. However, neither of the two investigated constructs starting from the common short open reading frame was translated into polypeptides. The finding suggested that AAA1 functions as a non-coding RNA gene.

This was further confirmed in cell transfection experiments. The expression constructs containing the open reading frame with C-terminal tag sequences for detection were negative for immunofluorescence microscopy and Western blot analyses. In addition, cells transiently transfected with HA-AAA1 fusion construct failed to produce recombinant protein detectable by anti-HA antibodies.

Polyclonal antibodies were raised against the synthetic peptide YVRRNAGRQFSHC containing the sequence included in the constant region of AAA1. The antibodies demonstrated high affinity against the peptide and the constant region of AAA1 expressed as a GST fusion protein. However, no signals were detected in either Western blots (spleen, skeletal muscle, uterine muscle, colon muscle, colon epithelium, kidney, testis and prostate) or immunohistochemistry (BEAS-2B, A549, HepG2, Ptks2, 293 and myoblast cell lines positive for AAA1 mRNA).

3. CHARACTERIZATION OF GPRA AT mRNA LEVEL

3.1. Genomic structure and splice variants (I, II)

The full-length cDNA sequence of *GPRA* was assembled by using conventional RT-PCR with primers designed for predicted exons. The alternatively spliced transcripts were further determined by RACE-PCR using different sources of mRNA such as the human lung epithelial carcinoma cell line NCI-H358 (ATCC) and commercially available cDNAs as templates. Nested PCR amplification was utilized in producing full-length cDNAs of the various splice variants, which have the same initiation sites but use alternative exons in encoding the 3’ end of the gene.

The *GPRA* gene spans 0.2 Mb of the genomic contig NT_000380 (NT_007819). The open reading frame is distributed across 9 exons and the gene undertakes complex splicing events (Figure 4). The longest *GPRA* transcripts express cDNAs of 1116 bp and 1134 bp producing alternative 3' exons 9a and 9b and were named *GPRA-A* and *-B,* respectively. The 133-kb

segment spans from intron 2 to intron 5 of *GPRA*, but most of the strongly associated SNPs are within the long intron 2 comprising 93.8 kb of genomic DNA. However, there is one nonsynonymous polymorphism in the first extracellular loop of the receptor that replaces the Asn 107 with Ile.

The *C* variant is the shortest *GPRA* transcript including only the exons E1, E2 and E3a encoding a 94 aa-peptide. Several additional novel splice variants termed *D*, *E* and *F* were observed in the cloning process using PCR analysis with nested PCR specific for the *A* variant. Variants *GPRA-D* -*E* and -*F* encode 158-, 136- and 305-aa peptides, respectively, which have a deletion of either exon 3, 4 or both, respectively. The deletions result in an early stop codon for *GPRA-D* and -*E* variants, whereas *GPRA-F* shares the rest of the reading frame with *GPRA-A*. *GPRA-B*$_{short}$ encoding a 366-aa peptide has an in-frame deletion of 33 bp (11 aa) at the beginning of exon 3, while the rest of the downstream exons are the same as in *GPRA-B* (Figure 4).

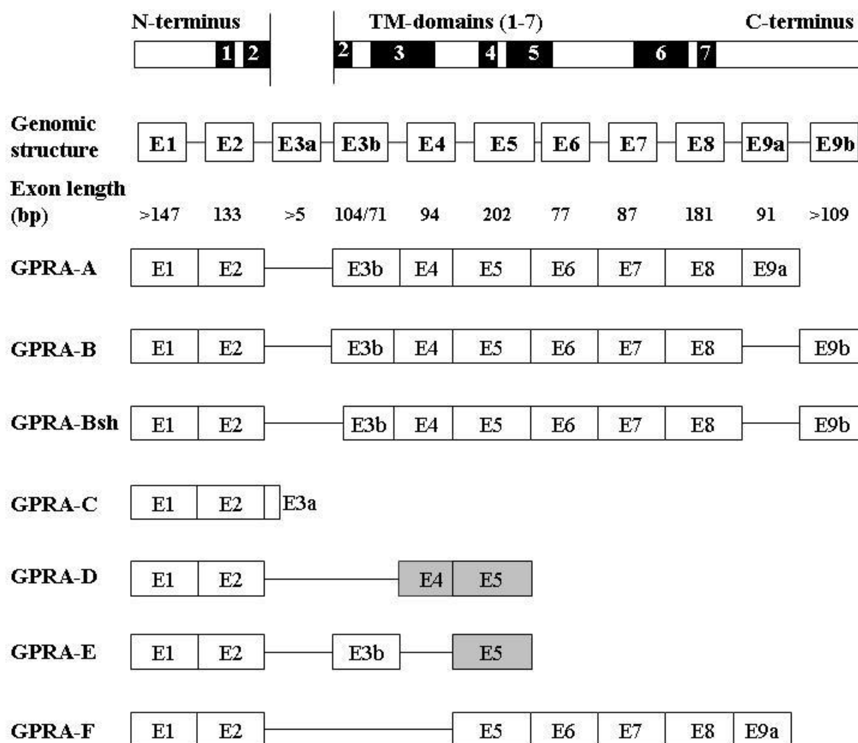

Figure 4. Exon structure of the *GPRA* variants *A-F*. Black areas represent transmembrane regions in the full-length receptor variants *A* and *B*. Gray areas indicate the alternative open reading frames producing truncated proteins. Modified from (II).

3.2. Expression profiling by RT-PCR and Northern blot hybridization (I, III, IV)

According to RT-PCR analysis, different *GPRA* variants are expressed in several human tissues including testis, brain, pituitary gland, placenta, lung, heart, thymus, and fetal heart. In Northern blot analysis of a commercial eight-lane membrane, 1285-bp full-length *GPRA-A* cDNA probe

detected a 2.4-kb transcript in spleen, thymus, prostate, testis, uterus, small intestine, colon and peripheral blood leukocyte lanes. In addition, a 2.4-kb band was observed in the lung tissue and a further 1.8 kb transcript in four tissues.

To address the role of GPRA in inflammation, mRNA levels of the mouse ortholog of *GPRA* (*Gpra*) were investigated in a mouse model of OVA-induced lung inflammation with additional mold exposure. According to the real-time RT-PCR analysis (Taqman assay), metacholine induced hyperreactivity was associated with elevated pulmonary Gpra mRNA levels in sensitized (n=7) mice when compared with unsensitized mice (n=8).

In order to identify the cell types expressing GPRA, mRNA expression of GPRA-A and -B was further characterized in fractionated human blood cells using real-time RT-PCR with SYBR Green chemistry. GPRA-A mRNA expression in peripheral blood mononuclear cells (PBMCs) was similar to bronchial epithelial and bronchial SMCs. The expression of GPRA-B mRNA was higher in PBMCs than in BEAS cells, but very weak in BSMCs. In fractionated leukocytes, monocytes and eosinophils expressed both GPRA-A and GPRA-B mRNA, whereas CD4+ T expressed mainly GPRA-B mRNA.

In order to examine the effects of monocyte and T-cell activation on GPRA expression, PBMCs were stimulated with LPS or anti-CD3 and anti-CD28 antibodies, respectively. LPS increased GPRA-A and -B mRNA expression 16 and 28 h after challenge, whereas T-cell activation with anti-CD3 and anti-CD28 antibodies decreased the expression 28 h after challenge. Thus, GPRA expression in PBMCs correlates to the maturation of monocytes to macrophages. The decreased expression of GPRA after T-cell activation may be a secondary effect produced by induced cytokine production.

The nasal epithelium displays characteristics comparable to more distal airways and can be used as a representative of pulmonary epithelium. GPRA mRNA expression was determined in samples taken from the nasal respiratory epithelium of term and preterm infants, and healthy adults using real-time PCR (Taqman assay). Strong signal (relative expression 353.3 ± 254.0, mean $\pm$ SD) was observed in all of the 10 adult samples, whereas the relative GPRA mRNA expression was weak (6.3 ± 3.8) in the 6 term infant and very weak or nonexistent (1.3 ± 1.7) in the 7 preterm infant samples at the age of 1-4 h. No significant differences were observed in the GPRA mRNA expression levels between the samples taken from the nasal epithelium at the age of 1-4 h and 21-48 h of age. We also studied GPRA mRNA expression in 4 preterm infants with BPD, although age-matched control samples were unavailable. In these samples, the GPRA expression was significantly higher (57.7 ± 32.4) than in the neonates sampled at the age of 1-4 h.

Taken together, these findings indicate that GPRA mRNA is expressed in leukocytes and in several peripheral organs containing epithelial and SMCs. GPRA mRNA levels are increased in a mouse model during inflammation.

3.3. Expression profiling by *in situ* hybridization (II)

GPRA mRNA expression was studied in paraffine-embedded bronchial and colon sections as well as tissue arrays containing 30 human tissues. Automated non-radioactive *in situ* hybridization with a digoxigenin-labeled non-isoform-selective *GPRA* antisense probe resulted in positive staining in epithelial cells of all tissues relevant to asthma and allergy including bronchus, the gastrointestinal tract and skin. In addition to colon epithelium, also inflammatory cells between the crypts expressed *GPRA*. In other lymphatic tissues, positive inflammatory cells were detected in tonsils but not in the thymus gland. The glandular epithelia (e.g. in the salivary gland and thymus) and submucosal epithelial cells of other peripherial tissues such as spleen, kidney, pancreas, prostate, uterus and breast showed strong staining. Peripheral nerves, cerebral cortex and cerebellum as well as the skeletal and the smooth muscle of various tissues were negative. However, staining of frozen sections of bronchial tissue from asthmatic patients resulted in strong mRNA expression also in the SMC layer suggesting a potential role for GPRA in bronchial hyperresponsiveness. Staining with the sense probe resulted in no signal.

4. CHARACTERIZATION OF GPRA AT PROTEIN LEVEL

4.1. The predicted structure of GPRA (I)

According to the TMpred and PredictProtein softwares for protein prediction, the conserved amino acid residues revealed similarities to the typical characteristics of the rhodopsin GPCRs. These included two conserved Cys residues in exoloops 1 and 2 potentially forming a disulfide bridge, the Asp-Arg-Tyr sequence (DRY motif) in the proximity of TM3, Asn-Pro-X-X-Tyr motif in TM7, and a Cys residue in the C-terminal region. The putative N-glycosylation site in Asn^4 was predicted by the PredictProtein software. GPRA-A and -B encode 366 and 371 amino-acid proteins with 7 TM domains containing mutually exclusive C-terminal intracellular tail sequences (Figure 5).

The closest structural homolog is the vasopressin receptor V1a, which has 27 % amino acid similarity to GPRA-A. Thus, GPRA belongs to the β-group of rhodopsin receptors (section C: 3.1). A GPRA homologue exists in insects suggesting that an ancestor of this receptor was present more than 700 million years ago. Because GPRA cannot be found in fish, it is possible that its function is essential only for land-living species. In man, expressed sequence tags (ESTs) for GPRA can be found both in central nervous system and peripheral tissues (Gloriam et al. 2005).

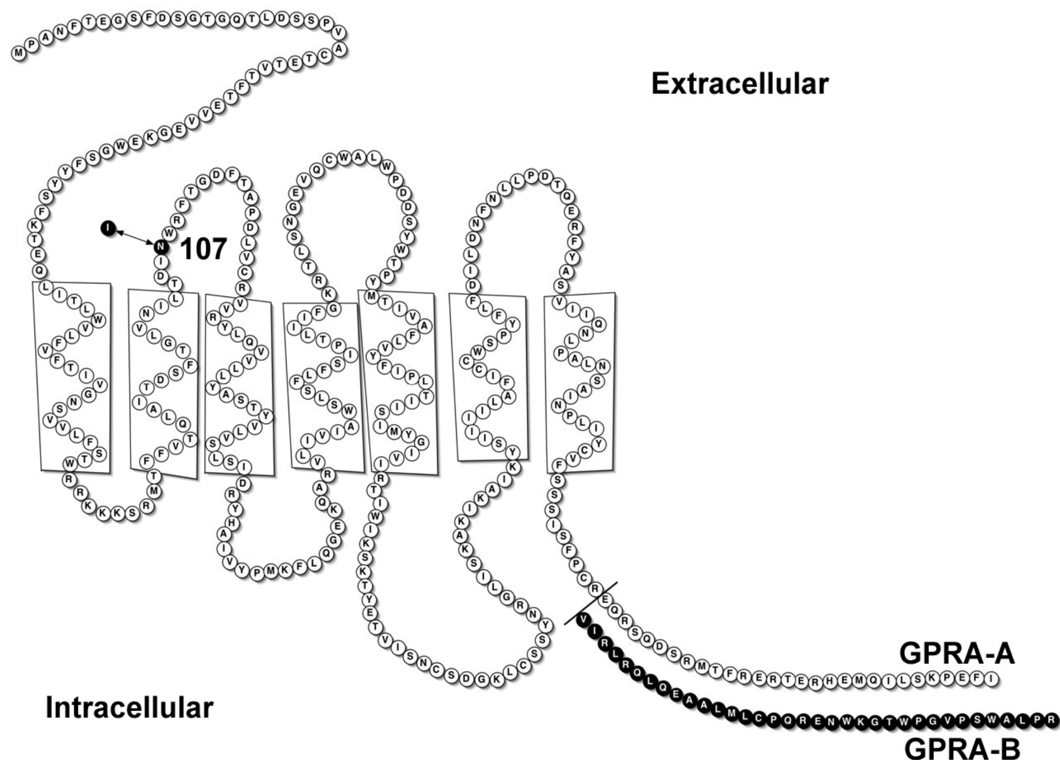

Figure 5. Predicted protein structure of GPRA-A and -B. GPRA-A and -B encode 366 and 371 amino-acid proteins with molecular weights of 42.7 and 43.1 kDa, respectively. The nonsynonymous Asn(107)Ile polymorphism is located in the GPRA exon 3 encoding the first exoloop of the receptor. Modified from Reinscheid et al. 2005.

4.2. Characterization of the GPRA antibodies (I, II, III)

Two different polyclonal antibodies detecting intracellular sequences against the two alternative C-terminal tails (anti-GPRA-A and anti-GPRA-B) of GPRA were raised in rabbit by immunization with the corresponding peptides conjugated to a KLH carrier protein. In order to use parallel antibodies, additional polyclonal antibodies detecting the third cytoloop (anti-GPRA-CL3) of the receptor and recognizing both GPRA-A and -B isoforms were raised in rabbit according to the same protocol. To further obtain polyclonal antibodies against extracellular epitopes of GPRA, a goat was immunized with a multiple antigen peptide containing the amino terminus (anti-GPRA-N) of GPRA and a KKG core. The sera were screened with an enzyme-linked immunosorbent assay using the uncoupled peptides immobilized onto the nitrocellulose filters as antigens. Positive antisera were purified with affinity chromatography using the corresponding peptides as antigens in order to remove any cross-reacting IgG and other contaminants. The antibodies were characterized by immunoblotting, peptide competition assays and immunohistochemistry utilizing assays based on colour reaction and fluorescence.

To determine whether the purified antibodies detect their peptide epitopes, the antibodies were characterized by immunoblotting of corresponding recombinant GPRA fragments produced in *Esterichia coli* as fusion proteins (III). The peptides were expressed in pGEX 4T-3 GST fusion

expression vector as GST fusion proteins except for the C-terminal tail of the A isoform, which was expressed as a DHFR fusion protein. Epitope-specific signals were detected with no cross-reactivity between the constructs or with the GST or DHFR or construct alone. For further verification, use of molar excess of the free peptide as a competitor prevented the signal detection in blocking experiments. Because the anti-GPRA-B and the anti-GPRA-N antibodies detected also shorter GPRA variants produced as a result of protein degradation, full-length GST-constructs were purified from the lysates with the aid of glutathione affinity chromatography. Immunoblotting of the purified GST-constructs with the corresponding antibodies resulted in detection of a single band as expected.

To show that the antibodies also recognize full-length receptor variants in immunoblotting, myc-tagged GPRA-A and -B constructs were transiently expressed in COS-1 cells (II). Cells were harvested 48 h post-transfection, the crude membrane preparations were isolated by ultracentrifugation and analyzed using anti-myc, anti-GPRA-A and anti-GPRA-B antibodies. The anti-GPRA-A and anti-GPRA-B antibodies recognized the same recombinant proteins as the anti-myc antibodies at about 35 to 50 kDa. Since the calculated molecular weights of GPRA-A and -B are 42.7 and 43.1 kDa, respectively, the results indicate that there are no extensive post-translational modifications in produced recombinant GPRA proteins.

The anti-GPRA antibodies were further studied in immunocytochemistry and immunohistochemistry settings utilizing the parallel antibodies and knowledge from mRNA expression studies. According to the results of these studies described in the following sections in more detail, anti-GPRA-N and anti-cytoloop-3 recognized the shared segments of the GPRA-A and -B isoforms. However, the affinities of the GPRA-N antibodies were overall weaker when compared with the cytoplasmic antibodies, which is typical for antibodies produced by immunization against epitopes previously recognized by the immune system, i.e. extracellular epitopes of the surface receptors.

4.3. Cellular localization of the splice variants (II)

In order to study the cellular localization of the different GPRA isoforms, COS-1 cells were transiently transfected with N-terminally conjugated myc-tagged or C-terminally HA-tagged pCMV-GPRA constructs. ELISA in both permeabilized and non-permeabilized cells as well as fluorescence microscopy was used for detection of the myc-tagged GPRA-constructs. According to the results, 71% of the produced GPRA-A and 52% of the GPRA-B were translocated to the plasma membrane, while all the five shorter variants remained in the intracellular compartments 48 h post-transfection.

In order to further study the specificities of the antibodies in immunocytochemistry, COS-1 cells grown on coverslips were transiently transfected with N-terminally conjugated myc-tagged or C-terminally HA-tagged pCMV-GPRA constructs, permeabilized, and then incubated with anti-GPRA-A, anti-GPRA-B, or anti-HA antibodies for 1 h. Thereafter, the cells were incubated with fluorescent goat anti-rabbit IgG or goat anti-mouse IgG antibodies for 30 min.

Samples were visualized with fluorescence microscopy. In consistence with the results obtained from the cell-based ELISA assay, anti-GPRA-A and -B antibodies detected the receptor on the plasma membrane as expected also from the topology of the receptor. However, all the shorter GPRA isoforms were maintained in the intracellular compartments as detected with anti-HA antibodies.

In order to examine the effect of the truncated GPRA isoforms on the full-length GPRA-A or -B translocation, COS-1 cells were co-transfected with myc-tagged GPRA-A or -B constructs and truncated variant constructs with increasing concentrations. Subsequently, the relative amount of the receptor on the plasma membrane was measured with the cell-based ELISA assay. According to the results, the GPRA-A and GPRA-B amount on the plasma membrane remained constant despite of the presence or absence of other splice variants. To conclude, shorter GPRA isoforms did not affect translocation of GPRA-A and -B and the role of the various GPRA transcripts remained unclear.

The expression of GPRA-A was further analyzed in AAA1 transfected COS-1 cells by utilizing immunohistochemistry with fluorescent labels and immunoblotting analysis. According to the results, AAA1 did not affect the expression of GPRA since no differences in GPRA expression were observed between the transfected and non-transfected cell samples at 24 h or 48 h. The results were reproduced with conventional RT-PCR analysis, which showed no differences in GPRA mRNA expression between AAA1 transfected and mock-transfected COS-1 cells 4 h and 24 h post-transfection.

4.4. Expression profiling by immunoblotting (II, III)

In immunoblotting experiments of human tissue lysates, detection with anti-GPRA-A antibodies revealed one intensive polypeptide band corresponding to molecular weight of approximately 50 kDa in SMC containing tissues such as uterine muscle, colon muscle and prostate, but also in colon epithelium. Spleen and testis were negative for GPRA-A. With GPRA-B antibodies, a 50-kDa polypeptide band was observed in all examined tissues, except in skeletal muscle. An additional 39-kDa band was visible in testis. Detection with the anti-GPRA-CL3 antibodies recognizing both isoforms A and B resulted in positive signals in overlapping polypeptide bands confirming the results.

In addition, inflammatory cells expressed GPRA. Detection with both anti-GPRA-A and GPRA-B antibodies revealed the 50-kDa polypeptide band in human sputum and PBMC samples. The additional 60-kDa band observed in sputum samples after anti-GPRA-A detection may represent a receptor form carrying differentially processed N-glycans, representing post-translational modification that is typical for GPCRs. In PBMC lysates, the amount of both GPRA isoforms was increased in LPS-treated PBMCs 48 h after challenge when compared with the unstimulated samples. Quantification of the band intensities demonstrated that the increases in GPRA-A and -B expression after LPS challenge were 1.4-fold and 1.6-fold, respectively,

whereas T-cell activation with anti- CD3/28 antibodies did not modulate GPRA-A or -B expression.

The calculated full-length molecular weights of the GPRA-A and GPRA-B proteins, 42.7 and 43.1, respectively, are in agreement with the observed results. For further verification of the antibody specificities, omission of the primary antibodies and blocking experiments with 10-fold molar excess of free peptide as a competitor were used to verify the correct polypeptide bands in immunoblotting.

4.5. Expression profiling by immuhistochemistry (I-IV)

4.5.1 Normal human tissues (I, II)

The protein expression of GPRA-A and -B was characterized using paraffine-embedded sections from bronchial, colon and skin tissue samples relevant to the pathogenesis of allergy and asthma. In addition, commercial tissue arrays containing 30 normal adult human tissue samples were used in further expression profiling of GPRA.

Immunostaining with the anti-GPRA-A antibodies resulted in weak immunoreactivity in the epithelium of most tissues studied. SMCs in the bronchial and arterial walls were constantly positive for GPRA-A in all tissues studied. Immunostaining with the anti-GPRA-B antibodies revealed ubiquitous protein expression in the glandular epithelia of bronchus, stomach, small intestine, colon, uterus, esophagus, spleen, kidney, pancreas, prostate, and breast. In the distal lung, both isoforms were present in alveolar macrophages and B isoform additionally in the septal cells lining the alveolar epithelium. Interestingly, GPRA-A was expressed in the basal surfaces of the colon epithelium and skin keratinocytes, whereas GPRA-B was expressed the apical surfaces of colon and in the terminally differentiating skin keratinocytes. However, in comparison to GPRA-B, the expression of GPRA-A was absent in some tissues such as stomach and small intestine. Consistent with immunoblotting, neither of the isoforms showed immunoreactivity in skeletal muscle.

The reliability of the results was further verified with the GPRA-N antibodies raised against the N-terminus of GPRA. Staining with the GPRA-N antibodies was overall weaker than with the GPRA-A and -B antibodies detecting intracellular epitopes of the receptor, but consistently, the antibodies recognized GPRA isoforms in exactly overlapping positions, i.e., in various epithelial cells and in SMCs. In the skin, both basal and apical cells of the stratified epithelium were stained with the GPRA-N antibodies, whereas the middle layer was negative for GPRA. An overall summary of GPRA-A and -B expression in different tissues is shown in Table 4, which also includes the expression of GPRA mRNA in the corresponding tissues.

Table 4. An overall summary of GPRA-A and -B expression in different tissues as assessed by immunohistochemistry and *in situ* hybridization. Anti-GPRA-N antibodies recognized epithelial and SMCs in overlapping locations to GPRA-A and –B expression.

	GPRA-A	GPRA-B	GPRA mRNA
LUNG	SMC, macrophages	SMC, macrophages, septal cells	-
BRONCHUS	SMC, epithelial cells	Epithelial cells	Epithelial cells
SKIN	Basal cells	Differentiated keratinocytes	Keratinocytes ±
ESOPHAGUS	Squamous epithelium, muscularis mucosae, lymphatic vessels	Arteria, lymphatic vessels	Squamous epithelium
STOMACH	SMC, neutrophils, glands	Glandular epithelia	Glandular epithelium
SMALL INTESTINE	SMC, epithelial cells ±	Epithelial cells	Epithelial cells
COLON	Basal cells, goblet cells	Epithelial cells	Epithelial cells, inflammatory cells
HEART MUSCLE	Arterioles, cardiomyocytes ±	Cardiomyocytes ±	Cardiomyocytes ±
SKELETAL MUSCLE	-	±	-
TONSIL	-	-	+
SPLEEN	-	Arterial wall	-
BONE MARROW	Mature neutrophils +	Immature neutrophils +, mature neutrophils -	-
THYMUS GLAND	-	-	Glandular epithelium
SALIVARY GLAND	Mucous acini +, serous acini -	Mucous acini +, serous acini -	Glandular epithelium
ADRENAL GLAND	Zona glomerulosa ±	-	+
THYROID GLAND	-	Epithelial cells	Glandular epithelium
PARATHYROID GLAND	Unrepresentative sample	Unrepresentative sample	±
PITUITATORY GLAND	Basophils +, acidophils ±	-	±
PANCREAS	The islet of Langerhans	-	Glandular epithelium
KIDNEY	Epithelium ±	Tubular epithelium ±	Epithelium
BREAST	Glandular epithelium	Glandular epithelium	-
OVARY	-	-	-
UTERUS	Glandular epithelium, SMC	Glandular epithelium, SMC	Glandular epithelium
PLACENTA	Arteria	-	±
PROSTATE	SMC, epithelium	SMC, epithium -	Epithelium
TESTIS	-	-	-
OMENTUM	-	-	-
CERVIX	Squamous epithelium ±	Squamous epithelium ±	-
PERIPHERAL NERVE	-	±	-
CEREBRAL CORTEX	-	-	-
CEREBELLUM	-	-	-

4.5.2 Pulmonary expression of GPRA in asthma, RDS and BPD (I, IV)

In order to clarify the role of GPRA expression in the pathogenesis of asthma, GPRA-A and -B immunoreactivity was examined in 8 paraffin-embedded bronchial biopsies from asthmatic patients and compared with the expression of 10 control samples from non-asthmatic subjects. The GPRA-A expression was similar in asthmatic and nonasthmatic subjects with the strongest expression in the bronchial SMCs and additional expression in the epithelial cells. In healthy subjects, GPRA-B was expressed in the epithelial cells but not in SMCs of the bronchial wall. Strikingly, GPRA-B was upregulated in the SMCs of asthmatic airways in each of the studied samples. Because the genetic risk effects of the GPRA haplotypes are estimated to account for over half of the Finnish asthma patients as opposed to approximately one third of the healthy controls, the clear-cut upregulation of GPRA-B in asthmatic patients is unlikely to be caused by a genetic drift. The findings, however, indicate that GPRA may be involved in the development or maintenance of BHR in asthmatic patients. The GPRA immunoreactivity in leukocytes suggested a further role for the receptor in inflammation.

To further explore the link between GPRA expression and BHR, GPRA-A and -B immunoreactivity was analyzed in paraffin-embedded autopsy samples of preterm infants with RDS and BPD showing clinical features of increased SMC contractility, and compared with midterm fetuses and term infants. In term infants who lived up to 24 h and died of extrapulmonary causes, GPRA-A and -B expression was weak but similar to the healthy adult controls. Neither GPRA-A nor GPRA-B was expressed in midterm fetal lung. Similarly to asthma, GPRA-B expression was upregulated in the SMCs of the large bronchi in RDS and BPD. Also the epithelial expression of GPRA-B in bronchi was slightly more intensive in RDS and BPD than in the full-term controls, whereas GPRA-A immunoreactivity did not differ from the staining of the full-term controls. These observations suggest that GPRA could contribute to the susceptibility to BHR.

4.5.3 Mouse model (III)

Paraffine-embedded lung tissue samples of classical OVA-induced murine model of asthma were analyzed by immunohistochemistry to examine the protein expression of GPRA during inflammation. Public mouse genome databases do not include the sequence for the B isoform and thus, only the anti-GPRA-A specific antibodies were utilized. However, the specificity of the immunostaining could be further verified with the GPRA-CL3 antibodies detecting intracellular third cytoloop common to all receptor isotypes. The corresponding murine epitope is identical to the human sequence except for the first amino acid.

In the lung tissue of naïve and unsensitized but OVA-challenged mice, GPRA-A was expressed in the epithelial cells of the bronchioles as expected from the human studies. In the OVA-challenged mice without sensitization, nonexistent to moderate immunostaining was also observed in alveolar macrophages. In the OVA-sensitized and -challenged mice, GPRA-A expression was decreased in the epithelial cells of the bronchioles, whereas the expression was

increased in macrophages when compared to the naïve and OVA-challenged groups without sensitization. The staining of the corresponding sections with the GPRA-A preimmune sera did not result in any immunoreactivity. Staining of the corresponding sections with the GPRA-CL3 antibodies resulted in identical immunoreactivity in overlapping locations to the anti-GPRA-A stainings.

In order to further confirm the GPRA expressing cell types, BAL samples from the corresponding study groups were stained. In the BAL fluid of naïve control mice, GPRA expression in the alveolar macrophages varied between nonexistent to very low. Moderate GPRA expression was observed in macrophages of OVA-challenged mice without sensitization. In the asthma model of OVA-sensitized and -challenged mice, GPRA staining in macrophages varied from mild to strong. Positive GPRA staining was also detected in eosinophils. These results confirmed the findings indicating upregulated GPRA expression in tissue infiltrated macrophages upon allergen challenge.

5. FUNCTIONAL STUDIES OF GPRA (II, III)

5.1. Expression of neuropeptide S, an endogenous ligand for GPRA (II, III)

We did not succeed in raising polyclonal antibodies against the polypeptide and therefore, NPS expression could only be assessed at mRNA level. According to the results obtained with quantitative real-time RT-PCR, NPS mRNA expression in blood cells resembled the expression profile of GPRA. Similarly to GPRA, the expression of NPS mRNA was upregulated in PBMCs 16 and 28 h after LPS stimulation and T-cell activation with anti-CD3 and anti-CD28 antibodies decreased NPS expression 28 h after challenge. Furthermore, *in situ* hybridization of bronchial and colon sections revealed that NPS mRNA is expressed in the epithelia in overlapping locations to the cognate receptor GPRA. These findings support the idea that NPS may activate GPRA by an autocrine or paracrine mechanism.

5.2. GPRA-A overexpressing cell line (II, III)

Human kidney epithelial cell line 293H did not express endogenous GPRA-A, as determined by real-time RT-PCR, and was used as a host in constructing a lineage stably overexpressing GPRA-A. After transfection with the corresponding GPRA-A DNA constructs, the clones were identified by RT-PCR and immunoblotting analyses. Three GPRA-A positive clones, two GPRA-A negative clones and the parental 293H cell line were selected for further experiments.

Nonradioactive GTP-binding assay was utilized in order to validate the signal transduction activities of the receptor. After 1 µM NPS challenge, GPRA-A positive cell clones displayed a 2-3 -fold increase in GTP-binding activity when compared with the GPRA-A negative cell clones. For measurements of cytoplasmic Ca^{2+} levels, the cells were labeled with Fluo-3 AM as an indicator. Ten to fifteen seconds after injection, NPS (1 µM) increased fluorescence levels indicating Ca^{2+} release to the cytoplasm of the GPRA-A overexpressing cells.

No change in Ca^{2+} levels was detected in parental 293H cells. In an ELISA-based assay, 20 min stimulation with 1 μM NPS resulted in a 36-40 % increase in basal cAMP levels in GPRA-A overexpressing cells but not in parental 293H cells. These findings indicate that NPS is able to activate its cognate receptor GPRA by increasing intracellular Ca^{2+} and cAMP accumulation.

To examine the effects of GPRA-A activation on cell growth, GPRA-A positive and negative cell clones were treated with NPS for 3 d, and the relative cell numbers were determined using the colorimetric proliferation assay. According to the results, the GPRA-A positive cells grew slower than the GPRA-A negative cells. The GPRA-A dependent cell growth was further inhibited by 1 μM NPS treatment ($p < 0.05$).

In consistence with the proliferation assay, cell counting at different time points indicated that the stably GPRA-A overexpressing cells grew slower than the negative control cells. After 2 and 4 d of culture, GPRA-A positive cells had grown 18% ($p < 0.01$) and 14% ($p < 0.05$) slower than the GPRA-A negative control cells, respectively. Addition of puromycin used as a selection marker for the construct intake had no influence on cell growth.

5.3. Macrophage cell line (III)

RT-PCR and immunohistochemistry experiments were used to demonstrate that murine macrophage cell line RAW 264.7 express GPRA at mRNA and protein levels, and was used as a model for GPRA-mediated macrophage functions. In phagocytosis assay, 1h prestimulation with NPS resulted in a dose-dependent and up to 10.8 -fold increase in cellular intake of fluorescein-labelled *Esterichia coli* ($p<0.001$) during 2 h incubation. When visualised with fluorescence microscopy, the increase was apparent in the intracellular compartments, whereas the nuclei of the cells did not emit any fluorescence.

In order to identify the specific signalling pathways involved in the NPS-mediated phagocytosis, specific inhibitors for intracellular Ca^{2+}, protein kinase C and protein kinase A were used. Incubation with each of the inhibitors resulted in a decrease in NPS-mediated phagocytosis when compared to the cells treated with NPS alone. The most effective inhibition was obtained with bisindolylmaleimide I (PKC inhibitor), which decreased the NPS -mediated phagocytosis approximately 70 % ($p < 0.05$). Use of the inhibitors did not decrease phagocytosis in cells not treated with NPS indicating that NPS-stimulated phagocytosis was individually dependent on all pathways involving intracellular Ca^{2+}, protein kinase C and cAMP.

Because directed cellular movement is characterized by a dynamic control of attachment and detachment of the cell surface adhesive receptors with their extracellular matrix ligands, we studied whether NPS modulates cell adhesion in RAW 264.7 cells. Maximal activation of the GPRA-A overexpressing cells and increased phagocytosis of the RAW cells was obtained with 1 μM NPS concentration and hence, it was selected for the cell adhesion and migration studies. Cells were preincubated in the presence or absence of NPS for 30 min and seeded onto fibronectin, collagen type I or poly-L lysine coated wells for 50 min. Cell adhesion on immobilized fibronectin was slightly but significantly ($p = 0.013$) decreased in the samples with

NPS preincubation. Poly-L-lysine was used as a matrix to support unspecific non-integrin - mediated adhesion, and did not affect NPS-mediated cell adhesion. For further demonstration of the importance of the immobilized matrix, collagen type I did not support adhesion of RAW 264.7 cells (data not shown). Because the observation of fibronectin binding to integrins $\alpha4\beta1$ and $\alpha5\beta1$ in hematopoietic cell-matrix adhesive interactions is well established (e.g. Gu et al. 2003), our results suggest that NPS-dependent cell detachment may be mediated by integrins.

In a quantitative fluorescence-based cell migration assay, a 2-fold increase ($p = 0.007$) in RAW cell migration was detected 2 h after NPS stimulation. The results were confirmed in another assay, where random migration of confluent RAW cells was measured 24 h after NPS stimulation. According to visual analysis, the migration of the cells was increased onto the denuded culture dish in the presence of NPS when compared to the untreated control cells.

6. GPRA AS A GENETIC REGULATOR OF RESPIRATORY DISTRESS SYNDROME (IV)

To study whether GPRA associates with RDS or BPD, the seven haplotype tagging SNPs in a 70.7-kb interval of the *GPRA* gene intron 2 were genotyped in a case-control setting of Finnish infants (Table 5). Because of the lack of unamplified genomic DNA from all of the subjects, DNA was extracted from dried blood spots, amplified with the improved primer preamplification (I-PEP-L) method and genotyped using the Sequenom platform (Hannelius et al. 2005, Sun et al. 2005). However, the amount of DNA was too low for proper amplification in some of the samples and therefore, these were excluded from further analysis decreasing the sample population from of 521 to 435 subjects.

Three age-matched case-control comparisons were made. The first comparison was performed in very preterm infants with gestational age (GA) < 32 weeks with ($n = 82$) or without RDS ($n = 42$), and the second in near-term infants born at $32 \leq GA < 35$ weeks with ($n = 43$) or without RDS ($n = 87$). In the third analysis, very preterm infants with BPD ($n = 37$) were separately compared with GA-matched controls with or without RDS.

In the second group consisting of near-term infants, haplotype H1 was underrepresented (OR 0.5; 95% CI 0.3-0.8; $P = 0.01$), whereas haplotype H4/H5 was overrepresented in the RDS group (OR 2.6; 95% CI 1.2-5.5; $P = 0.01$). Although the seven SNPs included in this study were selected on the basis of tagging the previously identified haplotypes H1-H8, we also examined possible associations for the individual SNPs with the outcomes mentioned above. At the allelic level, genotyping of each SNP except for the rs323917 tagging H7 resulted in significant differences in the allele frequences between cases and controls in near-term infants. The pairwise linkage disequilibrium values (D´) between the markers and the single-marker allele frequencies are presented in Figure 6. Corresponding associations were not found in the group of very preterm infants with RDS or BPD.

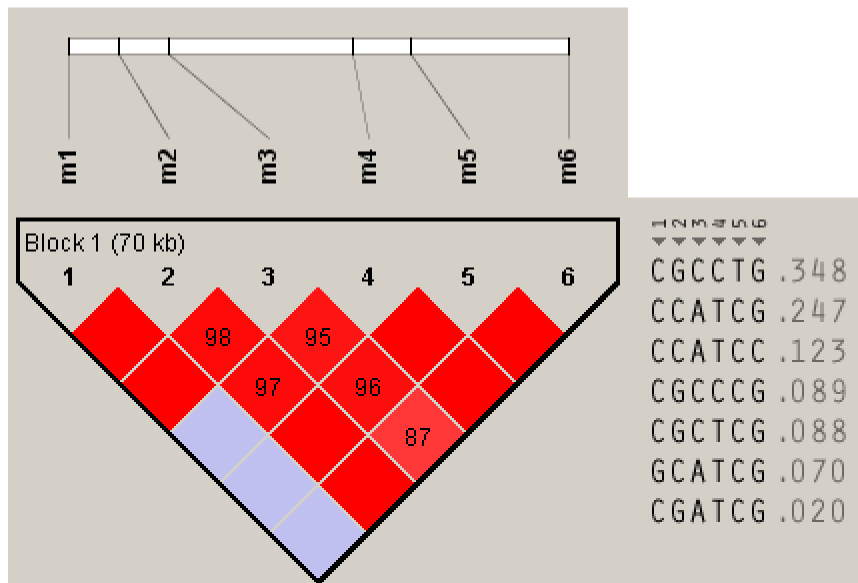

Figure 6. A Haploview illustration of the 70-kb haplotype block (Gabriel et. al 2002) in the *GPRA* gene. The numbers in each box correspond to the pair-wise linkage disequilibrium coefficient D' between respective SNPs. D' in the unmarked boxes corresponds to total linkage disequilibrium. All the samples were analyzed together and the numbers on the right represent haplotype frequencies within the whole study population. The SNPs are ordered according to their position in the gene (Figure 3, Table 5). The SNP 546333 tagging haplotype H4 was excluded from the studies because of low success rate, which made it virtually impossible to unambiguously distinguish between homozygotes and heterozygotes. In order to avoid bias in the allele/genotype distributions, we analyzed the haplotypes H4 and H5 as one (H4/H5).

Table 5. Single nucleotide polymorphisms (SNPs) within the 133-bp region defining the GPRA haplotypes H1-H8. In addition, the SNP rs324981 tagging for the Asn/Ile change in the *GPRA* exon 3 is presented. The asthma risk haplotypes in the Finnish studies are shown in gray.

	515224	522363	529556	546333	555608	563704	585883	591694
SNP	**rs323917**	**rs323922**	**rs324377**		**rs324384**	**rs324396**	**rs740347**	**rs324981**
	C/G	**G/C**	**A/C**	**G/A**	**T/C**	**C/T**	**G/C**	**Asn/Ile (A/T)**
H1	C	G	C	G	C	T	G	A
H2	C	C	A	G	T	C	G	T
H3	C	G	C	G	C	C	G	A
H4	C	C	A	A	T	C	C	T
H5	C	C	A	G	T	C	C	A/T
H6	C	G	C	G	T	C	G	T
H7	G	C	A	G	T	C	G	T
H8	C	G	A	G	T	C	G	

G. DISCUSSION

The starting point of the current study was the positional cloning of a novel asthma susceptibility locus on chromosome 7p14.3 and the identification of two potential genes within it. In this thesis work, we continued the work performed in our group and characterized both genes, *GPRA* and *AAA1* to some extent, focusing our interest ultimately to the expression and functions of GPRA.

1. GPRA expression and functions in peripheral tissues

A large fraction of the current data is based on the use of polyclonal antibodies, which have certain limitations and have to be carefully characterized before any definite conclusions can be drawn. The lack of knowledge from previous studies further impedes the interpretation of the data concerning the expression of a novel gene. Hence, the specificities of the anti-GPRA-A and -B antibodies were determined with special causion by using several parallel GPRA antibodies and commercial antibodies against different protein labels in several experimental techniques. In addition, the results were compared with the data obtained from the mRNA expression studies.

All the anti-GPRA antibodies detected their bacterial recombinant peptides with extremely high affinity in immunoblotting. The anti-GPRA-A and -B antibodies also recognized the same full-length receptor variants in immunoblotting and immunocytochemistry as the commercial antibodies against their peptide labels conjugated to the transfected GPRA constructs. As it seemed that the antibodies were specific for their intracellular epitopes, we utilized them in immunohistochemistry using either preimmune sera or species-matched IgG fractions as negative controls. The parallel GPRA antibodies against distinct epitopes of the receptor were utilized in confirming the results. Staining with the anti-GPRA-A, -B and -N antibodies resulted in staining of various epithelial surfaces as well as smooth muscle. Hence, we further demonstrated that peptide blocking eliminates the GPRA-A and -B immunoreactivity from the skin keratinocytes and subepithelial SMCs (P. Holopainen, unpublished results). For further confirmation of these results, use of several parallel techniques yielded compatible results. In agreement with immunohistochemistry, immunoblotting of human tissue lysates (including colon muscle and epithelium) with the anti-GPRA-B antibodies revealed polypeptide bands in tissues rich in epithelia, whereas detection with the anti-GPRA-A yielded chemiluminescence especially in the SMC containing tissues. For further verification, detection with the anti-cytoloop-3 antibodies recognizing both isoforms A and B resulted in positive signals in overlapping polypeptide bands and peptide blocking experiments removed the corresponding bands in immunoblotting.

Data obtained from GPRA mRNA expression studies were in line with the protein expression studies. Northern blotting analysis of several human tissues including lung, colon, small intestine and peripheral blood leukocytes resulted in detection of transcripts at correct molecular sizes. The nasal respiratory epithelium was used as a representative of pulmonary epithelium and displayed strong GPRA mRNA expression with quantitative real-time RT-PCR of

adult samples. Most importantly, characterization of GPRA mRNA expression with *in situ* hybidization yielded similar results to immunohistochemistry in overlapping locations to protein expression (Table 4). Most of the discrepancies between the results of the two methods are likely to originate from the dissimilar morphology between the small samples used in commercial tissue array stainings. Although the smooth muscle staining of GPRA-A was weakened and even removed upon peptide blocking experiments, the absence of GPRA mRNA expression in the *in situ* hybridization experiments still stays a major concern. A biological explanation for this discrepancy could involve the slower growth-rate of the SMCs in comparison to the rapidly regenerating epithelial cells, which could hamper the detection of smooth muscle -derived GPRA mRNA in the assay. Nevertheless, we could confirm the upregulated GPRA-B protein expression in SMCs of asthmatic patients at mRNA level.

These observations strongly argue that the GPRA antibodies recognize their correct epitopes, but do not totally exclude the possibility they could detect some other cross-reacting polypeptides, especially in techniques such as immunohistochemistry, in which the results are not easy to validate. This concerns especially the tissues of murine origin, although the homology between mouse and human GPRA-A epitopes was 79 % and the dissimilar amino acids possessed structural similarity. The polypeptides used for immunization contained no homology to any other known proteins than GPRA, but the cross-reactivity to epitopes not included in the current databases remains to be shown.

2. GPRA as a genetic marker of asthma

After our original association study of *GPRA* with asthma-related traits, there have been four replication studies, which have provided solid evidence for *GPRA* as a susceptibility gene for asthma and related traits. The first report did not find any associations with asthma, atopy, or total serum IgE values in a study conducted in a Korean case-control cohort consisting of over 800 subjects (Shin et al. 2004). However, instead of a complete haplotype analysis, only one SNP (rs323922) was genotyped. Further studies have suggested that rs323922 may not be the best choice for a haplotype tagging SNP. As shown, the genetic background among white and Asian populations as well as the different environment may result in different haplotypes.

In the second study, *GPRA* polymorphisms were shown to associate with asthma, rhinoconjunctivitis, and sensitization in two cohorts of European origin consisting of 3,113 and 800 children (Melen et al. 2005). The associated haplotypes could be allocated into risk (H5/H6) and nonrisk (H1/H3) groups. Especially the pattern of protective haplotypes was supported by allelic associations of two SNPs: rs324384 tagging the haplotypes H1/H3 and rs324396 tagging the haplotype H1. In parallelel with Melén and coworkers, Kormann and coworkers provided evidence that in addition to asthma and elevated serum IgE, the polymorphisms were also associated with BHR in a German study cohort (Kormann et al. 2005). The SNP546333 (Hopo546333) tagging the haplotype H4 increased the risk for asthma (odds ratio 1.40), and this

association was even more evident when concomitant asthma and BHR were present (odds ratio 2.38).

In the most recent study, the association of *GPRA* polymorphisms with airway responsiveness to metacholine was studied in a Chinese population isolate including 451 cases and 232 controls (Feng et al. 2006). The observed *GPRA* haplotype pattern was less polymorphic than in white populations and only three common haplotypes with no associations with asthma were identified. However, the AA homozygote of the SNP rs324981 flanking the nonsynonymous polymorphism in the *GPRA* exon 3 was shown to be protective against airway responsiveness to metacholine. Moreover, each additional copy of allele A reduced the risk by 21 %. An additional intronic SNP rs324987 in linkage disequilibrium with the SNP rs324981 also showed a similar association pattern.

In the European studies, the non-coding intronic SNPs within the 70-kb haplotype block accounted for the strongest associations. The nonsynonymous Asn(107)Ile polymorphism was located in the middle of this region, but didn´t initially appear as a haplotype tagging SNP. However, looking back to the sequencing data within the Finnish patients (Laitinen 2004, Table 5), Asn107Ile divides the observed risk and non-risk/protective haplotypes in the European studies into two separate groups: The individuals with the risk haplotypes carry the Ile^{107} variant whereas the subjects with the observed non-risk/protective haplotypes carry the Asn^{107} variant of the receptor (Figure 7).

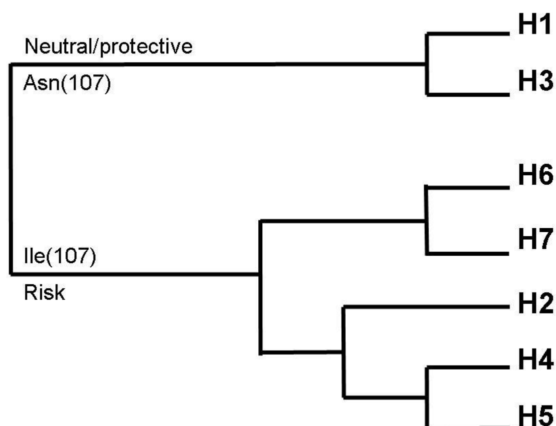

Figure 7. Phylogenetic tree of the seven *GPRA* haplotypes with frequences over 5 % in the studied European populations (Laitinen et al. 2004, Melen et al. 2005).

These association studies introduce GPRA as the best candidate gene and a drug target for asthma identified by genetic means so far. However, several important questions can be raised. Most importantly, how exactly do the polymorphisms in the GPRA gene link to the mechanisms involved in the complex pathogenesis of asthma, such as epithelial inflammation, immune mechanisms and BHR?

Reinscheid and co-authors provided one straightforward explanation. They reported that the SNP rs324981 giving rise to the Asn(107)Ile polymorphism resulted in a gain-of-function characterized by an increase in agonist potency without changing binding affinity to NPS in a GPRA overexpressing epithelial cell line (Reinscheid et al. 2005). On the basis of our results in asthmatic biopsies, the authors suggested that GPRA Ile^{107} variant could transmit enhanced contractile response in airway SMCs requiring lower agonist concentrations in asthmatic condition. Similar findings of polymorphism functions have been reported with the nonsynonymous SNP in the CysLTR and with the synonymous SNP in the PG receptor genes (section C: 3.3.1, Pillai et al. 2004, Oguma et al. 2004). Reinscheid and collegues also showed that NPS induced dose-dependent proliferation of Colo205 human colon cancer cells endogenously expressing GPRA and thus, suggested that the NPS-mediated proliferation could play a role in the remodelling events of SMCs in the developing asthma.

Further evidence of the importance of the Asn(107)Ile polymorphism was provided by Bernier and co-authors, who showed that residues 5-13 within the NPS peptide regulate GPRA activation (Bernier et al. 2006). The inhibitory effect of this regulatory region was absent in the Ile^{107} variant of the receptor suggesting an interaction between the NPS residues 5-13 and the receptor residue 107. The Ile^{107} variant of the receptor also showed increased cell-surface expression compared with the more common receptor variant, which could explain the increased signalling observed by Reinscheid et al.

3. GPRA as a marker of inflammatory diseases and respiratory distress syndrome

Gupte and co-authors showed that GPRA was expressed only in the hypothalamus and retina (Gupte et al. 2004). They suggested a role for the receptor in light detection and regulation of circadian rhythm, because the retinitis pigmentosa 9 (*RP9*) locus maps to the 7p14-15 locus. However, no association studies exist so far. According to our unpublished results, the anti-GPRA antibodies detect immunostaining in retina of man and murine origin consistent with the results of Gupte and co-authors. In addition to skin, gastrointestinal tract and lungs, also the outer parts of the eye are prone to allergic responses and thus, GPRA expression in the eye is in agreement with the theory of the receptor as a common mediator of allergic responses.

In addition to epithelial tissues lining outer and internal body surfaces that are normally populated with commensal non-dangerous microbes, we also showed that GPRA was expressed in macrophages. Just recently, the expression of GPRA in blood monocytes was confirmed using fluorescence activated cell sorter and the GPRA antibodies developed in our studies (Anna James, unpublished observations). According to our functional studies, the GPRA/NPS pathway appears to be involved in the complex signaling events of phagocytosis suggesting a function for GPRA in the innate immune system. This hypothesis is also supported by our genetic findings reporting association of GPRA with RDS, which has previously shown significant associations with genetic variants of the surfactant protein (SP) -A, -B, -C and -D genes (Hallman et al. 2002, Lahti et al. 2004, Nogee et al. 2004). SP-A binds to carbohydrate moieties on the surfaces of

viruses, bacteria and fungi and thus facilitates microbial aggregation and phagocytosis and killing of certain micro-organisms by alveolar macrophages (Pikaar et al. 1995, Eggleton 1999). In SP-D deficient (SP-D -/-) mice, SP-D is critical for the modulation of early stages of allergic inflammation in vivo. Allergen-induced Toll-like receptor 4 expression was increased in wild type mice, but not in SP-D -/- mice (Schaub et al. 2004). Thus, the expression pattern and the macrophage functions of the NPS/GPRA pathway show overlapping role with SP-A and SP-D in the first-line defence of the innate immunity (Crouch et al. 2000, Hohlfeld et al. 2002). Further evidence for these functions is supported by the results obtained from a recent microarray experiment carried out in our group (Vendelin et al., manuscript submitted). When GPRA-A overexpressing epithelial cells were stimulated with NPS and compared with the parental cell line not expressing GPRA-A, immune and defence responses to pest, pathogens and parasites were among the several upregulated gene pathways. The studies also supported our functional studies by showing that regulation of cell proliferation, chemotaxis and response to wounding were among the major upregulated pathways.

Interestingly, the newly cloned positional candidate genes for asthma have shown associations not only with AD, but also with IBD and MS (Noguchi et al. 2005, Nicolae et al. 2005). The immunomodulatory functions together with the expression pattern in the skin and throughout the digestive tract introduce the GPRA gene as a prime candidate for autoimmune diseases and allergic diseases other than asthma as well (Cookson 2004). However, no association of the GPRA polymorphisms with AD was found in a study conducted by Veal and collegues (Veal et al. 2004). This underlines the different etiopathogenesis of asthma and AD and may highlight the importance of inhaled allergens in the development of asthma. In genome wide scans, the 7p14 locus has shown significant linkages to MS (Sawcer et al. 1996) and to IBD in several studies (Satsangi et al. 1996, Cho et al. 1998, Rioux et al. 2000) as well as in a recent meta-analysis of genome scans for IBD (van Heel et al. 2004) suggesting that these diseases may share predisposing elements with asthma. NOD1 is a pattern recognition receptor located on chromosome 7p within 5 Mb of the reported peak of genetic linkage. An intronic insertion deletion polymorphism in the NOD1 gene was associated with asthma and high IgE (Hysi et al. 2005), but also to IBD (McGovern et al. 2005). NOD1 polymorphisms are also associated with atopic eczema and related phenotypes indicating that intracellular recognition of specific bacterial products may affect the presence of these diseases (Weidinger et al. 2005). However, this gene is unlikely to explain the observed linkage in the Finnish genome scan. Thus far, there are no publications reporting association of *GPRA* with allergic or autoimmune diseases of the gastrointestinal tract. Nevertheless, a forthcoming report indicates a strong association of *GPRA* haplotypes with IBD (Mauro D'Amato, manuscript submitted). The report also confirms the expression of GPRA in the gut by showing mRNA expression results from several biopsies obtained from mucosal surfaces with or without inflammation.

4. GPRA as a regulator of anxiety and sleep

We initially cloned the human *GPRA* gene from commercial cDNA samples originated from brain. During our studies, we also observed strong *GPRA* mRNA expression in the mouse hypothalamus and pituitary gland. Xu and co-workers also showed that GPRA is widely expressed in the brain with high levels in the hypothalamus (Xu et al. 2004). Unfortunately, the positive brain areas were not among the small tissue sample used in our microarray stainings. Additionally, Xu and co-workers showed that central administration of NPS promoted behavioral arousal and suppressed all stages of sleep in rodents. Furthermore, the peptide produced anxiolytic-like effects in experiments measuring behavioral responses to novelty or stress in rodents.

How could these events then be linked to inflammation and asthma? The first evidence is obtained from epidemiological studies. In a large study, psychobehavioral variables in early life, such as attention problems and overactivity, paved the way for the development of late-onset wheeze, and could even be linked to its development (Calam et al. 2005). Similarly, panic disorder and early childhood anxiety were suggested to predict subsequent asthma in adults (Hasler et al. 2005). The careful analyses have excluded the possibility of mere cause and effect phenomenon in these association studies. Nocturnal symptoms and overnight decrease in lung function are typical indications of clinical asthma and 75% of patients suffer from the symptoms at least once per week. Of those, approximately 40% experience cough and dyspnea on a nightly basis (Sutherland 2005). As a second body of evidence, increasing data indicates that these symptoms are related to circadian variations in airway inflammation and measurable physiologic outcomes, such as AHR.

Many important effector molecules in asthma are involved in sleep regulation (Majde and Krueger 2005). These include cytokines, nitric oxide, PGD2 (Hayaishi 2002), adenosine and numerous neuropeptides such as neuropeptide Y, whose receptor shares the same branch with *GPRA* in the phylogenetic tree of GPCRs. In addition to its functions in the CNS, neuropeptide Y also mediates immune functions in various inflammatory cells. On the other hand, the proinflammatory cytokine IL-1β is one of the most neurologically active molecules known. Furthermore, one of the most widely recognized effects of adenosine is its ability to control CNS functions in both physiological and pathophysiological conditions including asthma. In basal state, adenosine concentration in the extracellular space is very low, but increases after metabolically stressful conditions. Modest increases in extracellular adenosine concentrations activate specific adenosine GPCRs resulting in the modulation of many brain functions, most notably the regulation of sleep and arousal, locomotion, anxiety, cognition and memory (Haskó et al. 2005).

Microbial factors such as bacteria, LPS, and viral and synthetic double-stranded RNA, increase non–rapid-eye-movement sleep (NREM) and reduce REM in rabbits and induce IL-1, tumor necrosis factor α, IL-6 and other cytokines (Majde and Krueger 2005). According to the hypothesis, the sequence of events starts with microbial invasion into the peripherial tissues,

phagocytosis, and release of pathogen-associated molecular patterns into the tissue. They are detected by Toll-like receptors or scavenger receptors, such as MARCO (macrophage receptor with collagenous structure) (Elomaa et al. 1995, Arredouani et al. 2005), inducing local epithelium and phagocytes. These cells produce proinflammatory cytokine mediators to act on brain, where cytokines are able to induce other proinflammatory cytokines in the hypothalamus and elsewhere. As a result, this induces NREM sleep and release of mediators that may either dampen or induce the inflammatory events. Thus, the immunomodulatory effects of GPRA in the peripherial tissues observed in this work may not be contradictory to the results observed within the neuroscience community. It remains to be shown whether the functions of GPRA in peripherial tissues (and especially in SMC) are actually neurally mediated indirect actions.

5. Future challenges

There are several future challenges and important questions to be answered. What are the functions and the differences between GPRA-A and -B isoforms in various structural and inflammatory cells? What are the consequences of alternative splicing and dysregulation of isoform balance in asthma, and how exactly do they relate to the polymorphisms in the 7p14-p15 locus? At the time of writing, the findings quite strongly argue for an important role of the Asn107Ile polymorphism in the associations observed between GPRA and asthma-related traits. The findings among the Chinese patients do not only further support the hypothesis of the categorization of *GPRA* risk and non-risk/protective haplotypes, but also suggest direct involvement of the nonsynonymous polymorphism in the signal transduction of the receptor as a potential disease causing mechanism. The nonsynonymous SNP can also be found in non-asthmatic subjects and therefore, environmental factors may somehow trigger the hazardous effects of this SNP. Nevertheless, this interaction does not probably account for all of the genetic interactions. Additional genetic mechanisms of how the polymorphisms could affect the disease mechanisms include the regulation of allele-dependent splicing and/or the expression of the A and B isoforms. Future knock-down experiments with RNAi techniques and the development of GPRA antagonists will finally help in validating the functions of the various GPRA isoforms. Studies in *GPRA* deficient mice are on their way and will be helpful in clarifying the function and the biological role of GPRA. The replication and association studies of *GPRA* with other diseases as well as careful characterization of the GPRA functions in the various cells types will strengthen the understanding of the genetic mechanisms modifying the individual disease risk. The advanced techniques in the field of genetic analyses can be utilized in order to examine the interaction of *GPRA* with other positional candidate genes affecting asthma susceptibility. The effects of GPRA brain functions to asthmatic responses as well as the role of NPS as a possible mediator of these outcomes remain to be determined in future studies.

H. SUMMARY AND CONCLUSIONS

We have identified a novel asthma susceptibility gene (*GPRA*, G Protein coupled Receptor for Asthma susceptibility, alias *GPR154*, also known as neuropeptide S receptor) on chromosome 7p14.3 by means of positional cloning. *GPRA* haplotypes were associated with an increased risk of asthma related traits both in Finnish and Canadian cohorts. Asthma and respiratory distress syndrome (RDS) may share the same susceptibility and protective *GPRA* haplotypes for the individual risk of the disease.

GPRA is expressed in relevant tissues and immune cells regarding to asthma and allergic diseases. The universal expression pattern in the epithelial cells and the upregulated protein levels of GPRA in the bronchial smooth muscle cells both in asthma and in severe RDS turns *GPRA* into a potent candidate gene for other respiratory diseases as well and suggest a role for the receptor in bronchial hyperresponsiveness.

NPS, an endogenous agonist for GPRA, co-localizes with GPRA in the immune cells and in the bronchial epithelium. In a mouse macrophage cell line, NPS stimulates Gαs and Gαq -dependent phagocytosis of *Esterichia coli*. Combined with the results obtained from a mouse model of asthma, the findings suggest GPRA to participate in the removal of inhaled particles after antigen challenge.

In conclusion, the results suggest that in addition to the polymorphisms in the *GPRA* gene that influence on asthma susceptibility, the GPRA pathway is involved in the regulation of immune functions of asthma. *GPRA* gene variants may affect the individual risk for airway hyperresponsiveness in susceptible persons.

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Ville Pulkkinen

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