POSITIONAL CLONING AND PATHWAY ANALYSIS OF THE ASTHMA SUSCEPTIBILITY GENE, *NPSR1*

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ACADEMIC DISSERTATION

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To my family

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ABBREVIATIONS

AAA1	asthma-associated alternatively spliced gene 1
ADAM33	ADAM metallopeptidase domain 33
AHR	airway hyperresponsiveness
AM	alveolar macrophage
BAL	bronchoalveolar lavage fluid
BHR	bronchial hyperresponsiveness
bp	base pair
cAMP	cyclic adenoside monophosphate
cDNA	complementary DNA
CI	confidence interval
COPD	chronic obstructive pulmonary disease
COS	African green monkey kidney fibroblast-like cell line
DPP10	dipeptidyl-peptidase 10
GM-CSF	granylocyte macrophage colony stimulating factor
GPCR	G protein-coupled receptor
GPRA	G protein-coupled receptor for asthma susceptibility (synonymous to NPSR1, GPR154)
GPR154	G protein-coupled receptor 154 gene (synonymous to GPRA, NPSR1)
GO	Gene ontology
ECM	extracellular matrix
EL	extracellular loop
HEK	human epithelial kidney
HPM	haplotype pattern mining
IFNγ	interferon gamma
IgE	immunoglobulin E
ISH	*In situ* hybridization
LAR	late asthmatic reaction
IL	interleukin
LD	linkage disequilibrium
LOD	$\log_{10}$ of the likelihood ratio
LPS	lipopolysaccaride
MMP	matrix metallopeptidase (previously matrix metalloproteinase)
RACE	rapid amplification of cDNA ends
mRNA	messenger ribonucleic acid
NCI-H358	human lung epithelial carcinoma cell line
NKA	neurokinin A
NPL	non-parametric linkage
NPS	neuropeptide S
NPSR1	neuropeptide S receptor 1 (synonymous to GPRA, GPR154)
NPY	neuropeptide Y
OVA	ovalbumin, egg white protein
PHF11	PHD finger protein 11
RBM	reticular basement membrane
RT-PCR	reverse -transcription polymerase chain reaction

SCF	stem cell factor
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SP	substance P
TAC1	tachykinin, precursor 1
TDT	transmission disequilibrium test
TGFβ	transforming growth factor beta
TIMP3	TIMP metallopeptidase inhibitor 3
TM	transmembrane domain
TNFα	tumor necrosis factor alpha
Treg	regulatory T cell

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Laitinen T, Polvi A, Rydman P, **Vendelin J**, Pulkkinen V, Salmikangas P, Mäkelä S, Rehn M, Pirskanen A, Rautanen A, Zucchelli M, Gullstén H, Leino M, Alenius H, Petäys T, Haahtela T, Laitinen A, Laprise C, Hudson TJ, Laitinen LA, Kere J. Characterization of a common susceptibility locus for asthma-related traits. Science 304:300-304, 2004

II **Vendelin J**, Pulkkinen V, Rehn M, Pirskanen A, Räisänen-Sokolowski A, Laitinen A, Laitinen LA, Kere J, Laitinen T. Characterization of GPRA, a novel G protein-coupled receptor related to asthma. Am J Resp Cell Mol Biol 33:262-270, 2005

III **Vendelin J**, Bruce S, Holopainen P, Pulkkinen V, Rytilä P, Pirskanen A, Rehn M, Laitinen T, Laitinen LA, Haahtela T, Saarialho-Kere U, Laitinen A, Kere J. Downstream target genes of the Neuropeptide S-NPSR1 pathway. Hum Mol Genet 15:2923-2935, 2006

In addition, some unpublished data are presented.
The publications are referred to in the text by their Roman numerals

ABSTRACT

In the present study, we identified a novel asthma susceptibility gene, *NPSR1* (alias *GPRA*, *GPR154*), on chromosome 7p14.3 by the positional cloning strategy. An earlier significant linkage mapping result among Finnish Kainuu asthma families was confirmed in two independent cohorts: in asthma families from Quebec, Canada and in allergy families from North Karelia, Finland. The linkage region was narrowed down to a 133-kb segment by a hierarchial genotyping method. The observed 77-kb haplotype block showed 7 haplotypes (HI-H7) and a similar risk and nonrisk pattern in all three populations studied. All seven haplotypes occur in all three populations at frequences > 2%. Significant elevated relative risks were detected for elevated total IgE (immunoglobulin E) among H4 and H5 haplotype carriers, and for asthma among homozygous H2 carriers (1.4., 95% [CI] confidence interval 1.1-1.9 and 2.5, 95% CI 2.0-3.1, respectively).

NPSR1 belongs to the G protein-coupled receptor (GPCR) family with a topology of seven transmembrane domains. *NPSR1* has 9 exons, with the two main transcripts, A and B, encoding proteins of 371 and 377 amino acids, respectively. We detected a low but ubiquitous expression level of NPSR1-B in various tissues and endogenous cell lines while NPSR1-A has a more restricted expression pattern. Both isoforms were expressed in the lung epithelium. We observed aberrant expression levels of NPSR1-B in smooth muscle in asthmatic bronchi as compared to healthy. In an experimental mouse model, the induced lung inflammation resulted in elevated *Npsr1* levels. Furthermore, we demonstrated that the activation of NPSR1 with its endogenous agonist, neuropeptide S (NPS), resulted in a significant inhibition of the growth of NPSR1-A overexpressing stable cell lines.

To determine which target genes were regulated by the NPS-NPSR1 pathway, NPSR1-A overexpressing stable cell lines were stimulated with NPS, and differentially expressed genes were identified using the Affymetrix HGU133Plus2 GeneChip. A total of 104 genes were found significantly up-regulated and 42 down-regulated 6 h after NPS administration. By Gene Ontology enrichment analysis, the biological process terms, cell proliferation, morphogenesis and immune response were among the most altered. A TMM microarray database comparison suggested a common co-regulated pathway, which includes the *JUN/FOS* oncogene homologs, early growth response genes, nuclear receptor subfamily 4 members and dual specificity phosphatases. The expression of four up-regulated genes, *matrix metallopeptidase 10 (MMP10), INHBA (activin A), interleukin 8 (IL8) and EPH receptor A2 (EPHA2),* were verified and confirmed by quantitative reverse-transcriptase-PCR and for the MMP10 protein by immunoassay.

Immunohistochemical analyses revealed that MMP10 and TIMP metallopeptidase inhibitor 3 (TIMP3) were expressed in both bronchial epithelium and macrophages, and that eosinophils expressed MMP10 in asthmatic sputum samples.

In conclusion, we identified an asthma susceptibility gene, *NPSR1*, on chromosome 7p14.3. Neuropeptide S-NPSR1 represents a novel pathway that putatively regulates immune responses, and thus may have functional relevance in the pathogenesis of asthma.

INTRODUCTION

Prevalence of asthma has almost doubled in Western countries during the past decades. It is estimated that 5-15% of children and adolescents in all industrialized countries have asthma. Among children, asthma is one of the most common chronic diseases. The epidemic increase in asthma has been attributed to the Western lifestyle, including outdoor and indoor air pollution, childhood immunizations and cleaner living conditions. Asthma is apparently initiated by an inappropriate response of the specific immune system to inhaled antigens and allergens (Sengler et al., 2002; Cohn et al., 2004; Phipps et al., 2004). Even though the prevalence of asthma has been rapidly increasing, a recent epidemiological study among Swiss children and a cross-sectional survey among Italians suggest that the prevalence of asthma may level off (Galassi et al., 2006; Grize et al., 2006).

Asthma is a complex disease caused by the interaction of multiple disease susceptibility genes and environmental factors. In the field of genetics, there are two main strategies used to identify susceptibility genes in complex diseases: a candidate gene approach and a genome-wide screen approach. The candidate gene approach is hypothesis driven and based on the identification of polymorphisms within a gene of known function. The genome-wide screen approach involves the collection of well-defined populations/cohorts with a certain disease related phenotype(s), searching through all chromosomes until the approximate location of a susceptibility gene is discovered by linkage analysis, narrowing down the region of interest by fine-mapping, and genetic association analyses. The term “positional cloning” is used to describe the process whereby disease susceptibility genes are identified directly as a result of multistep genetic analysis without any prior knowledge of gene defects. The identification of susceptibility gene(s) is followed by functional studies to find out the consequences of genetic variations affecting disease pathogenesis.

There are several asthma susceptibility loci on different chromosomes that have been identified by the genome-wide linkage approach. However, only a few approaches led to the identification of novel positional candidate genes. One of the positional candidate genes for asthma is *ADAM33* on chromosome 20p13 (Van Eerdewegh et al., 2002), *PHF11* on 13q14 (Zhang et al., 2003) and *DPP10* on 2q14 (Allen et al., 2003).

The present study is based on the earlier genome-wide screen approach among a Finnish Kainuu subpopulation whereby a significant linkage was found on chromosome 7p14-p15. The strongest evidence of linkage was seen for high serum IgE [non-parametric linkage (NPL) score 3.9, P=0.0001] (Laitinen et al., 2001). This

locus was among those six that had been highlighted as possible loci by the genome-wide scan among Australian and British families (Daniels et al., 1996). In the present work, the susceptibility locus on chromosome 7p14-p15 was narrowed down by hierarchical genotyping, followed by the identification of two putative disease susceptibility genes. The linkage result was replicated for asthma in a French Canadian sample set, and for high IgE in Finnish North Karelian samples.

The functional studies focused on the characterization of NPSR1 (previously known as GPRA and GPR154), which belongs to the protein family of G protein-coupled receptors. *NPSR1* was at the time of our positional cloning an unknown gene. Thus, the gene structure, alternative splicing mechanism, and expression pattern in various endogenous cell lines and tissues were intensively studied in this thesis work. Furthermore, the identification of the endogenous ligand, neuropeptide S (NPS) in parallel studies by other research groups, enabled later studies on downstream signaling of the NPS-NPSR1 pathway. The time line of publications during this thesis work is shown in detail in Figure 1.

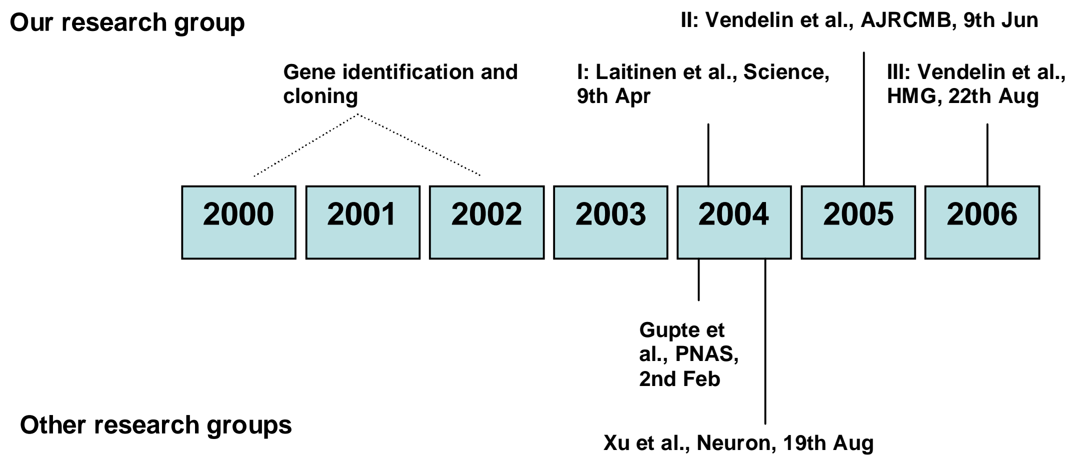

Figure 1. A time line of work and publications on the course of this thesis work

REVIEW OF THE LITERATURE

1. General features of asthma

Asthma is a chronic inflammatory disease of the airways characterized by a variable airflow obstruction and airway hyperresponsiveness (AHR), which is defined as an increased bronchoconstrictor response to nonspecific stimuli. These phenomena give rise to symptoms of wheeze, cough, chest tightness and breathlessness. In asthma, the airway wall is infiltrated with mononuclear cells, most of which are $CD4^+$ lymphocytes and eosinophils. Structural changes of the airway walls are a characteristic feature of asthma, with increased deposition of several extracellular matrix (ECM) proteins and collagens in the reticular basement membrane (RBM) and in the bronchial submucosa. Other features include an activation of smooth muscle, smooth muscle hypertrophy and hyperplasia, mucus hypersecretion and mast cell degranulation. In addition, there is vascular dilatation and angiogenesis, increased vascular permeability, and airway wall edema (Sengler et al., 2002; Cohn et al., 2004; Phipps et al., 2004).

2. Airway remodeling

In chronic asthma, the repair processes that restore normal structure and function of the airways become disturbed. Ineffective repair leads to airway remodeling, which refers to structural changes that occur in conjunction with, or because of, chronic airway inflammation. Airway remodeling involves airway wall thickening, subepithelial fibrosis, and an increase in smooth muscle, vascular proliferation, and mucous gland hyperplasia. Thus, airway remodeling involves the airway epithelium, RBM and associated fibroblast sheet (also called EMTU, for epithelial-mesenchymal trophic unit) as well as airway smooth muscle. It has been suggested that the airway remodeling in asthma may partially result from repeated acute activation of the EMTU by allergen challenge (Holgate et al., 2000; Tiddens et al., 2000; Phipps et al., 2004). An overview of airway remodeling and the related mediators is shown in Figure 2.

2.1. The role of matrix metallopeptidases in airway remodeling

Proteins of the matrix metallopeptidase (MMP) family are involved in the breakdown of extracellular matrix under normal physiological processes, such as embryonic development, reproduction and tissue remodeling. Most MMPs are secreted as inactive pro-proteins which are activated when cleaved by extracellular proteinases.

Elevated levels of MMP family members MMP2, MMP3, MMP9 and MMP12 have been detected in asthma (Kelly and Jarjour, 2003). Mattos et al. (2002) detected increased levels and activity of sputum MMP9 in patients with severe asthma compared with mild asthmatics and normal subjects. In addition, imbalance of MMPs and their specific tissue inhibitors, TIMPs such as MMP9/TIMP1, have been shown to be relevant in asthma (Matsumoto et al., 2005). Tang et al. (2006) detected significantly increased levels of MMP9 and TIMP1 in bronchoalveolar lavage (BAL) fluid of asthmatic children relative to the controls. Genetic studies have further demonstrated an association between *TIMP1* polymorphisms and asthma (Lose et al., 2005).

2.2. Structural changes of the airway epithelium

The normal bronchial epithelium is a stratified structure consisting of a columnar layer, comprising ciliated and secretory cells supported by basal cells. The epithelium has many important functions, including formation of the natural barrier against bacteria, viruses and toxic inhaled molecules. It contributes to the mucociliary clearance of inhaled matter, and modulates the bronchial smooth muscle by producing mediators and neurotransmitters (Tiddens et al., 1995; Holgate et al., 2000). In severe asthma, the bronchial epithelium is structurally disturbed so that columnar cells are separated from their basal attachments, and the ciliated cells appear to be the most destroyed cell type (Laitinen et al., 1985; Montefort et al., 1992). Epithelial shedding is characteristic of asthma and does not occur in other airway diseases such as chronic obstructive pulmonary disease (COPD) (Holgate et al., 2000). Thickening of the inner airway wall is another common feature of asthma. Thus, patients with severe asthma have thicker airways when compared with normal subjects or those with mild asthma. Airway wall thickening ranges from 10% to 300% of normal, leading to reduction in the airway luminal diameter (Homer and Elias, 2000; Elias, 2000; Cohn et al., 2004). The subbasement membrane (SBM) of asthmatics thickens as a result of deposition of collagen (types I, III and V), fibronectin, laminin $\alpha2$ and $\beta2$ chains, and tenascin in the lamina reticularis (Roche et al., 1989; Altraja et al., 1996; Laitinen et al., 1997). SBM thickening reflects that of the entire airway wall. The main source of the matrix proteins are myofibroblasts, whose numbers and activity are increased in asthma. Other factors contributing to the airway wall thickening is increases in microvascular networks and permeability (Fick et al., 1987; Brewster et al., 1990; Chung et al., 1990; Schratzberger et al., 1997).

2.3. Airway smooth muscle

The smooth muscle layer runs from the trachea to the smallest bronchioles. Smooth muscle makes up 5-10% of the bronchial wall of the small airways, but only 1-2% of the more central airway (Bosken et al., 1990; Tiddens et al., 1995). The primary

function of smooth muscle cells is to contract and alter the stiffness or diameter of the airways. Smooth muscle layers are interleaved with neurons and accessory cells are followed by an outer layer of connective tissue, containing fibroblasts, small blood vessels and various leukocytes, for example tissue macrophages. The mass of smooth muscle in adults with chronic asthma is increased and may occupy three times the normal area, predominantly because of the cell hyperplasia (Cohn et al., 2004; Singer et al., 2004; Tiddens et al., 2000). It has been shown that asthmatic airway smooth muscle cells grow at approximately twice the rate of the cells from healthy subjects. This leads to an increase in bronchial responsiveness by increasing the force in response to bronchoconstrictor stimuli and by reducing the airway diameter (Johnson et al., 2001; Tattersfield et al., 2002).

In asthmatics, airway smooth muscle putatively contributes to inflammation and airway remodeling by producing inflammatory mediators. These mediators include the chemokines eotaxin, interleukin8 (IL8), monocyte chemotactic protein-1 -2 and 3 (MCP-1, -2 and -3), macrophage inflammatory protein (MIP)1α and β, and RANTES; the cytokines IL1β, IL5, IL6, IL11 and granulocyte-macrophage-colony stimulating factor (GM-CSF); and other modulators such as cyclooxygenase-2 (COX-2), interferonγ (IFNγ), stem cell factor (SCF), tumor necrosis factor alpha (TNFα) and vascular endothelial growth factor (VEGF) (Singer et al., 2004). Furthermore, after allergen challenge and/or passive sensitization of SMC, the increased release of some matrix components, fibronectin, perlecan, laminin gamma1, and chondroitin sulfate have been detected in serum from asthmatic individuals (Johnson et al., 2001).

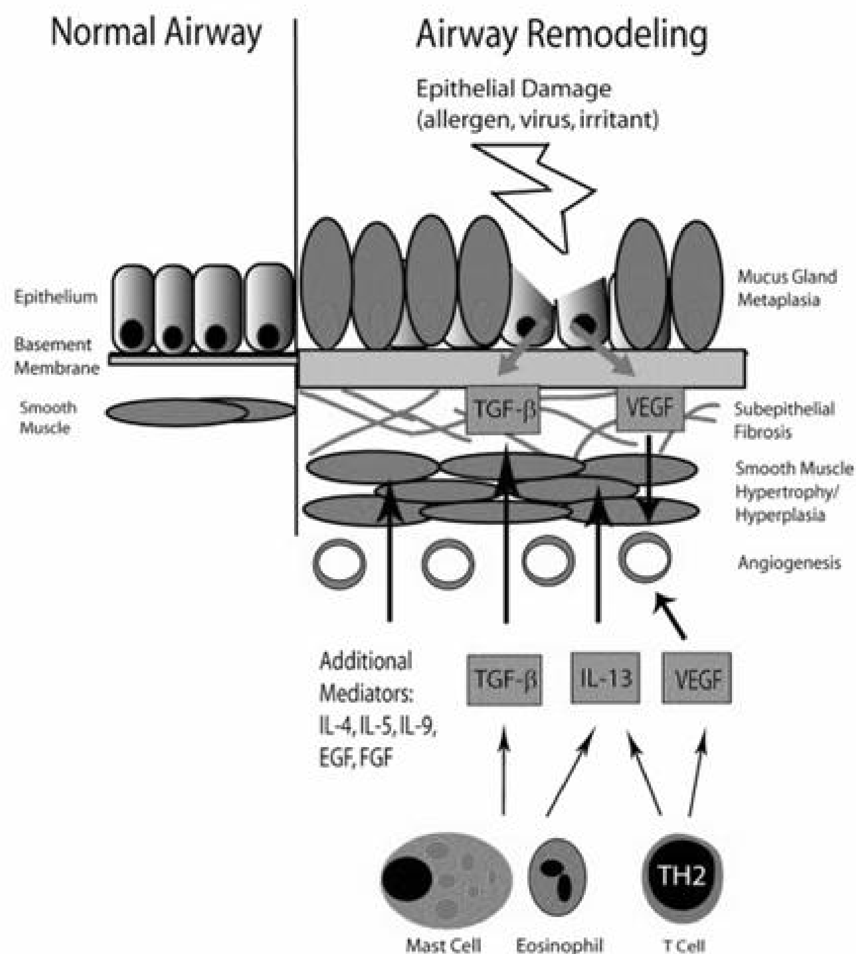

Figure 2. Airway remodeling. Inhaled allergens, viruses or irritants may induce a cascade of structural changes, collectively termed airway remodeling. These include epithelial cell mucus metaplasia, smooth muscle hypertrophy/hyperplasia, subepithelial fibrosis and angiogenesis. Studies with allergen induced remodeling in transgenic mice suggest an important role for TGFβ, VEGF and Th2 cytokines (IL5, IL9, IL13) released from inflammatory or structural cells. Abbreviations: TGFβ, transforming growth factor β; VEGF, vascular endothelial growth factor; EGF, endothelial growth factor; FGF, fibroblast growth factor. Reprinted from Doherty and Broide (2007).

3. Neurogenic inflammation in asthma

The inflammation that results from the release of substances, such as neuropeptides from airway nerves is called neurogenic inflammation. The neurogenic inflammatory effects have also been termed as “axon reflects”. The released bioactive substances act on target cells, such as mast cells, immune cells, and vascular smooth muscle cells, to produce inflammation (Barnes, 1986; De Swert and Joos, 2006). Results from several animal studies suggest that neurogenic inflammation may account for at least

some of the pathophysiology of asthma. Among the large variety of neuropeptides, those which are stored in and released from sensory nerve terminals prominently contribute to neurogenic inflammation. These sensory neuropeptides include substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide that are considered to be the major initiators of neurogenic inflammation in asthma. In addition, other biologically active peptides (e.g. neuropeptide tyrosine, vasoactive intestinal polypeptide or endogenous opioids) may modulate the inflammatory response (Groneberg et al., 2004).

3.1. Tachykinins

The tachykinin peptide hormone family include *TAC3*, which encodes neurokinin B, and *TAC1* (preprotachykinin gene), which encodes substance P and neurokinin A. The latter two are prominent neuropeptides released into the airways. Tachykinins exert their effects through the G protein-coupled receptors NK_1, NK_2 and NK_3. The tachykinins and their receptors are widely expressed in neuronal and non-neuronal cells in different human tissues. In the lung, *TAC1, TAC3* and the three tachykinin receptors are expressed at different levels in the peripheral airways, pulmonary arteries and veins, and bronchus (Pinto et al., 2004). However, a distinct subpopulation of primary afferent nerves is considered a principal source of SP and NKA (Lundberg et al., 1984). In addition, expression of SP in the airway epithelium, smooth muscle and in inflammatory cells has been detected (Lai et al., 1998; Chu et al., 2000; Maghni et al., 2003; De Swert and Joos, 2006). In animal models, the amount of tachykinins has been shown to increase in the airway neurons upon allergen challenge (Fischer et al., 1996; O'Connor et al., 2004; Dinh et al., 2005). Tachykinins have also been measured in bronchoalveolar lavage fluid (BAL), induced sputum and plasma in both healthy and asthmatic subjects. The amount of SP is increased in BAL fluid of atopic patients in comparison to non-allergic subjects (Nieber et al., 1992; Joos et al., 2003). Both SP and NKA are capable of contracting human bronchi and bronchioli, and they are potent vasodilators (De Swert and Joos, 2006).

Tachykinins have also a variety of immunomodulatory effects that putatively contribute to inflammatory processes. Substance P is produced by eosinophils, monocytes, macrophages, lymphocytes and dendritic cells. Inflammatory stimuli such as lipopolysaccaride (LPS) can upregulate tachykinins in these cells (Germonpre et al., 1999; Lambrecht et al., 1999). Substance P can induce degranulation of mast cells, causing release of TNFα, histamine and 5-hydroxytryptamine (Joos and Pauwels, 1993). The latter two are biogenic amines that are known to constrict pulmonary arteries and veins (Bradley et al., 1993). Other functions of tachykinins include inducing mucus secretion by submucosal glands, and vasodilation; inducing an increase in vascular permeability, stimulating cholinergic nerves, macrophages and

lymphocytes; and the chemo-attraction of eosinophils and neutrophils (Maggi, 1997; Joos et al., 2003).

3.2. Neuropeptides

3.2.1. Neuropeptide Y

Neuropeptde Y (NPY) is a 36 amino acid peptide, which is expressed throughout the body including the airways (Tatemoto et al., 1982). NPY exerts its effects through 5 different (Y1, Y2, Y4, Y5 and Y6) G protein-coupled receptors, some of which belong to the β group of Rhodopsin receptors (also known as class I of rhodopsin-like receptors (Berglund et al., 2003; Bjarnadottir et al., 2006). All NPY receptors are coupled to inhibitory G proteins (G_i) mediating inhibition of cAMP synthesis (Malmstrom, 2002). In the airways, NPY is present in sympathetic nerves, co-localizing with catecholamines (such as norepinephrine and epinephrine), the major class of sympathetic neurotranmitters (Lundberg et al., 1989). Upon activation of the sympathetic nervous system (e.g induced by stress, NPY together with other neuropeptides are released (Lundberg et al., 1989; Bedoui et al., 2003). NPY participates in the regulation of several physiological and psychological processes including vasoconstriction, energy balance and feeding, anxiety, depression and neuroendocrine secretion (Wahlestedt et al., 1985; Morris and Pavia, 1998; Kalra et al., 1999; Kask et al., 2002; Redrobe et al., 2002).

The exact role of NPY in allergic asthma has not been delineated so far, even though elevated levels of NPY have been detected in acute severe asthma in elderly patients (Dahlof et al., 1988). However, some earlier and recent studies with animal models highlight the importance of immunomodulatory functions of NPY that may also have relevance to asthma. Using isolated murine spleen lymphocytes, Kawamura et al. (1998) showed that NPY can induce IL4 production and decrease IFNγ production upon stimulation with CD3 antibodies. Using Y1-deficient (Y1(-/-)) mice, Wheway et al. (2005) showed that the Y1 receptor might act as a negative regulator of T cell activation as well as an activator of antigen presenting cell function. Furthermore, Y1 deficient mice had reduced numbers of B cells and increased numbers of naïve T cells. Using a monocyte/macrophage murine cell line (Raw 264.7). Ahmed et al. (2001) showed that NPY (as well as other neuropeptides such as vasoactive intestinal peptide, somatostatin and calcitonin gene-related peptide) suppressed the phagocytic and leishmanicidal capacities of macrophages at various concentrations.

3.2.2. Neuropeptide S

Neuropeptide S (NPS) is a 20 amino acid peptide cleaved from a larger precursor polypeptide. NPS precursor-like sequences are present in all tetrapods including

mammals, birds, reptiles and amphibians, but are absent from fish. NPS is a highly conserved peptide, with the first seven amino acids being perfectly conserved among all species (Reinscheid, 2007). NPS signals through the NPSR1 receptor by inducing both G_s and G_q pathways, thus eliciting intracellular cAMP and Ca^{2+} levels, respectively (Gupte et al., 2004). Expression of both the Nps precursor and the Npsr1 mRNAs has been determined in rat tissues. Both are expressed in various rat tissues with highest levels in different sections of the brain. The highest expression levels of Npsr1 mRNA were found in cortex, thalamus, hypothalamus and amygdala, while the NPS precursor was mainly expressed in brainstem nuclei. In addition, high expression of Nps and Npsr1 mRNA is found in endocrine tissues, including thyroid, mammary, and salivary glands, but a relatively low level of expression is found in rat lung tissue (Reinscheid et al., 2005). NPS may participate in regulating several different physiological and phychological functions. It has been shown to induce hyperlocomotion, increase arousal-like behaviour and wakefulness; and suppress all stages of sleep, anxiety (Xu et al., 2004) and food intake in rodents (Smith et al., 2006).

4. The role of immune cells in asthma

4.1. T lymphocytes

In both normal and asthmatic airway mucosa, the prominent cells are T lymphocytes, which are activated in response to antigen stimulation. They are subdivided into two major subsets according to their surface markers and distinct functions: CD4+ (T helper) and CD8+ (T cytotoxic) cells. CD4+ cells are further divided into Th1 and Th2 cells, depending on the type of cytokines they produce. Another subtype of CD4+ cells are regulatory T helper cells (also termed as Th3 cells or Tregs), which produce high levels of transforming growth factor β (TGFβ) and various amounts of IL4 and IL10 (Asano et al., 1996).

Asthma is associated with a shift in immune responses away from a Th1 (IFNγ) pattern toward a Th2 (IL4, IL5 and IL13) profile. CD4+ Th2 cells are commonly considered to initiate and perpetuate asthma. Tolerance to allergens is a mechanism that normally prevents Th2-biased immune responses. The activity and expansion of Th2 cells is controlled by regulatory T cells (Tregs). Tregs involved in regulating allergy and asthma consist of a family of related types of T cells, including natural CD25(+) Tregs as well as inducible forms of antigen-specific adaptive Tregs. Suppression by CD4(+)CD25(+) T cells is decreased in allergic individuals. Furthermore, CD4(+)CD25(+) T cells may contribute allergic responses by regulating airway eosinophilic inflammation. A key regulatory factor of Tregs is FOXP3, which,

upon expression is sufficient to induce regulatory T-cell phenotypes (Robinson et al., 2004; Schmidt-Weber and Blaser, 2005; Shi and Qin, 2005).

4.2. Eosinophils

Airway eosinophilia has been considered one of the central phenomena in asthma. Eosinophil numbers in sputum and airway wall correlate with disease severity. Airway eosinophilia is dependent on IL5 and STAT6 signaling. Experiments in mice have shown that in the absence of IL5, blood and BAL eosinophils are not increased in numbers in response to Th2 activation. In mice lacking IL4 and IL13 signaling, only a few eosinophils were measured in BAL or in tissue samples in response to Th2 cell activation in the airways. Eosinophils secrete among others MBP (major basic protein), ECP (eosinophil cationic protein), EP (eosinophil peroxidase), platelet-derived growth factor (PDGF) and several cytokines, including TNFα, GM-CSF, IL4, IL13 and IL5, as well as chemokines, including RANTES and eotaxin. Eosinophils enchance inflammation by producing cytokines and increase remodeling by stimulating subepithelial fibrosis (Cohn et al., 2004).

4.3. Alveolar macrophages

Alveolar macrophages (AM) are the predominant immune effector cells residing in the alveolar spaces and conducting airways of the lung. AMs are phagocytic cells, which are important in the immune regulation of the airways to protein allergens. Macrophages are the predominant cell type recovered in BAL in both non-asthmatic and asthmatic persons (Thepen et al., 1994). Alveolar macrophages are a heterogeneous pool containing different subpopulations with different phenotypes and functions (Campbell et al., 1986). The macrophages are prominent cells along the airway surface. They have putatively a dual role in both promoting and preventing inflammatory responses (Hamid et al., 2003). Alveolar macrophages suppress T cell activation and antigen presentation by dendritic cells (Holt et al., 1993; Schauble et al., 1993). Some recent findings show that AMs are capable of suppressing airway hyperresponsiveness, which is one of the characteristics of asthma (Careau and Bissonnette, 2004; Peters-Golden, 2004). Macrophages can perform accessory cell functions by presenting antigens. However, macrophages are less potent antigen presenting cells than for example, dendritic cells present in the airways (Langhoff and Steinman, 1989; Hamid et al., 2003).

AMs express the low-affinity receptor for IgE, FcεRII, the expression of which is increased in asthmatics, compared to healthy individuals (Melewicz et al., 1981). Macrophages can respond to antigens through FcεRII by releasing leukotriene B4, LTC4, PDGD2, superoxide anion, and lysosomal enzymes. The inflammatory

mediators produced by macrophages include platelet-activating factor, prostaglandin F2α and thromboxane. Pro-inflammatory cytokines produced by macrophages include IL1β, IFNγ, TNFα, IL6 and GM-CSF (Gosset et al., 1999; Hamid et al., 2003).

4.4. Mast cells

Mast cells, which originate from hematopoietic progenitor cells migrate into tissues where they complete their differentiation and maturation. Mast cells express the high affinity receptor for immunoglobulin E (IgE), FcεRI, on their surface. The crosslinking of IgE- FcεRI can induce mast cell activation and mediator release by at least four different mechanisms (Marone et al., 2005). Mast cells can respond to many different stimuli, such as SCF and LPS, via other surface receptors (e.g toll-like receptors, TLRs) that they express (Metcalfe et al., 1997; Okumura et al., 2003; Galli et al., 2005).

Upon activation, mast cells are capable of secreting a wide variety of different mediators, stored in their granules or synthesized *de novo*. Furthermore, some mediators are secreted continuously in the airways of asthmatics. Mast cell mediators include histamine; tryptases and chymase; heparin; lipid mediators such as LTC4 and PDGD2; chemokines and cytokines such as SCF, IL5, IL6, IL8, IL13, TGFβ1, TNFα and GM-CSF (Okayama et al., 2001; Galli et al., 2005).

Both human and mouse studies have implicated that Th2 cytokines regulate the mast cell infiltration into the lung that is a well-known characteristics of asthma. The increased numbers of mast cells have been detected in bronchial biopsy samples of both atopic and non-atopic asthmatics (Amin et al., 2000; Austen and Boyce, 2001). In the asthmatic lung, mast cells reside adjacent to blood vessels, in the bronchoalveolar space, beneath the basement membrane, surrounding the submucosal glands and scattered throughout the airway smooth muscle bundles (Casolaro et al., 1989; Brightling et al., 2002). A higher density of mast cells is seen during inflammation at mucosal sites, such as the respiratory mucosa (Boyce, 2003; Williams and Galli, 2000). Mast cells are primary effector cells of asthma: they are involved in acute symptoms and the early asthmatic response to allergen challenge (Corrigan and Kay, 1992).

A more complete list of mediators released from the immune cells (restricted to those dicussed in the text) during an asthmatic attack is seen in Table 1. In addition, basophils, neutrophils, dendritic cells, bronchial epithelial cells, airway smooth muscle cells and endothelial cells release mediators during an asthmatic attack (Bloemen and Verstraelen et al., 2007).

Table1. Mediators released from immune cells during induction phase, early asthmatic reaction and late asthmatic reaction.). Abbreviations: EDN=eosinophil-derived neurotoxin, PAF=platelet activating factor, ROS=reactive oxygen species, NO=nitric oxide. Modified from Bloemen and Verstraelen et al. (2007

Induction phase	
T cells	Cytokines (IL-4, IL-5, IL-9, IL-13)
Early asthmatic reaction	
Mast cells	Histamine; proteases (tryptase, chymase, carboxypeptidase); proteoglycans (heparin, chondroitin sulphate E); prostaglandins (PGD2); leukotrienes (LTC4); cytokines (TNF-α, IL-3, IL-4, IL-5, IL-6, IL-8, IL-16, GM-CSF); chemokines (CCL2, CCL3, CCL11)
Late asthmatic reaction	
Eosinophils	MBP; ECP; EDN; EP; leukotrienes (cys-LTs: LTC4, LTD4, LTE4); cytokines (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-11, IL-12, TNF-α, TGF-α, TGF-β, GM-CSF); chemokines (CXCL8, CCL3, CCL5)
T cells	Cytokines (IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, GM-CSF); chemokines (CCL1, CCL22)
Macrophages	Cytokines (IL-1, IL-6, IFN-γ, TNF-α); chemokines (CXCL8); lipids; PAF; ROS; NO

5. Environmental factors influencing the pathogenesis of asthma and related diseases

5.1. Risk factors

Sensitization to allergens is one of the main mechanisms leading to the development of asthma and other allergic disorders in genetically predisposed individuals. The most common allergens include house dust mite, grass pollen and cat (Arshad et al., 2001).

One of the most well-known environmental factors is exposure to tobacco smoke. Many reports have shown the association between environmental tobacco smoke (ETS) and asthma. It has been reported that continuous ETS exposure approximately doubles the prevalence of asthma among children (Gortmaker et al., 1982; Weitzman et al., 1990). Large epidemiological studies show that prevalence of asthma and wheezing was increased with ETS exposures. Furthermore, smoke exposure was associated with increased asthma severity and worsened lung function in a nationally representative group of the US children with asthma (Gergen et al., 1998; Mannino et al., 2001).

Air pollutants have a more complex role in predisposing to asthma. It has been shown that asthma symptoms are exacerbated to varying degrees by exposure to particulates, sulphur dioxide and nitrogen oxides. However, the substantial reduction of air pollutants over a time period in which asthma prevalence has increased in many industrialized countries argues against pollutants being a major causal effect (Tattersfield et al., 2002).

The role of viral respiratory infections in the development of asthma has been intensively studied. Previous findings have shown that respiratory viruses, the most common of which is rhinovirus, are present in most patients hospitalized for life-threatening and acute non life-threatening asthma. It has also been demonstrated that children with recurrent virally induced wheezing episodes during infancy are at higher risk for developing asthma. However, the exact mechanism is still unclear, even if it is known that viral infections lead to enhanced airway inflammation and can cause airway hyperresponsiveness (Tan, 2005; Proud and Chow, 2006). Furthermore, infections induced by respiratory viral pathogens are less frequent today than in the past while the incidence of asthma has increased (Umetsu et al., 2002).

Currently, one of the most common theories that try to explain the increased prevalence of asthma and related disorders is the hygiene hypothesis, which states that an excessively hygienic environment in early childhood may predispose to asthma, allergies and other autoimmune disorders. According to the hygiene hypothesis, numerous infections early in life favor the development of a Th1 pattern, whereas fewer infections shift the immune system towards a Th2 pattern (Strachan, 1989; Johnson et al., 2002).

5.2. Protective factors

Farming environment is one of the putative protective factors for allergic diseases. Several protective factors related to farming environment have been suggested, such as development of tolerance due to increased microbial stimulation in stables where livestock is kept, and a more traditional lifestyle, for example diet such as farm milk and housing conditions (Braun-Fahrlander, 2000; Riedler et al., 2000; Von Ehrenstein et al., 2000). However, the major environmental factor explaining the protective effect of the farming environment may not have been identified yet.

The role of parasitic infections caused by helminths (parasitic worms) has been widely studied as a protective factor for asthma and other allergic diseases. Some recent studies using a murine model of atopic or allergic asthma show that a parasitic infection can suppress allergen-induced eosinophilia, eotaxin levels, bronchial hyperreactivity and Th2 responses in an IL10 dependent manner (Wohlleben et al., 2004; Kitagaki et al., 2006). In a recent study, exposure to helminths in Central

European children, as measured by antibody levels reactive to helminth parasites, was found to be more frequent in children of farming households compared to children of non-farming households. However, this finding did not explain the protective effect of farming against atopic diseases (Karadag et al., 2006).

Gastrointestinal exposure to bacteria and bacterial products has been suggested to have a significant effect on the maturation of the immune system and thus protection against the development of asthma. It has been shown that an increased incidence of allergy is associated with a reduced prevalence of colonization with bifidobacteria and lactobacillus strains in the gastrointestinal tract (Bjorksten et al., 1999; Umetsu et al., 2002).

5.3. Chronic obstructive pulmonary disease (COPD)

COPD is a slowly progressing and mainly an irreversible disorder associated with substantial morbidity and mortality. There are several phenotypes under the single clinical COPD diagnosis i.e. those with predominant airway obstruction (obstructive bronchiolitis) and those with emphysema (parenchymal destruction). The airway limitation related to COPD is determined by reductions in quantitative spirometric indices, including forced expiratory volume at 1 second (FEV1) and the ratio of FEV1 to forced vital capacity (FVC) (Silverman et al., 2002b; Rabe et al., 2007).

Cigarette smoke is the most important risk factor for the development of COPD. It accounts for 80-90% of COPD cases in the United States. However, only 15-20% of heavy smokers develop clinically significant airway obstruction, which suggests a genetic susceptibility to the development of the disease. It should be noted, that smoking is also common among asthmatics. It is estimated, that in developed countries, one-fifth to one-third of adults having asthma are smokers (Sethi and Rochester, 2000; Petty, 2002, Thomson, 2007).

Both pulmonary and systemic inflammation related to COPD, are caused by inhalation of noxious particles, such as cigarette smoke. Inflammatory events trigger both innate and adaptive immunity. An increase in both CD8+ and CD4+ lymphocytes has been reported in patients with COPD. However, serum levels of C reactive protein (CRP) are often increased in patients with COPD independent of cigarette smoke (Rabe et al., 2007).

Asthma is much more reversible than COPD in its response to therapy, such as bronchodilators and corticosteroid drugs. Furthermore, COPD tends to be more inexorably progressive than asthma. However, smokers with chronic asthma are less sensitive to beneficial effects of corticosteroid treatment compared with non-smoking asthmatics (Petty, 2002; Thomson, 2007).

5.3.1. Genetics of COPD

The genetics of COPD is still poorly understood. So far, the only confirmed genetic risk factor for COPD is severe alpha 1-antitrypsin deficiency, which is an autosomal recessive genetic disorder (Larsson, 1978; Tobin et al., 1983). However, during recent years, some reports on the issue have been published. Ning et al. (2004) studied COPD pathogenesis by serial analysis of gene expression (SAGE) and microarray analysis among smokers. They found 327 differentially expressed genes by SAGE and 261 by microarray analysis between two groups of smokers. Among differentially expressed genes were transcription factors, growth factors and related proteins: *EGR1, FOS, CTGF, CYR61, CX3CL1, TGFB1* and *PDGFRA*. Furthermore, they localized expression of *EGR1, CTGF* and *CYR61* to alveolar epithelial cells, airway epithelial cells, and stromal and inflammatory cells of the smokers. Demeo et al. (2006) integrated results from microarray studies of murine lung development and human COPD gene expression. In addition, based on their earlier linkage results on chromosome 2q (Silverman et al., 2002a), they identified *SERPINE2* as a susceptibility gene for COPD in a family-based association study of 127 pedigrees. They suggested that *SERPINE2* is influenced by gene-by-smoking interaction, and polymorphic variants in the *SERPINE2* gene could contribute to the development of COPD through alterations in matrix metallopeptidase pathways. SERPINE2 belongs to the serpin family of proteins, as alpha 1-antitrypsin. Furthermore, the region on chromosome 2q33 has shown overlapping linkage to asthma-related traits (Postma et al., 2005).

6. Murine models of asthma

None of the current mouse models duplicate all features of human asthma. However, one of the most widely used murine models for acute asthma/airway inflammation is an ovalbumin (OVA) sensitization/challenging protocol and modifications thereof. Using mice has several advantages. Due to their small size, mice are easy to handle and inexpensive. Mice have numerous inbred strains and there are species-specific reagents available. IgE is a major class of anaphylactic antibody and the mouse demonstrates airway hyperresponsiveness to nonspecific stimuli. Disadvantages of mice include poorly developed airway smooth muscle, weak responses to histamine and vasculature as an anaphylactic target (Karol, 1994).

6.1. Mouse lung inflammation by challenging with ovalbumin (OVA)

In murine models for acute asthma/airway inflammation, mice are sensitized with ovalbumin (OVA) and thereafter challenged with aerosolized OVA. Prior sensitization to OVA is in most cases done by intraperitoneal injection by an adjuvant containing (e.g. alum) or adjuvant-free protocol. Thereafter, (e.g. at day 14) mice are

exposed daily to aerosolized OVA by periodical inhalation for a few days or longer. In some cases, a non-surgical technique with multiple intratracheal instillations of OVA has been used. These protocols have repeatedly demonstrated some human asthma-like responses: increased infiltration of neutrophils, eosinophils or lymphocytes into the lungs, greater airway responsiveness to non-specific stimuli like methacholine, excessive mucus production and elevated levels of Th2 -type cytokines and serum IgE (Blyth et al., 1996; Krinzman et al., 1996; Hamelmann et al., 1999). However, OVA sensitized and challenged mice may lack some features of chronic asthma: mucosal inflammation, recruitment of eosinophils into the epithelial layer, sub-epithelial fibrosis and epithelial changes. Mice need to be challenged with OVA repeatedly for several weeks, even for up to 8 weeks to induce some of the chronic asthma-like symptoms (Temelkovski et al., 1998; Kumar et al., 2004).

6.2. Mouse lung inflammation by challenging with *Stachybotrys chartarum*

Stachybotrys chartarum is a damp building mould that has been associated with pulmonary health problems including asthma. It may impact humans through both immunologic and toxic mechanisms (Barnes et al., 2002). Murine models using *S. chartum* as the sensitizing agent have been developed, and they represent one of the modified murine models for asthma. In their mouse model, Leino et al. (2003) exposed BALB/C mice intranasally for 3 weeks to spores of a satratoxin-producing and non-producing *S. chartum* strain. They observed a dose-dependent increase in inflammatory cells, mostly macrophages and neutrophils, in BAL fluids after intranasal challenge of the spores. Infiltration of the inflammatory cells was associated with several pro-inflammatory cytokine (IL1beta, IL6, TNFalpha) and leukocyte attracting chemokine (CCL3/MIP1alpha, CCL4/MIP1beta, CCL2/MCP1) mRNA levels in the lungs. The former pro-inflammatory cytokines are known products of macrophages. There were no differencies between satratoxin-producing and non-producing *S. chartum* strains in BAL, but CXCL5/LIX mRNA levels were higher after exposure to satratoxin-producing spores. They concluded that components other than satratoxins are mediating the development of the inflammatory response in their model. Unlike in OVA-mouse models, bronchial responsiveness to methacholine, IgE, IgG2a and IgG1 antibody, and Th1 and Th2 cell levels were not changed after mould exposure.

Viana et al. (2002) used an extract of *S. chartum* to challenge BALB/c mice, to induce asthma-like responses. In this experiment, the crude antigen preparation of a combined mixture of five different *S. chartum* isolates (SCE-1) was used. Female mice were sensitized by involuntary aspiration of SCE-1 extract and subsequently re-exposed for up to 4 weeks. Mice receiving four doses of SCE-1 had increased BAL and serum IgE levels, significant influxes of lymphocytes and eosinophils, and increased levels of the Th2 cytokine IL-5. In contrast, animals exposed to only one

dose of SCE-1 demonstrated nonspecific inflammatory responses, but did not have elevations in levels of IgE, IL-5, or eosinophilia in BAL. In both cases, there was no bronchial hyperresponsiveness to methacholine.

Some differences in responses (e.g. elevated IgE and IL5 levels) compared to the mouse model of Leino et al. (2003), may be due to different doses, higher numbers of strains and differences between *S. chartum* strains used to induce inflammation. Furthermore, Viena et al. (2002) did not separate toxin producing and non-producing strains, and some endotoxin levels were measured in extracts.

6.3. Advantages of the guinea-pig model compared with the mouse model

In some cases, the guinea-pig model is better than the mouse model. An important advantage in guinea-pigs compared to mice is that lung is their major shock organ with their airways and tracheal smooth muscle responding to histamine. Guinea-pigs demonstrate both early and late asthmatic reactions (LAR). There is eosinophilic inflammation during LAR and neutrophil influx to lung following LAR. Major disadvantages of the guinea-pig model are the existence of few inbred stains and species-specific reagents. Furthermore, IgG_1 is the major anaphylactic antibody (Karol, 1994).

Bronchoconstriction is one of the hallmarks of asthma. The guinea-pig trachea model has been used to study contractile effects of different agents. Bäck et al. (2001) utilized a guinea-pig trachea model to examine the effects of contractions to cysteinyl-leukotriene metabolism. Briefly, they used spirally cut trachea that was divided into four equal preparations in organ baths containing Tyrode's solution and gassed with CO_2 in O_2.

7. Identification of disease susceptibility genes

7.1. Linkage analysis

Linkage analysis tests for co-segregation between the disease phenotype (trait) and DNA markers. Thus, the linkage is a tendency of two closely located loci in the genome to be inherited together more often than independently of each other. The linkage method is either parametric, which directly estimates the recombination fraction assuming a Mendelian inheritance model, or non-parametric, which indirectly tests for excess allele sharing among affected relatives. The non-parametric method is commonly used to detect quantitative trait loci (QTLs) in complex diseases (Weeks and Lathrop 1995; Vink and Boomsma, 2002).

In linkage analyses, a large number of highly polymorphic microsatellite markers of known locations evenly dispersed throughout the whole genome are chosen, and the alleles are determined in individuals from multiple generations. The LOD score ($\log_{10}$ of the likelihood ratio) value is used to estimate the strength of parametric linkage whereas the NPL (non-parametric linkage) score is commonly used for non-parametric linkage. Evidence of linkage is present when maximal score values exceed a pre-defined threshold, which depends on the size of the genome and the number of markers (Kruglyak and Lander, 1995; Ott and Hoh, 2000).

The chromosomal region surrounding a marker with a significantly high LOD- or NPL score will be selected for fine-mapping, where a denser set of markers are used to narrow down the susceptibility region in a single chromosome. If the region is sufficiently small, for example 100-200 kb, it may be fully sequenced in study samples to identify the genetic polymorphisms related to a disease (Kere and Laitinen, 2004).

7.2. Association analysis

Association studies aim to compare an association between a disease and a specific allele in groups of unrelated cases (patients) and controls (healthy subjects) to assess the relative allele frequencies of genotypes. The frequencies of the two variant forms (alleles) of a SNP or microsatellites are of primary interest for the identification of disease susceptibility genes. Basically, SNPs can be either anonymous variants within or between genes (i.e. uncharacterized in respect to protein coding or gene function) or functional, causal mutations (Cardon and Palmer, 2003).

There are two common types of association analyses: population-based and family-based case-control approaches. The latter one includes extended pedigrees, relative-pairs, parent-child trios and nuclear families. The population-based and family-based association studies differ on how controls are selected. In population-based methods, a large set of samples are randomly selected from the "at-risk" population. The family based case-control approach uses healthy biological relatives of cases as controls (Ackerman et al., 2005; Bull et al., 2005). The TDT (transmission disequilibrium test) is a commonly used family-based method, which can be utilized to test for association in the presence of linkage. The TDT compares the frequency of transmission versus non-transmission of specific marker alleles from parents to offsprings (Spielman et al., 1993; Spielman and Ewens, 1996).

The indirect association method employs linkage disequilibrium (LD) mapping. LD can be determined as a measure of the degree of non-random association of two markers, i.e. alleles at adjacent loci (Collins, 2000; Collins et al., 2004). If LD exists, the alleles at adjacent markers are in association with the disease at the population

level. LD mapping includes a search for a common ancestral haplotype inherited by the affected individuals in the population. Haplotypes are characterized by a block of sequence within which there is a high LD among common SNPs, but between which there is low LD. LD is decayed through gene conversion and recombination over time, and the strength of LD depends on the age of the mutations and on the history of human population size and structure. One of the general hypotheses in LD-based association studies is the common disease/common variant (CD/CV) hypothesis, which states that genetic susceptibility for common diseases is often influenced by relatively common predisposing alleles (Reich and Lander, 2001).

7.3. Gene prediction and identification

As a result of the Human Genome project (HGP) and the parallel genome project by Celera Genomics (Lander et al., 2001; Venter et al., 2001) the identification of human disease genes has become a less laborious and time consuming process. The availability of the complete human genome sequence data (International Human Genome Sequencing Consortium, 2004) together with the complete sequences of several other organisms (Gibbs et al., 2004; Mouse Genome Sequencing Consortium et al., 2002) enables more specific database searches and the use of comparative genomics. In the past, physical maps were constructed using overlapping genomic clones i.e. BACs (bacterial artificial clones) to cover the linkage regions. Gene-prediction software programs, for example Genscan (Burge and Karlin, 1997) and Fgene (Solovyev and Salamov, 1997), were intensively used to predict protein-coding exons and genes. These programs search for conserved exon-intron structures, such as acceptor and donor splice sites, or specific signals for 5' (a TATA box and/or translation start codon) and 3' exons (a stop codon and/or polyadenylation signal) However, gene-prediction programs have several limitations. They sometimes fail to detect the correct exon-intron boundaries, miss exons or detect false exons (Claverie, 1997). Therefore, the best results are obtained using a combination of several different gene-prediction programs, and each prediction needs experimental verification.

An overview of positional cloning procedure is shown in Figure 3.

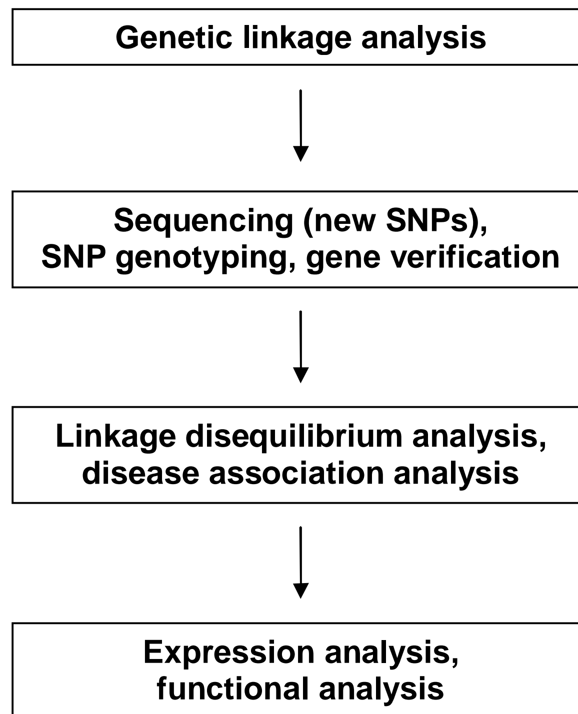

Figure 3. A positional cloning procedure. Genetic linkage analysis implicates susceptibility loci. These are narrowed down by genetic association analysis using microsatellite markers and single-nucleotide polymorphisms (SNPs) at very high density (i.e. SNP per 1-5 kb). Patient DNAs are sequenced to discover putative new susceptibility SNPs. Gene structures are verified experimentally, on the basis of sequence predictions and database information. Linkage disequilibrium between SNPs and disease associations are analyzed after genotyping patients and controls. The implicated genes are assessed for expression patterns and for functional differences between patients and controls. Modified from Kere and Laitinen, (2004)

8. Asthma susceptibility loci and positional candidate genes

Asthma is caused by an interaction of several susceptibility genes and environmental factors and therefore it is a complex disease. An overview of all positionally cloned asthma susceptibility genes is shown in Table 2.

8.1. *ADAM metallopeptidase domain 33 (ADAM33)* on chromosome 20p13

A genome-wide linkage scan that was performed on 460 Caucasian families identified a locus on chromosome 20p13 that showed a significant evidence of linkage to asthma [$\log_{10}$ of the likelihood ratio (LOD), 2.94)] and bronchial hyperresponsiveness

(BHR) (LOD, 3.93). The affected sib-pair (affected children having the same biological parents) families were collected in the USA and in the United Kingdom. In total, 40 genes were identified in a 2.5 Mb 90% confidence interval region spanning the peak of linkage. The genes were prioritized based on their potential function, expression in the relevant tissues and location with respect to the peak LOD score for BHR. Association analysis using a case-control study design was performed. Analyses of 135 nucleotide polymorphisms (SNPs) in 23 genes (spanning the 90% confidence interval) revealed that *ADAM33* was most significantly associated with asthma. Transmission disequilibrium test (TDT) (which uses family based controls) and haplotype analyses supported a positive association with asthma (P = 0.04–0.000003) (Van Eerdewegh et al., 2002).

ADAM33 (ADAM metallopeptidase domain 33) belongs to the family of type I transmembrane metallopeptidases (former metalloproteinases), the members of which have been implicated in a variety of biological functions. About half of the thirty four ADAM proteins identified to date, including ADAM33, were predicted to be active proteinases based on the presence of the zink binding motif and a glutamic acid in the catalytic domain (Becherer and Blobel, 2003; Black and White, 1998). ADAM33 has been demonstrated to possess catalytic activity (Zou et al., 2004). The structure of ADAM proteins is conserved and characterized by eight domains: the N-terminal secretion signal sequence, pro- and catalytically active domains, a disintegrin-like domain, the cysteine-rich domain, EGF domain, transmembrane domain and cytoplasmic domain. ADAMs can potentially interact with integrins (via disintegrin-like domain), syndecans (via cysteine-rich and EGF domains) and the SH3 domain containing proteins, such as the Src family proteins (via binding site in cytoplasmic tail (Seals and Courtneidge, 2003).

ADAM33 is expressed in smooth muscle, myofibroblasts and fibroblast of asthmatic airways as demonstrated by Holgate et al. (2005) using *in situ* hybridization. Furthermore, they found that ADAM33 is preferentially expressed in mesenchymal cells of the airways, adjacent to the basement membrane. They further suggested that ADAM33 might affect mesenchymal cell migration, differentiation and proliferation. Alteration of its activity may underlie abnormalities in the function of smooth muscle cells and fibroblasts linked in airway remodeling and BHR. So far, no experimental evidence has been shown to support these suggestions. Furthermore, the effects of polymorphisms on the functional properties of ADAM33 are not known, even though two asthma-associated SNPs in the catalytic domain are of interest. Therefore, the exact role of *ADAM33* in asthma remains to be elucidated

To date, at least eight association studies, including samples from 14 populations, have been carried out to replicate the original association of *ADAM33* to asthma. A positive association with diverse asthma phenotypes (with the lowest p-value 0.0009)

was found in five studies (Howard et al., 2003; Jongepier et al., 2004; Raby et al., 2004; Werner et al., 2004; Hirota et al., 2006). However, no single SNP was associated across all populations. In addition, some haplotype analysis carried out revealed that no single haplotype accounted for asthma susceptibility risk.

8.2. *Dipeptidyl-peptidase 10 (DPP10)* on chromosome 2q14

The locus on chromosome 2q14-q32 has earlier been linked to asthma and related phenotypes by at least four linkage studies (Daniels et al., 1996; Hizawa et al., 1998; Wjst et al., 1999; Koppelman et al., 2002a). A positional candidate gene for asthma, *dipeptidyl-peptidasae 10 (DPP10)*, was identified on 2q14 (Allen et al., 2003).This linkage study contained 244 families and 1122 subjects, including 293 asthmatic children and 103 asthmatic sibling pairs collected in Australia and in the United Kingdom. For a replication study, 129 severe adult asthmatics, 49 severe childhood asthmatics and 92 mild asthmatics were collected in London, UK. The total serum IgE concentration was used as a quantitative measure of atopy. An association to asthma was found and replicated. The surrounding region was sequenced and a high-density SNP linkage disequilibrium (LD) map was constructed. The strongest association was limited to the 5' parts of the *DPP10* gene, which represented the only gene expressed from the region. The polymorphic sites are located in the intron and promoter regions of *DPP10,* suggesting that they influence the expression and/or splicing of *DPP10* mRNA (Allen et al., 2003).

DPP10 belongs to a family of proteins characterized by structural similarity to dipeptidyl-peptidase 4 (DPP4), which is a membrane bound enzyme belonging to the S9B prolyl oligopeptidase class of serine proteases. DPP10 is highly homologous with the subfamily member DPP6 (also known as DPPX). Both of these proteins lack serine, which is replaced by other residues in their catalytic active site, suggesting that they may not act as enzymes *in vivo* (Allen et al., 2003).

DPP10 is expressed strongly in the brain, pancreas, spinal cord and adrenal glands. DPP10 is prominently expressed in neurons of the brain, and in nodose and dorsal root ganglia in the airways (Ren et al., 2005; Zagha et al., 2005). Nodose ganglion neurons project afferent nerves to lung and airways, controlling the sensitivity of bronchi to variety of stimuli (Carr and Undem, 2003).

DPP10 modulates Kv4-mediated A-type potassium channels (voltage-gated K^+ channels), which are responsible for a large portion of the rapidly inactivating outward K^+ current (A-type current) in many neurons (Zagha et al., 2005).Based on the expression pattern of DPP10 and its modulating activity of Kv4 channels, DPP10 may affect the abundance or gating properties of Kv4 channels in the neurons of the

airways. DPP10 may also function in other cell types such as T cells (Ren et al., 2005).

To date, no replication association studies have been reported that would confirm the association of *DPP10* with asthma or related phenotypes.

8.3. *PHD finger protein 11 (PDF11)* on chromosome 13q14

The chromosomal locus 13q14 has been linked to atopy and total IgE serum levels by multiple genome-wide screens (Daniels et al., 1996; Hizawa et al., 1998; Kimura et al., 1999; Beyer et al., 2000). Association [P (corrected) <0.005] between total serum IgE levels (LnIgE) and a microsatellite marker on 13q14 was implicated by Anderson et al. (2002). Zhang et al. (2003) used nuclear families from population sample sets collected in Australia and in the UK (including both atopic and non-atopic members). Additionally, nuclear families with atopic dermatitis were included in the studies. Case-control analyses included adults and children with severe asthma and individuals with mild asthma. They constructed a high-density SNP map around the associated region. Error checking and haplotype generation were also carried out. The region of an association to LnIgE centered on the gene *PHF11.* With stepwise analyses the most significantly associated SNPs were found on introns IX and V, and the 3' untranslated region of *PHF11*. It should be noted, that there is a histone H3 methyltransferase gene, *SETDB2* (SET domain, bifurcated 2) and *RCBTB1*, which is a regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 1, in the close proximity of *PHF11*. The positive association was replicated in atopic dermatitis sample sets by genotyping six markers. In addition, transmission disequilibrium test with combined family materials showed a positive association to asthma.

PHF11 contains two PHD zinc finger domains suggesting its function as a transcription factor. PHF11 is expressed in many tissues. In addition, preferential expression in immune-related tissues, and a lower expression in the lung and brain was shown (Zhang et al., 2003). The exact function of *PHF11* and the effects of polymorphisms related to asthma or atopy remain to be elucidated.

To date, one replication study is published showing the association of two polymorhisms in the *PHF11* gene to atopic dermatitis in Australian population by TDT (with p values 0.029 and 0.007) (Jang et al., 2005). In addition, one recent study shows a weak association (P=0.03-0.05) of the *PHF11* locus with asthma (Hersh et al., 2007).

8.4. *HLA-G histocompatibility antigen, class I, G (HLA-G)* on chromosome 6p21

Linkage to asthma or related phenotypes on chromosome 6p21 has been reported in at least seven different genome-wide screens (Table 1). Nicolae et al (2005), carried out a genome-wide linkage study with families in the "Collaborative Study on the Genetics of Asthma" (1997a; Xu et al., 2001) and found the strongest linkage signal on chromosome 6p21 at marker D6S1281 (LOD=3.6). This marker is located at a distance of 2.5 cM from the human leukocyte antigen (HLA) complex cluster. The region between the HLA cluster and the marker is relatively gene-rich, including at least 20 known or predicted genes.

Fine-mapping and association analyses were carried out. A SNP linkage disequilibrium (LD) map was constructed and the pairwise combinations of SNPs were analyzed using the TDT. The strongest association to asthma was observed with polymorphisms in *HLA-G*. A significant association to BHR was found in the Hutterites, a founder population of European descent, ($p<0.05$) in studies with a case-control design.

HLA-G is a Major Histocompatibility Complex class I molecule that is defined as a non-classic HLA class I antigen, characterized by different isoforms (membrane-bound or soluble molecules), low polymorphism and restricted tissue expression under non-pathological conditions (Ober and Aldrich, 1997). In the asthmatic lung, the soluble isoform of HLA-G is expressed in bronchial epithelial cells (Nicolae et al., 2005). Furthermore, HLA-G is expressed in adult macrophages, dendritic cells, and myoblasts in response to inflammation (Yang et al., 1996; Khosrotehrani et al., 2001; Wiendl et al., 2003). Recently, it was reported that peripheral blood monocytes of asthmatic patients have decreased soluble HLA-G production that may be caused by decreased IL-10 production (Rizzo et al., 2005).

To date, no replication studies have been published to confirm the association of *HLA-G* with asthma or related traits. However, the expression of HLA-G in immunologically important tissues and cell lines (as described above) and its function in the inhibition of NK and T-cell effector functions (Rouas-Freiss et al., 1997; Le Gal et al., 1999;) support an importance of this molecule in immunoregulation in general.

8.5. *The beta2-adrenergic receptor gene (ADRB2)* on chromosome 5q31-q32

The chromosomal locus 5q31-q33 has been linked to asthma or atopy by many genome-wide linkage studies, which have utilized several asthma-associated quantitative traits: bronchial hyperresponsivenesss, blood eosinophil counts, percentage of positive skin prick test, and total and serum specific serum IgE levels (Xu et al., 1995; Postma et al., 1995; Palmer et al., 1998; Webb et al., 2007).

However, genetic linkage to 5q suggests the presence of at least two genes influencing allergy and asthma (Xu et al., 1995). *The ADRB2* (adrenergic, beta-2-, receptor, surface) gene located on chromosome 5q31-q32 is one of the strongest candidates for asthma. It encodes beta-2-adrenergic receptor, a member of the G protein-coupled receptor superfamily. The receptor is directly associated with one of its effectors, the class C L-type calcium channel Ca(V)1.2. (Yang-Feng et al., 1990; Davare et al., 2001). Beta-2 -adrenergic receptor (β_2AR) is expressed in airway smooth muscle cells.

Reihsaus et al. (1993) found in their association study of ADRB2 nine different SNPs in the coding region. Two SNPs occur at high allelic frequencies in the general population and correspond to substitutions of arginine for glycine at amino acid position 16 (Arg16Gly) and glutamine for glutamate at amino acid position 27 (Gln27Glu) (Reihsaus et al., 1993). These polymorphic sites in ADRB2 have been later extensively studied for their possible association with asthma or related phenotypes. There are reports showing association of the Gly16 polymorhisms in asthma-related phenotypes: nocturnal asthma (Turki et al., 1995; Santillan et al., 2003; Yin et al., 2006), asthma severity (Holloway et al., 2001) and bronchial hyperresponsiveness (D'amato et al., 1998). Two reports also show association of Glu27 to elevated IgE levels in asthmatic families and to childhood asthma, respectively (Dewar et al., 1997; Hopes et al., 1998). A recent meta-analysis of 28 published studies confirmed the association between the Gly16 polymorphism and nocturnal asthma, but found no association between the Arg16Gly or Gln27Glu variants and overall asthma susceptibility or bronchial hyperresponsiveness (Contopoulos-Ioannidis et al., 2005). Thus, the results of the studies showing association to asthma are controversial. A recent large-scale study suggests the existence of additional genetic variants besides Gly16Arg that may be important in determining asthma phenotypes. The data suggest that the length of a poly-C repeat (+1269) in the 3' untranslated region of ADRB2 may influence lung function, and may be important in delineating variation in beta-agonist responses, especially in African Americans (Hawkins et al., 2006).

Table 2. Positionally cloned asthma susceptibility genes. It should be noted, that *NPSR1* (alias *GPRA, GPR154*) on chromosome 7p14 is presented in this thesis work. Modified from Kere and Laitinen, (2004).

Chr	Gene name	Primary functions	Suggested role in asthma	Effect of polymorphisms	Reference(s) to genetic linkage	Reference to cloning
20p13	*ADAM33*	Metallopeptidase	Airway remodelling by fibroblasts and smooth muscle hyperreactivity	Both amino acid substitutions and 3′ noncoding changes	Van Eerdewegh et al., 2002	Van Eerdewegh et al., 2002
13q14	*PHF11*	Zinc finger transcription factor	Immunoregulation, especially of B lymphocytes	Noncoding; regulation of alternative splicing	Daniels et al., 1996; Hizawa et al., 1998; Kimura et al., 1999; Beyer et al., 2000	Zhang et al., 2003
2q14	*DPP10*	Dipeptidyl peptidase	Cytokine processing, especially in T cells	Noncoding; altered transcription factor binding to promoter, alternative splicing	Daniels et al., 1996; Hizawa et al., 1998; Wjst et al., 1999; Koppelman et al., 2002b	Allen et al., 2003
6p21	*HLA-G*	HLA class I antigen	Inhibition of NK and T-cell effector functions, immunosuppresive functions	Amino acid substitutions in the coding region	Daniels et al., 1996; 1997b; Ober et al., 1999; Wjst et al., 1999; Yokouchi et al., 2000; Xu et al., 2001; Koppelman et al., 2002b	Ellis et al., 1990
5q31-q32	*ADRB2*	G protein-coupled receptor	Regulate reactivity of airway smooth muscle in response to airway inflammation	Amino acid substitutions in the coding region; altered receptor regulation	1997b; Ober et al., 1998; Ober et al., 2000; Yokouchi et al., 2000; Haagerup et al., 2002; Blumenthal et al., 2004	Kobilka et al., 1987

9. G protein-coupled receptors

9.1. General features

G protein-coupled receptors (GPCRs) constitute the largest protein family in the human genome. It is estimated that ~ 3% of the human genes (more than 800 genes) encode for these types of receptors. GPCRs are plasma membrane bound receptors, which share the overall structure of an extracellular N terminus, an intracellular C terminus and 7 α-helically arranged transmembrane domains (TMs) connected with

three alternating extra- (E1-E3) and intracellular (I1-I3) peptide loops. TMs consist of stretches of consecutive 25-35 amino acid residues with a high degree of hydrophobicity. Another feature for GPCRs is their ability to interact with a heterotrimeric G-protein. The main function of GPCRs is to recognize a diversity of extracellular ligands and to transduce their signals into the cell (Bockaert and Pin, 1999).

In the absence of agonist, GPCRs are in the low affinity state. Upon agonist binding they go through conformational changes that enable their interaction with heterotrimeric G proteins (GTP-binding proteins) located on the inner side of the plasmamembrane. G proteins are composed of an α subunit and tightly associated β and γ subunits. Activation leads to dissociation of the α subunit from the βγ dimer, which both activate several membranous or cytosolic effector proteins (see Figure 4). GDP is released from the G protein and is replaced by GTP. The α subunit is responsible for GTP and GDP binding and for GTP hydrolysis. To date, at least 28 different α subunits have been identified. Heterotrimeric G proteins can be divided into 4 main families based on the degree of primary sequence similarities of their α subunits: G_s, G_i, G_q and G_{12} (Pierce et al., 2002).

Gα and Gβγ can directly interact with enzymes or ion channels, increasing or decreasing their products or ionic currents, which are the secondary messengers. The following interactions are known: G_s proteins couple to stimulation of adenylyl cyclase; G_i proteins couple to inhibition of adenylyl cyclase and stimulation of ion channels, for example G protein-coupled inwardly rectifying potassium (GIRK) channels; G_q proteins couple to activation of phospholipase Cβ; and G_{12} couples to activation of guanine nucleotide exchange factors (GEFs). GEFs are distinguished from other regulatory factors by their ability to interact preferentially with the nucleotide-depleted state of G proteins. GEFs may interact with RHO GTPases. The secondary messengers include, for example cyclic nucleotides (cAMP, cGMP), phoshatidylinositide metabolites (IP_3, DAG, PIP3), and ions (Ca^{2+}, K^+) (Pierce et al., 2002; Kristiansen, 2004; Landry et al., 2006). Overview is shown in Figure 4.

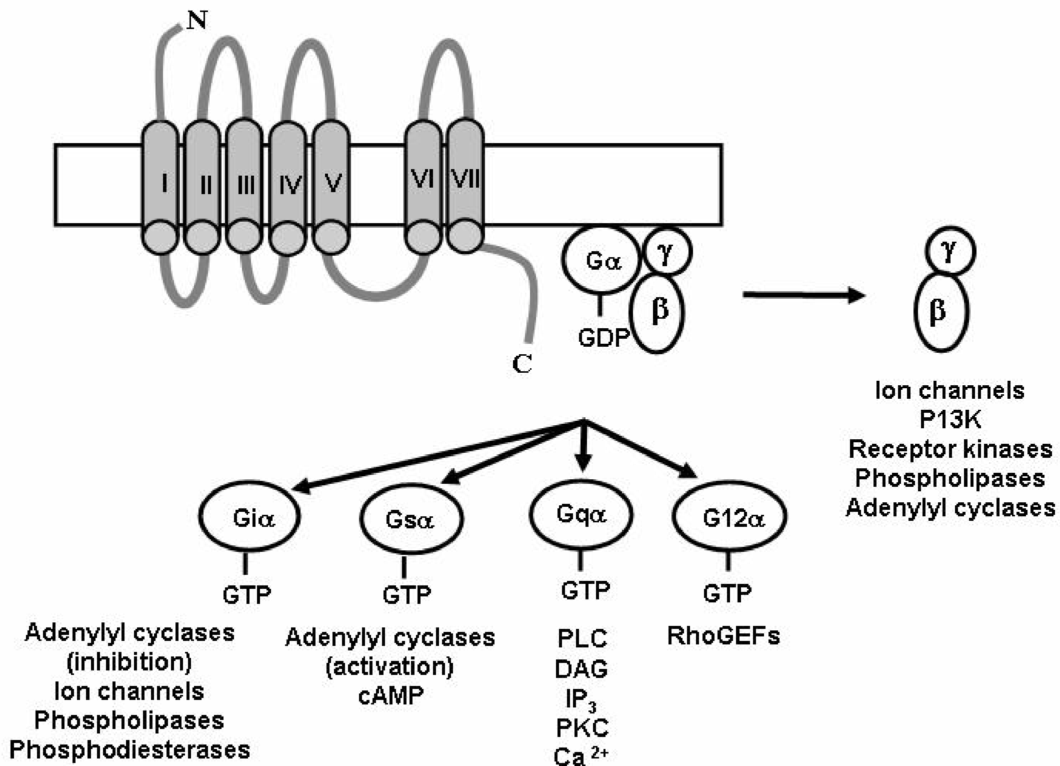

Figure 4. G protein-coupled receptor pathways. G protein-coupled receptors consist of 7 transmembrane domains. Upon activation they interact with heterotrimeric G proteins that leads to dissociation of the α subunit from the βγ dimer. GDP is released from the G protein and is replaced by GTP. The four most common secondary signaling pathways are presented. In addition, Gβγ subunit can directly interact with ion channels, receptors kinases, etc. Abbreviations: DAG, diacyl glycerol; GDP/GTP, guanoside di/triphosphate; IP_3, inositol 1, 4, 5 –triphosphate; PKC, phosphokinase C; PLC, phospholipace C; RhoGEF, Rho GTPase-guanine nucleotide exchange factor; PI3K, phosphatidylinositol 3-kinase.

9.2. Classification of GPCRs

Several classification systems have been used to divide GPCRs into different subfamilies. Classification of GPCRs can be based on amino acid sequence similarity, ligand binding properties, physiological or structural features. One of the most frequently used classification systems uses classes (clans) A, B, C, D, E and F. There is generally over 25% amino acid sequence homology in the TM core region and a distinctive set of highly conserved residues and motifs within each family. The A-F system covers all GPCRs in both vertebrates and invertebrates. Classes D, E, family IV in class A and class F containing fungal pheromone receptors, fungal cAMP receptors, invertebrate opsin receptors and archaebacterial opsin receptors, do not exist in humans. Thus, there are three main GPCR subfamilies (A-C) in humans

according to the A-F classification (Attwood and Findlay, 1994; Kolakowski, 1994; Pierce et al., 2002).

Fredriksson et al. (2003) presented a novel classification system for GPCRs, named GRAFS based on the first letters of the 5 different subfamily categories: glutamate, rhodopsin, adhesion, Frizzled/taste2 and secretin. Three of the families, the rhodopsin (A), secretin (B), and glutamate (C) families correspond to the A-F system, whereas the two other families, adhesion and frizzled/taste2, are not included in the A-F classification. Fredriksson et al. (2003) used strict phylogenetic criteria with alignments of TMs, the common regions to all GPCRs. Later they expanded their phylogenetic analysis to include a more comprehensive repertoire of GPCRs in human and mouse (Bjarnadottir et al., 2006). Their study represents the first overall mapping of GPCRs in the human genome. The recent improvements in accuracy of the human genome sequence information (International Human Genome Sequencing Consortium, 2004), and expansion of the relevant databases (i.e. Refseq, ENSEMBL) has enabled the identification of novel GPCR family members and assessment of their correct identity. In addition, the mouse genome assembly has become more complete, enabling more accurate sequence comparisons between the two species. Therefore, a more exact classification of GPCRs in the human genome has become possible.

9.3. Rhodopsin family

Rhodopsin family (or class A in A-F classification) is the largest GPCR class, containing the receptors for light (rhodopsin), odorants, small endogenous agonists (i.e. biogenic amines, nucleotides, melatonin), endogenous peptides (i.e. neurotransmitters, hormones and paracrines), glycoprotein hormones (i.e. the luteinizing hormone, chorionic gonadotrophin, follicle stimulating hormone and thyroid stimulating hormone) and protease-activating receptors (i.e. PAR_1, PAR_2 and PAR_4) (Kristiansen, 2004).

Sequence alignment reveals approximately 20 amino acids which are highly conserved in most rhodopsin class A family receptors. These include the following: Asp residue at the TM2, two Cys residues in E1 and E2, the Asp-Arg-Tyr (DRY) – motif at the TM3/ I2-transition, a Tyr residue in TM5, Pro in TM6 and Asn-Pro-X-X-Tyr in TM7 (Rana et al., 2001).

In the phylogenetic tree of the GRAFS system, the rhodopsin family, with a total of 701 members, can be further subdivided into four main groups (α, β, γ and δ) and 13 branches. The α-Group contains prostaglandin, amine, opsin, melatonin, and MECA receptor clusters. The γ-Group contains SOG, MCH, and the chemochine receptor clusters. The δ-Group contains MAS-related, glycoprotein, purin and the olfactory receptor clusters (Fredriksson et al., 2003; Bjarnadottir et al., 2006).

9.3.1. The β-Group of rhodopsin receptors

The β-Group includes 36 receptors. All the known ligands for the receptors of this group are peptides. The group contains, among others, the neuropeptide FF receptors (NPFFs), the tachykinin receptors (TACRs), the neuropeptide Y receptors (NPYRs), arginine vasopressin receptors (AVPRs), the gonadotrophin-releasing hormone receptors, the oxytocin receptor (OXTR) and the NPSR1/GPR154 receptor (Bjarnadottir et al., 2006; Fredriksson et al., 2003). For the majority of peptide receptors studied, peptide agonists have been shown to interact directly with residues in the N terminus and extracellular loops (Gether, 2000).

9.4. G protein-coupled receptor mutations

Many different GPCR mutations can elicit a wide spectrum of disease phenotypes/or differential drug efficacies. The association of GPCR mutations with a specific disease phenotype can be traced in cases where amino acid substitution alters a receptor's ability to undergo activation, coupling or ligand desensitization. However, similar amino acid substitutions can induce disparate effects on binding and/or signaling in different GPCRs, which makes the identification of correlation between a disease phenotype and a specific substitution more difficult. One of the most used *in vitro* methods to study GPCR variant pharmacology is site-directed mutagenesis (Thompson et al., 2005).

9.4.1. Mutations in N- terminus

The length of the N-terminus in GPCRs can range from 154 residues in the calcitonin receptor to 36 residues in the rhodopsin receptor. The N-terminus has several important features, including asparagine residues and motifs for N-glycosylation, which influences intracellular trafficking of the receptors to the plasma membrane (Petaja-Repo et al., 2000; Rana et al., 2001).

The N-terminus of some GPCRs also contains residues involved in ligand binding, activation and down-regulation. The mutations, Arg16Gly, and Gln27Glu are located in the N-terminus of the β_2 adrenergic receptor (ADRB2). The variants were shown to have enhanced agonist-mediated down-regulation, suggesting that the variant receptors may be removed from the cell surface more rapidly than the wild-type. In asthmatics, the Arg16Gly variant may alter beta 2-adrenoceptor expression (Green et al., 1994; Dewar et al., 1997).

Constitutively active mutants (CAMs) of GPCRs encode for receptors capable of enhanced signaling, because they are activated without exposure to ligand. The CAMs of rhodopsin are responsible for retinitis pigmentosa. The CAMs include several N-terminal variants. The Thr4Lys and Asn15Ser variants are known to affect N-glycosylation (Bunge et al., 1993; Sullivan et al., 1993).

9.4.2. Mutations in extracellular loops (ELs)

The EL domains participate in ligand binding and ligand specificity as shown in studies of melanocortin 1 receptor, the chemokine receptor CCR5 and the purinergic receptor P2Y1 by site-directed mutagenesis (Chhajlani et al., 1996; Samson et al., 1997; Thompson et al., 2005). Studies of the PRY1 receptor have also revealed that a disulfide bridge between EL2 and the upper part of transmembrane 3 is required for the proper trafficking of the P2Y1 receptor to the cell surface (Hoffmann et al., 1999). The Arg202Cys variant of the V2 vasopressin receptor, found in a patient with nephrogenic diabetes mellitus (NDI), has impaired binding and decreased activation of adenylyl cyclase by vasopressin. In addition, Gly185Cys in V2 receptor (in EL2) causing NDI has impared ligand binding (Tsukaguchi et al., 1995; Schulein et al., 2001).

9.4.3. Mutations in C-terminus

The intracellular carboxy-terminal (C-terminal) domain regulates GPCR signaling. Serine and/or threonine residues serve as sites for G protein receptor kinase (GRK)-mediated phosphorylation and receptor desensitization. A possible cysteine residue in C-terminus serves as a site for palmitoylation. The G protein-binding domain exists upstream of the palmitoylated cysteine residue and downstream of the TMVII. Furthermore, the C-terminus is involved in interactions with the other GPCR signaling mediating proteins (Rana et al., 2001). An Arg334/Cys substitution at the putative palmitoylation site is known in the $P2Y_2$ receptor. This substitution affects the secondary messenger accumulation that may have pharmacological relevance (Janssens et al., 1999). The G protein-mediated signalling may be disturbed if an amino acid substitution exists in the site regulating G protein coupling. This type of substitution (Ser290Arg) in endothelin B receptor was found in a Japanese patient with Hirschsprung's disease. As a result of it, there was a decreased intracellular calcium level and decreased inhibition of adenylyl cyclase activity in the patient (Tanaka et al., 1998).

AIMS OF THE STUDY

1. To identify the putative asthma susceptibility gene on chromosome 7p14-p15, previously implicated by a genome-wide linkage scan in Finnish families

2. To characterize the structure and the expression pattern of the asthma susceptibility gene in different endogenous cell lines and tissues

3. To characterize the function of the new gene by molecular biology methods

4. To reveal downstream target genes of the new gene by microarray analysis using a cell line model

MATERIALS AND METHODS

1. Identification of asthma locus on chromosome 7p14-p15 and susceptibility genes

1.1. Study subjects (I)

The patient recruitment and verification of asthma diagnoses in the Kainuu (a total of 254 families and 1015 study subjects), North-Karelia and Quebec (Canada) study populations have been described earlier (Laitinen et al., 1997; Kauppi et al., 1998; Laitinen et al., 2001). Microsatellite genotyping was performed in all of the 86 pedigrees in the original genome scan (Laitinen et al., 2001) and an additional 103 trios (a total of 853 study subjects). SNP genotyping was done in the subset of families (106 trios, 361 study subjects) that was informative in Haplotype pattern mining (HPM) analysis (Toivonen et al., 2000) for high serum IgE level. 193 trios from the Northeastern Quebec region were included into the association analysis. 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. Based on total serum IgE level (Diagnostics CAP FEIA, Kabi Pharmacia), the study individuals from Kainuu and North-Karelia were divided into two groups: high IgE responders (IgE >100 kU/L) and low IgE responders (IgE ≤100 kU/L). For Canadian families, asthma was used as phenotype (Laitinen et al., 2001).

1.2. Genotyping, SNP discovery, haplotype pattern mining and sequencing (I)

To create a dense map of polymorphic markers spread evenly across the linkage region on chromosome 7p14-p15, the publicly available genomic sequences for potentially polymorphic tandem repeats were screened (Polvi et al., 2002). All 76 microsatellite markers and 8 deletion/insertion polymorphisms found in the critical region were genotyped using fluorescently labeled primers in gel electrophoresis on an ABI377 sequencer. SNP genotyping was done using two different methods: single base pair extension or PCR, followed by restriction enzyme digestion. In total, 8 individuals were re-sequenced for non-repeated DNA segments by direct sequencing of PCR products.

A genotyping scheme was adopted whereby the density of increased number of markers was used, with intermediate analyses to guide further genotyping. A final density of markers was one SNP per 1-5 kb. More specifically, if genetic association

analysis suggested that a haplotype occurred in patients more often than in controls, additional markers were genotyped to either exclude or support the identity-by-descent of the haplotypes observed in unrelated patients. For the haplotypes to be identical by descent, all newly typed markers would have to be identically shared between them. An overview of hierarchical genotyping is presented in Figure 5.

1.2.1. Statistical and computational analyses

For all population samples, haplotypes obtained from trios were used as input for the HPM software (Toivonen et al., 2000). Linkage disequilibrium between SNP markers was calculated and the graphical output was produced by the Haploblocks software (Zucchelli & Kere, unpublished).

1.2.2. Sequencing

To fully explore the genetic variation in associated haplotypes, non-repetitive DNA segments in this interval (from position 506,401 to 638,799 in the public sequence NT_000380) were sequenced in one patient homozygous for the susceptibility haplotype and in one control subject homozygous for the most common (non-risk) haplotype. These sequences were then compared to the public sequence (NT_000380).

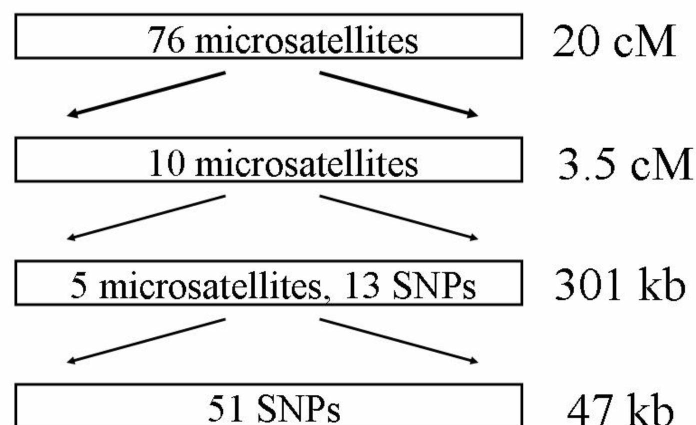

Figure 5. **A hierarchical genotyping strategy.** The linkage region of 20 cM implicated previously by Laitinen et al. (2001) was refined by genotyping 76 microsatellite markers in families from Kainuu. Haplotype patterns spanning 12 microsatellite markers within 3.5 cM were associated by a permutation test implemented in HPM software (Toivonen et al., 2000). At the next round of fine-mapping, 10 additional microsatellites implicated a 301 kb haplotype pattern. A further five microsatellites and 13 SNPs were genotyped, implicating a 47 kb haplotype pattern. Thereafter, 51 SNPs were analyzed across the region of the best association.

2. Gene identification

2.1. Exon prediction (I)

The gene-specific primers were designed using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) for all exons predicted by GENSCAN (http://genes.mit.edu/GENSCAN.html) using genomic clones AC005493.1 and AC005826.1 as one block (Polvi et al., 2002).

2.2. Reverse transcriptase-PCR and rapid amplification of cDNA ends (I)

Reverse transcriptase-PCR (RT-PCR) amplifications were performed using human lung, brain, testis, placenta and thymus mRNAs (Invitrogen) as templates. In addition, rapid amplification of cDNA ends (RACE-PCR) was performed to generate 3' and 5' cDNA ends using human testis cDNA and human Marathon Ready Fetal Thymus cDNA (Clontech) as templates according the manufactures protocol for the SMART RACE cDNA Amplification Kit (Invitrogen). The RACE-PCR products were cloned using pGEM-T Easy Vector system (Promega) or TOPO TA cloning kit (Invitrogen) Plasmid DNA was purified using QIAprep Spin miniprep kit (Qiagen). The purified RT-PCR products and the cloned RACE-PCR products were verified with automated sequencing with the dye-termination chemistry (ABI Prism3100, Applied Biosystems).

2.3. Northern hybridization (I)

Human multiple tissue 8-lane Northern blots (Clontech) were hybridized using $\alpha[_{32}P]$-dCTP random-labeled 1285 bp cDNA probe (comprising the full-length *NPSR1*). Hybridizations were performed in ExpressHyb solution (Clontech) for 3 h at 65°C. The membranes were washed with 2 x SSC/0.05 % SDS and thereafter with 0.1 xSSC/0.1% SDS for several hours. The membranes were exposed to X-ray film at –20°C for 3d (*NPSR1*) and for 1 h (β-actin control).

2.4. Cloning of *NPSR1-A* and *NPSR1-B*

Nested PCR amplification with two set of primers was used in cloning the full-length cDNAs for *NPSR1* (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, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Finnzymes), 0.52 µM of primers (I, supplementary data, table S5), 5% DMSO 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 reaplified by 30 cycles under the same conditions as above. 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).

2.5. Culture of NCI-H358 cell line (I-II)

Human lung epithelial carcinoma cell line NCI-H358 (ATCC) was cultured in RPMI 1640 medium (Gibco BRL) supplemented with 1 mM sodium puryvate (Gibco BRL), 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), and 1% penicillin/streptomycin. Cell lines were cultured at 37°C in a CO_2-conditioned, humidified incubator.

2.6. Characterization of the alternatively spliced *NPSR1* transcripts (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 a 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); 1 µM of each primers (II) and 0.5 U AmpliTaqGold (Applied Biosystems) 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 and sequence verified as described above (in chapter 2.4.).

3. Expression studies

3.1. NPSR1-A and NPSR1-B specific antibodies (I-III)

Antibodies specific for the two alternative carboxy termini of NPSR1 proteins were raised by immunizing rabbits with the following peptides: CREQRSQDSRMTFRERTER (residues 341-359 of variant A) and CPQRENWKGTWPGVPSWALPR (residues 357-377 of variant B) (Sigma Genosys Ltd). The specificity of antibodies purified by affinity chromatography was tested by comparing their reactivity against recombinant proteins by western blotting and by blocking experiments. In addition, two non-isoform specific polyclonal antibodies were produced. Antibodies against the amino terminus were raised by immunizing a

goat with the peptide TEGSFDSSGTGQTLDSSPVAKKG (corresponding to the residues 6–25 of NPSR1) (University of Oulu, Finland). Rabbit antibodies were produced against the third cytoloop SSYNRGLISK (corresponding to the residues 258–267 of NPSR1; Sigma Genosys Ltd). Antisera were purified by affinity chromatography with Sulfolink (N-terminus) and Ultralink Immobilization (cytoloop-3) kits according to the manufacturer's (Pierce) instructions. Blocking experiments using molar excess of free peptide as a competitor were also performed to demonstrate antibody specificities. Monoclonal anti-myc and anti-HA antibodies were purchased from Berkeley antibody company. Horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories Inc.

3.2. Culture of cell lines (I-II)

COS-1 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL/Invitrogen) supplemented with 10% fetal bovine serum (Perbio), 1% penicillin/streptomycin (Gibco BRL) and 1x non-essential amino acids (Gibco BRL). BEAS-2B cell line, which originates from normal human bronchial epithelium, was cultured in Basal Medium (Cambrex) supplemented with Bullet Kit (Cambrex). Myoblast cells (isolated from normal human skeletal muscle) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 15% fetal bovine serum (Perbio), 4% Ultroseer G (BioSepra, Fremont, CA) and 1% penicillin/streptomycin. The other endogenous cell lines used for gene expression studies were cultured according to the protocols from ATCC. All cell lines were cultured at 37°C in a CO_2-conditioned, humidified incubator.

3.3. Western blot analysis (I-II)

Human cell lysates were obtained by homogenizing the cell samples in RIPA-buffer (1x phosphate-buffered saline [PBS], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with Mini Complete protease inhibitors (Roche). Human tissue lysates from spleen, skeletal muscle, uterine muscle, colon muscle, colon epithelium, testes, and prostate were obtained by mechanically homogenizing frozen tissue samples in 10 mM Tris HCl, 100mM NaCl, 2% Triton X-100 buffer with Complete Mini protease inhibitors (Roche). The amount of protein was measured with the BCA Protein Assay Reagent kit (Pierce).

For crude membrane preparations, transfected COS-1 cells were harvested in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5). The membrane fractions were separated by suspending cell pellets in TE buffer/0.32 mM sucrose, homogenizing mechanically, and centrifuging for 15 min at 380 x *g* at 4°C. Supernatant was further

centrifuged for 30 min at 40,600 x *g* at 4°C. The pellet was suspended into sucrose-free TE buffer and centrifuged as above.

Lysates were run on 12.5% SDS-PAGE gels and electroblotted to the PVDF membranes according to standard procedures. Nonspecific protein binding was prevented by incubating the membrane with 5% milk/ 0.1% Tween 20/TBS (TBST) for 1 h at room temperature. Thereafter, membranes were incubated with anti–NPSR1-A, anti–NPSR1-B, or anti–NPSR1-cytoloop-3 antibodies for 1 h at 37°C, washed with TBST, and then incubated with a dilution of 1:2,000 of HRP-conjugated anti-rabbit IgG antibody in 5% milk/ 0.1% TBST for 30 min at room temperature. The protein bands were visualized by using an ECL detection kit (Amersham Biosciences).

3.4. Immunohistochemistry (I-II)

Formalin fixed, paraffin-embedded specimens of normal adult human bronchus, skin and colon, and human normal tissue array slides (MaxArray, Zymed Laboratories Inc.) containing 30 different tissues were used for immunohistochemistry. In addition, bronchial biopsies from 8 asthma patients (who used either sodium cromoglycate or short-acting beta2-agonist medication) and 10 control subjects were studied. The bronchoscopic examination and biopsy-taking (Laitinen A et al., 1997) have been approved by the appropriate Ethical Review Board.

Tissues 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. Immunohistochemical analyses were performed using the ABC method (Vectastain Elite ABC kit, Vector Laboratories). Omission of primary antibody and staining with preimmune sera were used as negative controls for parallel sections.

3.5. In situ hybridization (II)

Antisense and sense probes of *NPSR1* (full-length NPSR1-A cDNA in pCMV-Script vector) and *NPS* 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)

Nonradioactive *in situ* hybridization on tissue sections was performed with Ventana Discovery device (Ventana Medical Systems). In brief, the samples were frozen sections or sections that were deparaffinized with heat treatment followed by post-fixation and RiboClear pretreatment. 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 included a 20 min incubation with biotinylated anti-DIG antibody (Jackson ImmunoResearch Laboratories) and a 2 h incubation with the BCIP/NBT substrate. After the colour reaction the slides were washed, dehydrated and mounted with Mountex (HistoLab, Sweden). All reagents for Discovery^{TM} were provided by Ventana Medical Systems, except for protease K (Roche), which was used at a concentration of 350 ng/µl.

4. Experimental mouse model (I)

Female BALB/c mice were sensitized with 2 intraperintoneal injections of ovalbumin (20 µg OVA absorbed to 2 mg of Alum; Sigma-Aldrich) on days 1 and 10 (saline for control) in combination with 7 intranasal applications of *Stachybotrys chartarum* mould (NRRL 6084) over a period of 22 days. Sensitized (N=7) and control (N=8) mice were challenged with inhalation of aerosolized OVA on days 20-22 (Leino et al., 2003). Whole lungs were harvested and homogenized for RNA extraction. *Npsr1* levels were measured by quantitative real time-PCR.

5. Cell localization of the NPSR1 isoforms (II)

5.1. Construction of expression vectors (II)

The cDNAs encoding different *NPSR1* isoforms were subcloned from the pCR 2.1 TOPO -vector into the pCVM-Script expression vector (Stratagene) by restriction enzyme digestion. N-terminally Myc-tagged and C-terminally HA-tagged expression constructs were generated by PCR using the corresponding non-tagged cDNA in the pCMV-Script vector as a template. The resulted PCR products were subcloned into the pCMV-Tag3A vector (Stratagene). All expression constructs were verified by sequencing.

5.2. Transient transfections (II)

COS-1 cells were transiently transfected using Fugene6 transfection reagent (Roche) according to the manufacturer's protocol. The empty pCMV vector, ß-gal-pCMV, and myc-tagged luciferace-pCMV vectors (Stratagene) were used as controls. In transfections, 12 µl of Fugene6 and 2.5 µg of DNA (NPSR1-C, -D, -E, -F, and -B$_{short}$ vectors) or 8 µl Fugene6 and 2.0 µg of DNA (NPSR1-A and -B vectors) were used for 5 x 10^6 cells in 35-mm cell culture dishes.

5.3. 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% milk powder and 1% goat normal serum at 37°C for 30 min. Cells were then incubated with 1:1,000 dilution of anti-myc antibodies for 1 h at 37°C, washed three times with TBS and thereafter incubated with a dilution of 1:2,000 of HRP-conjugated anti-mouse IgG antibodies for 30 min at room temperature. TMB-substrate (Sigma Genosys) was added to cells for 3–6 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 to detect the total expression level of the corresponding construct by adding 0.5% TX-100 in PBS for 10–15 min after fixation. The results were normalized using the absorbance values obtained from pCMV and ß-gal control experiments.

5.4. Immunofluorescence microscopy (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% BSA at room temperature for 30 min, and then incubated in PBST/0.1% BSA with anti–NPSR1-A, anti–NPSR1-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 under fluorescence microscopy.

6. NPSR1 activation

6.1. NPSR1-A and NPSR1-B overexpressing stable cell lines (II-III)

To construct stable cells, NPSR1-A and NPSR1-B were cloned into a pQM vector under CMV or SRalpha promoters (produced by Quattromed AS, Estonia). Human epithelial kidney (HEK)-293H cell line was selected as the parental cell line, because it does not express endogenous NPSR1-A, and has a low endogenous level of NPSR1-B expression. The cells were transfected with Lipofectamine 2000 (Gibco BRL/Invitrogen) and clones were cultured under puromycin selection. NPSR1-A positive clones were characterized by RT-PCR or quantitative RT-PCR and by Western blotting. Stable cell lines and parental HEK-293H cells (ATCC) were cultured at 37°C in a humidified 5% CO_2 incubator in 293 SFM II medium (Gibco BRL/Invitrogen) supplemented with 1% penicillin/streptomycin. Stable cell lines

[therafter reffered as NPSR1-A and NPSR1-B (positive) cells] were constantly cultured under puromycin (0.8 µg/ml; Sigma-Aldrich) selection.

6.2. Neuropeptide S (II-III)

In all cases, we activated NPSR1 with synthetic neuropeptide S (NPS, SFRNGVGTGMKKTSFQRAKS, purchased from MedProbe, Norway). NPS was stored at –20 °C in 7% HAc stock solution to ensure its stability, or alternatively it was stored in sterile H2O at –20 °C, and remained stable as far as no additional freeze-thaw cycles were done.

6.3. NPSR1 activation assay (II)

NPSR1 activation in parental HEK-293H cells, in three NPSR1-A– and in five NPSR1-B–positive clones, and in two NPSR1-A–negative clones was determined by nonradioactive GTP-Eu binding assay (Perkin Elmer) according to the instructions of the manufacturer. NPSR1 activation was measured by comparing the GTP binding in the absence and presence of NPS (1 µM).

7. BrdU proliferation and apoptosis assays (III)

Two different NPSR1-A cell clones and a parental HEK-293H cell line (negative for NPSR1-A) was studied. Cells ($2x10^4$cells/well) were cultured in a 96-well round-bottom plate for 3 d with or without NPS (1 µM). Cells were labeled with BrdU for 14 h whereafter proliferation was analyzed by colorimetric Cell Proliferation ELISA, BrdU assay (Roche) according to the manufacturer's instructions. The proliferation inhibiting reagent, cyclohexamide (100 µg/ml; Sigma-Aldrich) was used as negative control.

Two different NPSR1-A positive cell lines and a parental HEK-293H cell line were cultured on glass slides for 1 to 2 d in puromycin-free medium with or without NPS (1 µM; $1x10^5$ cells per assay). The degree of apoptosis was visualized using DeadEnd Colorimetric TUNEL System according to the manufacturer's (Promega) instructions.

8. Studies of NPSR1 downstream target genes by microarray analyses (III)

8.1. Microarray sample preparation and hybridizations

The NPSR1-A cells (clone A3) with the highest NPS-induced GTP-binding activity (II) and parental HEK-293H cells were used in this experiment. The cells were seeded at a density of 1×10^6 cells/ml. The NPSR1-A cells were stimulated with NPS (2 µM) for 6 h. In parallel, NPSR1-A cells were cultured without stimulation and parental HEK-293 cells were treated with NPS (2 µM; negative control). Total RNAs were isolated with RNeasy Mini Kit (Qiagen) according to the instructions of the manufacturer. The RNAs from duplicate samples were stored at -70°C in 70% EtOH and 3 mM NaAc until used. RNA concentrations were measured by a UV spectrophotometer and the quality determined using the RNA Nano LabChip kit on the Agilent 2100 Bioanalyzer (Agilent Technologies).

Total RNA (8 µg) from each sample was used for target cDNA synthesis according to the Affymetrix protocol. Six hybridizations (duplicate sample sets) and scannings (Affymetrix GeneChip Scanner 3000) were carried out using standard Affymetrix protocols for gene expression technology (www.affymetrix.com) at the Karolinska Institutet Bioinformatics and Expression Analysis Core Facility. HumanGenome U133 plus 2.0 (HGU133plus2) array targeting over 47,000 transcripts and variants were used in the experiment.

8.2. Microarray data analysis

The normalization and statistical analyses of the microarray data were performed using the statistical software R (www.R-project. org; by implementing the packages Affy, limma, HGU133plus2, and kth). Background signals were subtracted using the robust multiarray average-method, and the quantile method was used to normalize the logarithm 2 (log2)-intensity distributions between arrays**.** Log2 expression values were calculated for each probe set on the basis of its Perfect Match values, by fitting an additive model using Tukey´s medianpolish procedure.

A linear model was fitted to the expression data for each probe using the least squares method. Pair-wise comparisons between all groups were made, and coefficients and estimated standard errors were computed based on the fitted linear models. The

estimated coefficients and standard errors were used to compute moderated t-statistics and log-odds of differential expression (B-values), using empirical Bayes shrinkage of the standard errors towards a common value. The prior assumption of the extent of differential expression used was 0.01.

8.3. Gene Ontology (GO) enrichment analysis

Annotation of the differentially expressed probes to GO terms and subsequent enrichment analysis of terms under the GO class of "Biological Process" were performed using the EASEonline annotation tool (http://apps1.niaid.nih.gov/david/), in the up- and down-regulated lists separately. All probes on the HGU133plus2 array were used as background in the enrichment analyses. The EASE score (E-score) was used to identify enriched categories. The score is a conservative adjustment of p-values generated by the one-tailed Fisher's exact test that penalizes the significance of categories supported by few genes. An EASE-score below 0.05 was considered significant.

8.4. TMM Microarray database comparison

To search for co-regulated genes among those significant differentially expressed (NPS stimulated NPSR1-A cells versus unstimulated NPSR1-A cells and NPS stimulated HEK-293H cells), we queried the TMM Microarray database (http://benzer.ubic.ca/cgi-bin/find-links.cgi) to find out which other genes have shown similar expression patterns in publicly available microarray data sets. The TMM Microarray database is currently based on information from 100 human microarray experiments, which have been filtered for unreliable features by the database curators. Co-expression has been defined by using several cut-offs such as Bonferroni-corrected p-values for the Pearson correlation coefficient, and requirements for the magnitude of the correlation to be among the top 5% or the bottom 5% in the experiment. Ultimately, this resulted in that genes with absolute correlations below 0.6-0.7 were not considered. A threshold of 3 was used in our analysis, meaning that the correlation had been observed for each considered pair of genes in at least three independent experiments. For all genes in our list, we compared the overlap between their TMM correlated genes and the complete list of differentially expressed genes to find instances of potential co-regulation. To define putative pathways, we required the gene to be correlated with at least two other genes in the group.

8.5. NPS stimulation and quantitative reverse transcriptase-PCR

In the first experiments, NPSR1-A cells and HEK-293H cells were seeded at $1x10^6$ cells/ml and treated with increasing concentrations of NPS (0.001-5 μM) for 6 h. Secondly, the same numbers of NPSR1-A cells were stimulated with NPS (0.1 μM)

for 1, 2, 4, 6 and 10 h. Unstimulated cell samples were collected in parallel. Total cellular RNA was isolated with the RNeasy Mini Kit (Qiagen), and eluted in 30 µl of ddH_2O. Reverse transcription was performed with TaqMan reverse transcription reagents (Applied Biosystems) with random hexamers according to the manufacturer's protocol.

The expression of *matrix metallopeptidase 10 (MMP10), interleukin 8 (IL8), INHBA (activin A)* and *EPH receptor A2 (EPHA2)* was confirmed with quantitative RT-PCR using the TaqMan (for *MMP10* using *GAPDH* control) or SYBR Green (for *INHBA, IL8* and *EPHA2*) methods. The PCR primers and probes were designed using Primer ExPress software version 1.2 (Applied Biosystems). The primers and the probe for the control gene, GAPDH were purchased from Applied Biosystems. The quantitative PCR amplifications were performed in a total volume of 25 µl, containing 7 µl of 1:10 diluted cDNA template, 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems) or 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of each primer and 100 nM of MMP-10 and GAPDH probes. The quantitative PCR was performed with 7500 Fast Real-Time PCR System (Applied Biosystems). The following reaction conditions were used: 50°C for 2 min and 94°C for 10 min; followed by 45 cycles of 92°C for 14 sec and 1 min at 60°C. The dissociation stage was added to SYBR Green reactions to confirm the specificity of the primers. All assays were carried out in triplicate. Relative quantification and calculation of the range of confidence was performed with the comparative ΔΔCT method as described by Livak and Schmittgen, (2001).

8.6. Matrix metallopeptidase 10 (MMP10) and TIMP3 antibodies

Mouse monoclonal anti-MMP10 and anti-TIMP3 antibodies were purchased from Novocastra Laboratories Ltd. and Calbiochem, respectively. MMP10 antibodies recognize amino acid residues 342-476 corresponding to the hemopexin domain of MMP10 and therefore detect both a pro-peptide containing latent protein and an active form of MMP10.

8.7. Human MMP10 immunoassay

The NPSR1-A cells ($5x10^5$ cells/ml) were stimulated with increasing concentrations of NPS (0.1-10,000 nM) and supernatants were collected at 24 and 48 h, and stored at -20°C until used. Total MMP10 concentrations were analyzed in duplicate samples with an ELISA-based Human (total) MMP10 Immunoassay (R&D Systems, UK) according to the manufacturer's instructions.

8.8. Immunohistochemistry

8.8.1. Bronchus tissue samples

Expression and localization of MMP10 and TIMP3 were studied in formalin-fixed, paraffin-embedded bronchus tissue sections from asthmatics and normal healthy controls using mouse monoclonal anti-MMP10 (1:200) and anti-TIMP3 (1:300) antibodies. The slides were blocked with 3% H_2O_2 for 10 min at RT, and thereafter heated at 95°C in water bath in DakoCytomation Target Retrieval Solution for 20 min (MMP10) or pre-treated at 37°C with 1% trypsin (TIMP3). Immunohistochemical analyses were performed using the DakoCytomation StreptABCoplex/HRP Duet method (DakoCytomation, Denmark). Diaminobenzidine (DAB) and AEC High Sensitivity Substrate Chromogen were used as chromogens for TIMP3 and MMP10, respectively, and Mayer's Hemalun solution as counterstain. Negative controls were performed with mouse immunoglobulins. Expression and localization of MMP10 and TIMP3 were studied in formalin-fixed, paraffin-embedded bronchus tissue sections from asthmatics and normal healthy controls using mouse monoclonal anti-MMP10 (1:200) and anti-TIMP3 (1:300) antibodies. The slides were blocked with 3% H_2O_2 for 10 min at RT, and thereafter heated at 95°C in water bath in DakoCytomation Target Retrieval Solution for 20 min (in the case of MMP10) or pre-treated at 37°C with 1% trypsin (TIMP3). Immunohistochemical analyses were performed using the DakoCytomation StreptABCoplex/HRP Duet method (DakoCytomation, Glostrup, Denmark). Diaminobenzidine (DAB) and AEC High Sensitivity Substrate Chromogen were used as chromogens for TIMP3 and MMP10, respectively, and Mayer's Hemalun solution was used as a counterstain. Negative controls were performed with mouse immunoglobulins.

8.8.2. Sputum samples

Expression of MMP10 was also studied in sputum samples of asthmatic patients and healthy controls**.** Cytospin preprations of sputum samples (Rytilä et al., 2000) were used for immunocytochemistry. The slides were fixed with 3% PFA for 5 min, washed two times with PBS for 5 min, blocked with 3% H_2O_2 for 10 min and thereafter washed with PBS for 5 min. The slides were incubated with mouse monoclonal anti-MMP10 (1:250) antibodies for 2 h at RT and thereafter with the secondary anti-mouse antibodies for 35 min. DAB was used in as chromogen and Mayer's Hemalun solution as counterstain. Negative controls were performed with mouse immunoglobulins. Expression and localization of MMP10 and TIMP3 were studied in formalin-fixed, paraffin-embedded bronchus tissue sections from asthmatics and normal healthy controls using mouse monoclonal anti-MMP10 (1:200) and anti-TIMP3 (1:300) antibodies. The slides were blocked with 3% H_2O_2 for 10 min

at RT, and thereafter heated at 95°C in water bath in DakoCytomation Target Retrieval Solution for 20 min (in the case of MMP10) or pre-treated at 37°C with 1% trypsin (TIMP3). Immunohistochemical analyses were performed using the DakoCytomation StreptABCoplex/HRP Duet method (DakoCytomation, Denmark). Diaminobenzidine (DAB) and AEC High Sensitivity Substrate Chromogen were used as chromogens for TIMP3 and MMP10, respectively, and Mayer's Hemalun solution as counterstain. Negative controls were performed with mouse immunoglobulins.

RESULTS

1. Identification of asthma risk and non-risk haplotypes (I)

Successive rounds of genotyping and analysis by the haplotype pattern mining (HPM) algorithm (Toivonen et al., 2000) suggested a strong association of a conserved haplotype pattern spanning between the markers NM51 and SNP563704, separated by 47 kb.

In total, a133-kb region was sequenced around this segment from a homozygous patient with asthma. Eighty polymorphisms (72 previously unknown SNPs and 8 deletion or insertion polymorphisms) specifying the susceptibility haplotype were identified by comparison to the public genomic sequence. Within this segment there was strong linkage disequilibrium between the markers. A permutation test for association showed $P \leq 0.01$ for all 43 markers in this 133-kb segment (10,000 permutations). For comparison, the nominal *P* value by χ^2 association test was 0.00001 for the best associated haplotype pattern.

In all three populations, 13 SNPs across the most conserved 77-kb segment formed seven alternative haplotypes (with frequencies >2% in the population). The hypothesis that the related haplotypes (identified either on the basis of high IgE in Kainuu or North Karelia, or asthma in Quebec) together conferred risk in all three populations was tested. The risk haplotypes could be tagged by SNP522363 and, indeed, associated with significant risk ($P = 0.004$ for all data combined, all three populations contributing), consistent with the common disease/common variant hypothesis. The relative risk for high serum IgE among H4 or H5 carriers in Kainuu was 1.4 (95% confidence interval 1.1 to 1.9, $P = 0.01$), and for asthma among homozygous H2 carriers in Quebec, it was 2.5 (95% confidence interval 2.0 to 3.1, $P = 0.0009$). Corresponding transmission disequilibrium test yielded $P = 0.05$ for Kainuu families ($n = 86$ trios). To assess whether genetic linkage to chromosome 7p could be explained by these haplotypes, parent-offspring transmissions and sibling-pair sharing of high IgE in Kainuu families were considered. One of the risk haplotypes cosegregated in 26 of 51 transmissions (51%) and was shared in 26 of 40 sibling pairs (65%), suggesting that a majority of the linkage signal was because of the observed risk haplotypes (Figure 6).

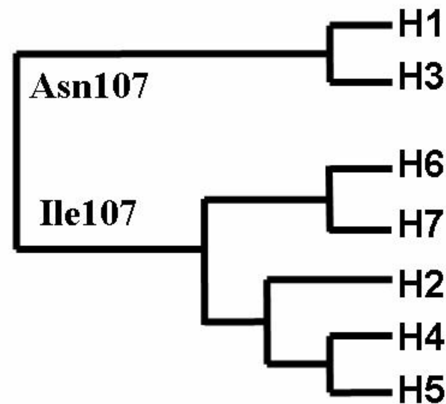

Figure 6. Phylogenetic analysis of haplotypes H1 to H7, within a 77 kb segment in Kainuu, North Karelia and Quebec. The haplotypes occur in all three population as frequencies > 2%. H4 and H5 are the most common risk-associated haplotypes in Kainuu, H7 in North Karelia, and H2 among French Canadians. H1, H3 and H6 are non-risk haplotypes in all three populations. The haplotypes can be divided into risk (often with Ile107) and non-risk (often with Asn107). Modified from (I) based on data from Melen et al. (2005). The difference between the two phylogenetic trees results from different numbers of SNPs used to construct the trees. This tree is based on a larger number of variations and is therefore more accurate.

2. Discovery of *NPSR1* (alias *GPRA*) and *AAA1* (I)

The results strongly implicated the 133-kb genomic segment as a susceptibility locus for asthma-related phenotypes. Two genes were identified in this region, one with exons 3 to 5 and the other with exons 3 to 10 lying within the susceptibility haplotype. Structures of both indicated complex alternative splicing of the mRNAs, suggesting translation to varying protein isoforms. One of the genes was predicted to belong to the G protein–coupled receptor family, and was named *GPRA* (for *G protein–coupled receptor for asthma susceptibility*). Later the gene was named *GPR154* and is presently named *NPSR1* by the Human Gene Nomenclature Committee. The full-length *NPSR1* gene has nine exons. The two main transcripts of *NPSR1* (A and B) had alternative 3' exons encoding proteins of 371 and 377 amino acids, respectively. The sequences of all predicted isoforms of the other gene, *AAA1* (*asthma-associated alternatively spliced gene 1*) showed only weak homologies to any previously identified proteins. *AAA1* has at least 18 exons (numbered 1 to 18) with complex alternative splicing. *AAA1* spans a total of 500 kb of genomic sequence. Several lines of evidence suggested that *AAA1* may not represent a protein-coding gene, although its expression was modified by the haplotype. Its longest open reading frame comprised only 74 potential amino acids, and *in vitro* translation failed to yield a stable

polypeptide. Transiently transfected cells did not produce recombinant protein. Polyclonal peptide antibodies detected the antigen but no proteins in Western blots or immunohistochemistry. The exonic structures of *NPSR1* and *AAA1* are shown in Figure 7.

2.1. Coding SNP of *NPSR1* alters amino acid (Asn107Ile) (I)

Both genes displayed coding polymorphisms in the asthma susceptibility haplotype**.** In NPSR1, SNP591694 changed an amino acid (Asn107Ile) in the first exoloop lining the putative ligand-binding pocket.

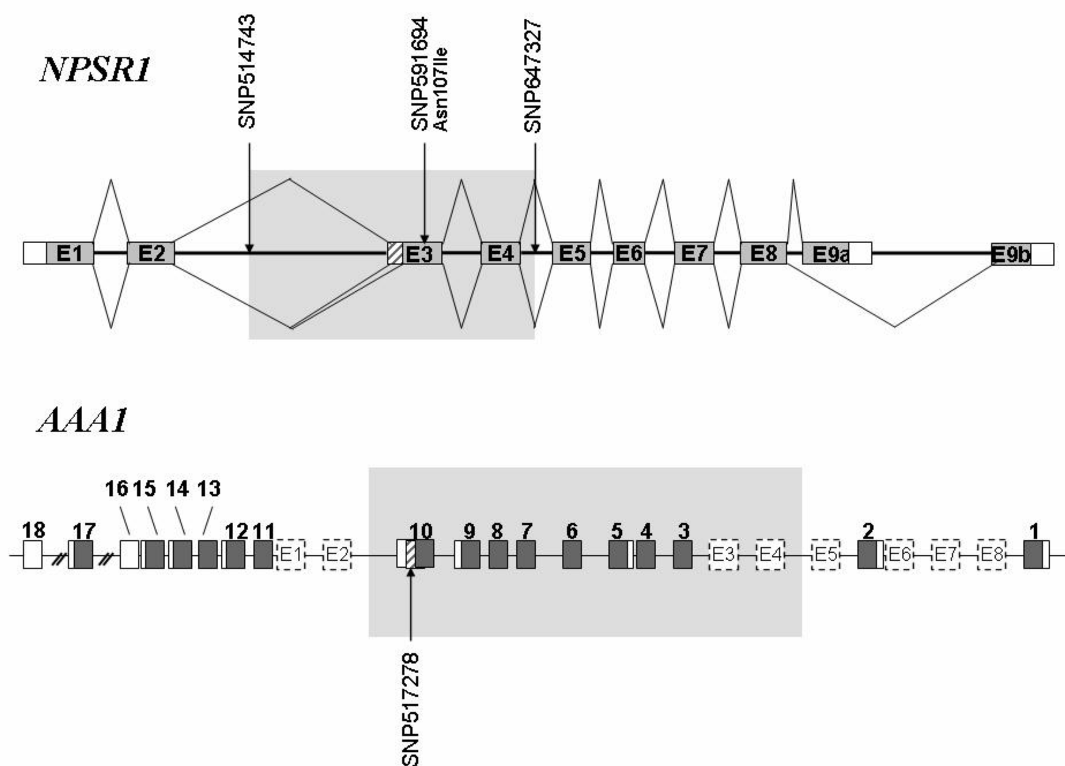

Figure 7. The exonic structures of *NPSR1* and *AAA1*. The gray box represents a conserved haplotype block of 133 kb. The placement of the *NPSR1* exons (EI-E8) compared with AAA1 is shown. Reprinted from (I).

3. Northern blot hybridization (I)

In Northern blot hybridizations, a 1285-bp full-length *NPSR1-A* cDNA probe was used. A 2.4-kb transcript of the *NPSR1* gene was detected in all nine tissues (spleen, thymus, prostate, testis, uterus, small intestine, colon, peripheral blood leukocyte and lung) assayed and a 1.8-kb transcript in four tissues.

4. Characterization of the splice variants of *NPSR1* (II)

To analyze the alternative *NPSR1* transcripts, RT-PCR analyses was performed using mRNA isolated from the human bronchoalveolar carcinoma cell line, NCI-H358. Five alternatively spliced mRNAs were identified in addition to *NPSR1-A* and *–B***.** The shortest transcript, *NPSR1-C* (encoding a 94 aa peptide), had only three exons. Variants *NPSR1-D* (encoding a 158 aa peptide) and *-E* (encoding a 136 aa peptide) had a deletion of exon 3 or 4, respectively, resulting in an early stop codon. *NPSR1-F* (encoding a 305 aa peptide) lacks both exons 3 and 4, but preserves the rest of the reading frame of *NPSR1-A*. *NPSR1-Bshort* (encoding a 366 aa peptide) has an in-frame deletion of 33 bp (11 aa) at the beginning of exon 3, whereas the rest of the downstream exons are the same as in *NPSR1-B* (Figure 8).

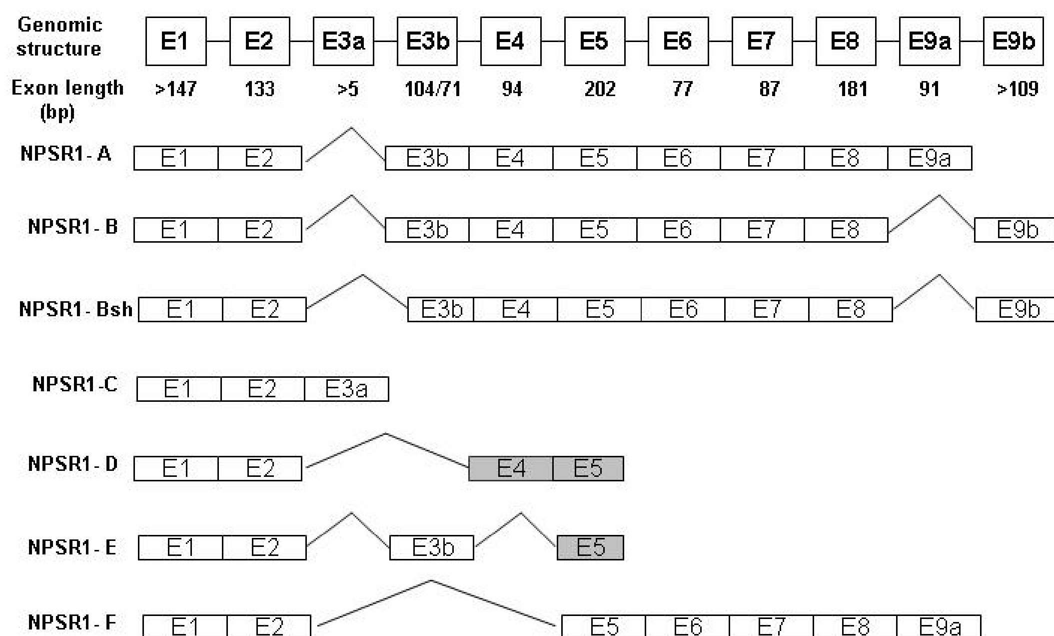

Figure 8. Schematic representation of the *NPSR1-A*, *NPSR1-B*, *NPSR1-Bshort*, *NPSR1-C*, *NPSR1-D*, *NPSR1-E*, and *NPSR1-F* transcripts. *NPSR1-A* and *-B* encode the full-length G protein–coupled receptor proteins with 7 TM domains. The other variants encode for truncated proteins. *Gray shading* indicates the alternate open reading frames. Exon lengths are shown below exons. The figure is modified from (II).

5. Expression profiling of NPSR1 and NPS

5.1. Specificity of NPSR1 antibodies (I-II)

To study expression of NPSR1, four different polyclonal antibodies were raised by immunization in rabbit or goat. The antibodies were characterized by peptide competition assays, by immunohistochemistry, and by Western blotting of cell lysates and tissues. Anti–NPSR1-A and -B antibodies were specific for the two alternative carboxy termini. Anti–NPSR1-N and anti–cytoloop-3 recognized the shared segments of the NPSR1-A and -B isoforms.

5.2. Expression of NPSR1-B increased in asthmatic smooth muscle (I-II)

Polyclonal antibodies raised against the different carboxyl termini of the A and B isoforms of the predicted NPSR1 protein were used. In bronchial biopsies, the isoform patterns were distinct between asthma patients and control samples. Most clearly, there was strong expression of the B isoform in smooth muscle cells in asthmatic airways compared with an absence of such staining in control samples. Staining for the B isoform in epithelial cells varied between healthy individuals but was consistently stronger in the asthma samples than in controls. The A isoform showed no consistent differences. The results were consistent among all the eight asthma patients and ten control subjects.

A surprising result with ISH in normal tissues was a negative staining in smooth muscle, contrary to our immunohistochemical staining with isoform A antibodies. This could be due to low expression of the corresponding mRNA, which is below the detectable level in nonradioactive ISH. To study this, we analyzed bronchial tissue section from patients with asthma ($n = 3$), presuming that up-regulation of the *NPSR1-B* isoform leads to a detectable ISH signal in the smooth muscle layer. As a result, the *NPSR1*-specific antisense probe showed a strong expression also at mRNA level in the smooth muscle layer.

5.3. Expression pattern of *NPSR1* at mRNA level (II)

Thirty human cell lines (including BEAS-2B, HL-60, MG-63, BE(2)-C, HEPG2, CCD-25Lu, and U-937) representing different tissue origins were tested for the *NPSR1* expression by RT-PCR. *NPSR1-B* was ubiquitously expressed in all cell lines studied, whereas *NPSR1-A* was expressed in myoblasts (of skeletal muscle origin), but not in any other cell line.

In situ hybridization (ISH) of paraffine sections of 30 human tissues with a *NPSR1* specific antisense probe resulted in positive staining of epithelial cells in the gastrointestinal tract (esophagus, stomach, small intestine, colon), and skin. In addition, strong staining was observed in the submucosal epithelial cells of spleen, kidney, pancreas, prostate, uterus, breast, and in some glandular epithelia (e.g., that of salivary gland). In heart muscle, weak staining was observed. When the corresponding sections were stained with the sense probe, no specific signal was seen.

5.4. Expression pattern of NPSR1 at protein level (I-II)

When NPSR1 expression was studied in human tissues by Western blot analyses, NPSR1-B had a broader expression pattern than the A isoform. Analyses with anti–NPSR1-A antibodies reveled one intensive polypeptide band corresponding to molecular weight of ~50 kD in smooth muscle containing tissues: uterine muscle, colon muscle, and prostate, but not in spleen and testis, which are both rich in epithelial cells. However, the band in colon epithelium demonstrates that epithelium-derived NPSR1-A also exists. With NPSR1-B antibodies, a 50 kD polypeptide band was detected in all studied tissues, except skeletal muscle. An additional 39 kD band was detectable in testis. The results were verified by anti–cytoloop-3 antibodies, which recognize both isoforms A and B in overlapping locations. As expected, all the studied tissues except skeletal muscle were positive for NPSR1.

Immunostaining of 30 normal adult human tissue samples with anti–NPSR1-B antibodies revealed ubiquitous protein expression in glandular epithelia of bronchus, stomach, small intestine, colon, uterus, esophagus, spleen, kidney, pancreas, prostate, and breast. Anti–NPSR1-A antibodies showed weak staining in the epithelium of most tissues studied. However, compared with NPSR1-B, the expression of NPSR1-A was absent in some tissues, such as stomach and small intestine. Smooth muscle cell layer in bronchial and arterial walls was constantly positive for NPSR1-A in all tissues studied. Interestingly, NPSR1-A was expressed in the basal and NPSR1-B in the apical surfaces of the colon epithelium and skin keratinocytes.

5.5. NPS is expressed in the epithelia of human bronci and colon (II)

The NPS mRNA was detected in the epithelium of human colon and bronchi in overlapping locations as compared with NPSR1 by ISH. Hybridization of the corresponding sections with the sense probe did not show any specific signal.

6. *Npsr1* mRNA was significantly up-regulated in mouse lung after ovalbumin/*Stachybotrys chartarum* challenge (I)

Npsr1 mRNA was significantly up-regulated in mouse lung after sensitization and challenge with a combination of ovalbumin and *Stachybotrys chartarum* mould over a period of 22 d, compared with nonsensitized mice.

7. Cellular localization of the NPSR1 isoforms (II)

N-terminally conjugated myc-tagged pCMV-NPSR1 constructs were used in transient transfections of COS-1 cells to study the expression and localization of different NPSR1 isoforms and particularly their translocation to the plasma membrane. The amount of myc-tagged protein was measured with a cell-based ELISA assay. When non-permeabilized cells were used in the assay, the anti-myc antibodies detected the recombinant receptor successfully translocated into the plasma membrane. When cells were permeabilized, the total amount of the recombinant protein produced was measured. According to our assays, 71% of the produced NPSR1-A and 52% of the NPSR1-B were translocated to the plasma membrane, whereas all the five shorter variants stayed in the intracellular compartments.

Cellular localization of different NPSR1 variants was further examined by immunofluorescence microscopy. COS-1 cells grown on glass slides were transiently transfected with a myc-tagged NPSR1 constructs. Consistent with the results obtained by a cell-based ELISA assay, only NPSR1-A and -B were translocated into the plasma membrane, while all the shorter NPSR1 isoforms were retained in the intracellular compartments.

8. NPSR1-A mediates signals for inhibition of cell proliferation (III)

Biological activity of NPSR1-A and –B overexpression in HEK-293H cell clones was first verified by the GTP-binding assay. Parental HEK-293H cells, three NPSR1-A–positive, five NPSR1-B–positive, and two NPSR1-A–negative clones were studied in the absence and presence of NPS (1 µM). Activated NPSR1-A–positive clones had 2–3 fold increases in GTP-binding activity compared with negative clones. NPSR1-B clones were not activated by NPS.

Proliferation rate of the NPSR1-A cells was significantly ($p<0.05$) lower (2.6±0.7 fold change) when compared to the NPSR1-A negative cells (3.2±0.4 fold change), as measured with BrdU proliferation assay after three days of culture. The addition of NPS agonist did not increase the effect. The results represent averages of two different NPSR1-A positive clones and one NPSR1-A negative clone (transfection negative cells). Each cell line was cultured in triplicate wells and the experiment was

replicated three times. Cyclohexamide (100 µg/ml) treated cells were used as negative control (1-fold change).

The degree of apoptosis was also studied in NPSR1-A positive clones and HEK-293H parental cells with or without NPS treatment using a death end tunel detection system. There was no increase in the level of apoptosis detected between different cell samples.

9. Downstream target genes of NPSR1 (III)

9.1. Microarray results

To study downstream signaling of NPSR1-A upon NPS stimulation, we compared the NPS stimulated NPSR1-A cells with non-stimulated cell lines and NPS treated HEK-293H cells. Duplicate samples were assayed with the HGU133plus2 array, containing over 47,000 transcripts.

A B-value larger than 7 was chosen to define the cut-off level. At this cut-off, there was a maximal number of differentially expressed genes in NPS stimulated vs. unstimulated NPSR1-A cells, and NPS stimulated NPSR1-A, vs. NPS stimulated HEK-293H, respectively. All the genes selected by this criterium have a false discovery rate-adjusted $p<0.0001$. Of these genes, 104 were found to be up-regulated and 42 down-regulated. According to the orientation of these genes in the log2 intensity versus log2 fold-change dimensions, it is evident that all of these genes had approximately 2.1-fold or larger changes. Forty eight genes had at least a 4-fold changes (Table 3).

Table 3. Differentially expressed genes with fold changes larger than 4. Modified from (III).

	Gene Symbol	Ref Seq	Gene Name	NPSR1-A, stimulated vs. NPSR1-A
Up-regulated	*CGA*	NM_000735	*glycoprotein hormones, alpha polypeptide*	25.6
	SV2C	XM_043493	*synaptic vesicle glycoprotein 2C*	22.3
	TFPI2	NM_006528	*tissue factor pathway inhibitor 2*	13.0
	EGR3	NM_004430	*early growth response 3*	13.8
	INHBA	NM_002192	*inhibin, beta A (activin A, activin AB alpha polypeptide)*	10.2
	ARC	NM_015193	*activity-regulated cytoskeleton-associated protein*	15.0
	MMP10	NM_002425	*matrix metalloproteinase 10 (stromelysin 2)*	11.3
	NR4A1	NM_002135	*nuclear receptor subfamily 4, group A, member 1*	9.3
	GEM	NM_005261	*GTP binding protein overexpressed in skeletal muscle*	10.4
	PCK1	NM_002591	*phosphoenolpyruvate carboxykinase 1 (soluble)*	8.4
	EMP1	NM_001423	*epithelial membrane protein 1*	8.8
	GPR50	NM_004224	*G protein-coupled receptor 50*	7.4
	FOS	NM_005252	*v-fos FBJ murine osteosarcoma viral oncogene homolog*	9.7
	EGR2	NM_000399	*early growth response 2 (Krox-20 homolog, Drosophila)*	6.9
	AREG	NM_001657	*amphiregulin (schwannoma-derived growth factor)*	7.0
	CTGF	NM_001901	*connective tissue growth factor*	6.3
	EGR1	NM_001964	*early growth response 1*	7.8
	TRIB1	NM_025195	*tribbles homolog 1 (Drosophila)*	7.6
	TAC1	NM_003182	*tachykinin, precursor*	9.3
	ZCCHC12	NM_173798	*zinc finger, CCHC domain containing 12*	4.9
	LOC387763	XM_373497	*hypothetical LOC387763*	6.7
	SERPINB2	NM_002575	*serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2*	4.9
	STC1	NM_003155	*stanniocalcin 1*	4.5
	GLIPR1	NM_006851	*GLI pathogenesis-related 1 (glioma)*	4.8
	NR4A3	NM_006981	*nuclear receptor subfamily 4, group A, member 3*	4.9
	FOSL1	NM_005438	*FOS-like antigen 1*	5.2
	CYR61	NM_001554	*cysteine-rich, angiogenic inducer, 61*	4.9
	TNC	NM_002160	*tenascin C (hexabrachion)*	4.7
	GADD45B	NM_015675	*growth arrest and DNA-damage-inducible, beta*	5.6
	MAFB	NM_005461	*v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)*	3.5
	MGC61598	NM_00100435	*similar to ankyrin-repeat protein Nrarp*	4.6
	HRB2	NM_007043	*HIV-1 rev binding protein 2*	4.7
	MAFF	NM_012323	*v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)*	4.9
	FOSB	NM_006732	*FBJ murine osteosarcoma viral oncogene homolog B*	4.6
	RGC32	NM_014059	*response gene to complement 32*	3.1
	ELL2	NM_012081	*elongation factor, RNA polymerase II, 2*	3.9
	IL6R	NM_000565	*interleukin 6 receptor*	4.6
	DUSP1	NM_004417	*dual specificity phosphatase 1*	4.6
	NR4A2	NM_006186	*nuclear receptor subfamily 4, group A, member 2*	4.0
	BHLHB2	NM_003670	*basic helix-loop-helix domain containing, class B, 2*	4.1
	KLF10	NM_005655	*Kruppel-like factor 10*	4.0
	SERPINE2	NM_006216	*serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2*	3.4
	BMP2	NM_001200	*bone morphogenetic protein 2*	2.9
	KIAA1199	NM_018689	*KIAA1199*	3.9
	HSPA5	NM_005347	*heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)*	4.3
Down-regulated	*KBTBD7*	NM_032138	*kelch repeat and BTB (POZ) domain containing 7*	-5.6
	SLC16A14	NM_152527	*solute carrier family 16 (monocarboxylic acid transporters), member 14*	-5.4
	TXNIP	NM_006472	*thioredoxin interacting protein*	-3.5

9.2. Gene Ontology pathway analysis shows significantly up-regulated pathways

To look for enrichment of GO terms in the class Biological Process, the up-and down-regulated groups of genes were analyzed separately in the EASE software, using the total gene-list from the HGU133plus2 array as reference. For the up-regulated genes, the most enriched term was *cell proliferation* (E-score=0.0006). Another enriched group, *transcription, DNA-dependent* (E-score=0.009), reflects the abundance of up-regulated early response genes in this assay. Other significantly enriched groups were *morphogenesis* (E-score=0.001), *immune response* (E-score=0.02), *cell communication* (E-score=0.02), *response to pest/pathogen/parasite/* (E-score=0.03), and *chemotaxis* (E-score=0.04). The enriched term *response to stimulus* connects all the enriched terms related to immunity and inflammation and consists of 20 genes presented here: *BTG2, CD24, CXCL2, CTGF, CYR61, DUSP1, FOSL1, GNAS, GEM, INHBA, IL6R, IL8, ERRFI1, NR4A2, PTGS2, STC1, TAC1, TIMP3, TCF8* and *FOS*. In the group of down-regulated genes, no terms were enriched in the EASE analysis.

9.3. TMM Microarray database analysis reveals a common NPSR1-A-regulated pathway

We queried the TMM microarray database with all differentially expressed genes to determine if any had previously been correlated with each other at a stringency level of at least three microarray experiments. When the complete list of differentially expressed genes was compared to the genes in the TMM results, we found a set of 43 co-regulated genes (of total 146), correlated with a minimum of two other genes in the group (mean 9.4 genes, median 7 genes). The majority of these genes are clearly early response genes (i.e., transcription factors). Sixteen genes showed correlations to more than 10 genes in this group, and are listed below where the genes with the highest number of correlations given first: *CYR61, JUNB, DUSP1, NR4A2, DUSP5, EGR1, NR4A1, FOSB, FOS, IER2, CXCL2, BTG2, EGR2, GADD45B, NR4A3,* and *EGR3*

9.4. Expression of *MMP10*, *INHBA*, *EPHA2* and *IL8* is NPS concentration dependent

The differential expression of *MMP10*, *INHBA, EPHA2* and *IL8* was confirmed with quantitative RT-PCR. In the first RT-PCR assays, the NPSR1-A cells were stimulated with NPS (2 μM) for 6 h, and expression of *EPHA2, MMP10, IL8* and *INHBA* in the NPSR1-A cells (with or without 2 μM NPS stimulation) was compared with their expresson level in HEK-293H cells. Expression of *INHBA* showed the highest change after NPS stimulation (77-fold change). Expression levels of *IL8, MMP10* and *EPHA2* were increased 53-fold, 47-fold and 7.5-fold, respectively, in the stimulated NPSR1-A cells compared to un-stimulated HEK-293H cells.

In a second set of experiments, the NPSR1-A cells were stimulated for 6 h with increasing concentrations (0.001-5 µM) of NPS and the expression levels of *IL8, INHBA, EPHA2* and *MMP10* were compared to the expression in unstimulated NPSR1-A cells. Expression was NPS concentration dependent. In the case of *MMP10*, the peak expression level was seen with 0.1 µM NPS, while the peak expression levels were with 1 µM NPS for the other genes.

In addition, the NPSR1-A cells were stimulated for 1, 2, 4, 6 and 10 h with NPS (0.1 µM), and the expression levels of *INHBA, EPHA2* and *MMP10* were compared to the expression in unstimulated NPSR1-A cells collected at the same time points. *INHBA* levels were significantly above the basal levels even as early as at 1 h (3-fold change) and the highest expression level was detected at 4 h (28-fold change). *MMP10* expression levels increased more slowly when compared with *INHBA* levels. The highest expression was detected at 10 h (26-fold change). Expression levels of *EPHA2* remained low across different early time points, but a significant increase was detected at 10 h (8-fold change).

9.5. Total MMP10 protein levels of NPSR1-A cells were increased upon NPS stimulation

The NPSR1-A cells were stimulated with 0.1-10,000 nM NPS for 24 and 48 h. Total MMP10 levels were detected with Human MMP-10 (total) Immunoassay. NPS dose-response was detected at both time points. The highest protein levels (3.8-5 ng/ml) were detected upon 0.1 NPS µM stimulation. There was no significant increase in protein concentrations at 48 h as compared to 24 h.

9.6. Expression of MMP10, TIMP3 and NPSR1-A in the bronchus

MMP10 and *TIMP3,* that were up-regulated by NPS-NPSR1-A activation, were selected because of their potential involvement in tissue remodelling seen in asthma. Their expression patterns were studied in asthmatic and normal bronchial tissue sections. Strong expression of TIMP3 was observed in the lung epithelium, and in some cases, specific staining in the basal cell layer could be detected. In addition, strong staining of TIMP3 was seen in sub-epithelial glands and some staining was also seen in the endothelial structures. MMP10 staining was positive in the epithelium, endothelium and smooth muscle, and faint staining was seen also in the sub-epithelial glands. However, the staining pattern differed between different samples and some samples lacked smooth muscle staining. By visual inspection, we could not detect major changes in the expression levels of TIMP3 and MMP10 between asthmatics and controls. However, NPSR1-A was also expressed in the lung epithelium.

9.7. Expression of MMP10 in sputum samples of asthmatic patients and healthy controls

Sputum samples from five asthmatic and three control individuals were stained for MMP10. There were no significant differences between asthmatics and controls: macrophages were MMP10 positive while epithelial cells and neutrophils were negative. In addition, some MMP10 positive eosinophils were detected in asthmatic samples. Negative controls incubated with mouse immunoglubulins showed no staining.

DISCUSSION

1. *NPSR1* as an asthma susceptibility gene

Previous results from our group had implicated a locus on chromosome 7p14-p15 in the genetic susceptibility to either high IgE values, asthma or both (Laitinen et al., 2001). I joined the group to find the putative susceptibility gene and if successful, to study its role in the pathogenesis of asthma. Accompanying the positional cloning of a new G protein-coupled receptor in this locus, we show strong genetic evidence to support the role of *NPSR1* as a novel asthma susceptibility gene. First, the original linkage and association results from the Kainuu subpopulation were replicated in two other cohorts: French-Canadians and Finnish North-Karelians.

We found the strongest disease associations to non-coding SNPs (single nucleotide polymorphisms) within *NPSR1* introns, and all but one nonsynonymous SNP, rs324981, (in exon 3 encoding the first exoloop of the receptor and giving rise to Asn107Ile) in the susceptibility haplotypes are non-coding. Initially, Asn107Ile didn't appear as a haplotype tagging SNP. However, when we re-examined the sequencing data within the Finnish patients, we observed that subjects with the risk haplotypes carry the Ile^{107} variant whereas the subjects with non-risk/protective haplotypes carry the Asn^{107} variant (unpublished data). Reinscheid et al. (2005) reported that the Asn107Ile polymorphism in a NPSR1 overexpressing epithelial cell line resulted in a gain-of–function, by increasing agonist potency. Bernier et al (2006) demonstrated an increased cell surface expression with the Ile^{107} variant. For the time being, it is still unclear whether the genetic effect predisposing to asthma is mediated by the amino acid variation, allele-specific regulation of expression, allele–specific regulation of splicing, or a combination of these mechanisms.

Further support for our genetic findings had later been provided by five independent replication studies confirming a positive association of *NPSR1* with either asthma, high serum IgE, sensitization or bronchial hyperreactivity in seven European populations, in one Chinese population, and in European-American and Puerto Rican populations (Melen et al., 2005; Kormann et al., 2005; Feng et al., 2006; Malerba et al., 2007; Hersh et al. 2007). One replication study among a Korean population, found no association with asthma, atopy or total serum IgE. However, this study included only one SNP (rs323922), and may therefore lack statistical power (Shin et al., 2004).

Recently, an association study of five cloned asthma susceptibility genes, *ADAM33, DPP10, NPSR1, HLA-G* and the *PHF11* locus (including the genes *SETDB2* and *RCBTB1*), replicated an association of *NPSR1* between three SNPs and asthma in two

family-based sample sets: European-American children and Hispanic (Puerto Rican) children (Hersh et al., 2007). The most significant associations (p=0.003 and p=0.0006) were observed with SNP rs1379928, located 5' to the risk haplotype. In total, 98 linkage disequilibrium (LD)-tagging SNPs in five genes were genotyped. The SNPs were tested for association with asthma and two intermediate phenotypes: AHR and total serum immunoglobulin E levels. Strikingly, SNP level replication was found only for *NPSR1*. Weak evidence for locus-level association with asthma was found in the *PHF11* locus**.** A summary of all replication studies of *NPSR1* is seen in Table 4.

Table 4. Replication studies of *NPSR1*. In studies, where different phenotypes were considered, the number of the largest subcohort (Nr. of cases) is shown. This table is modified from Kere, Allergy Frontiers, Springer, in print

Population	Nr. of cases	Nr. of SNPs	Best p or OR value, phenotype	Reference
Korea, adults	439	1	none	Shin et al., 2004
5 European countries,children	1087	7	OR=1.47, allergic asthma	Melen et al., 2005
Germany, children	671	6	OR=3.5, asthma+BHR	Kormann et al., 2005
China,mixed	715	8	OR=0.61 (protective haplotype)	Feng et al., 2006
Italy, mixed	511	7	p=0.008, high IgE	Malerba et al., 2007
European Americans, children Puerto Rico, children	497 439	26	p=0.0006, asthma p=0.003, asthma	Hersh et al., 2007

2. Genome-wide linkage versus genome-wide association studies

The asthma susceptibility loci presented or reviewed in this thesis were all found by genome-wide linkage studies. In the recent years, there has been a rapid expansion of detailed genomic information due to the completion of both the human genome

sequencing and the mapping of human haplotypes of SNPs (International HapMap Consortium, 2005; International Human Genome Sequencing Consortium, 2004). In addition, cost-effective genotyping methods (i.e. dense genotyping chips) have been developed that can assay hundreds of thousands SNPs simultaneously. Therefore, genome-wide association studies for thousands of cases and controls have become technically and financially feasible. Large and well-characterized clinical samples are also available for many common diseases.

Recently, Moffatt et al. (2007) published the first genome-wide association study in the field of asthma, whereby the effects of more than 317,000 SNPs were studied in 944 patients with childhood onset asthma and 1243 non-asthmatics, using family and case-control panels (collected in the UK and Germany). Multiple markers on chromosome 17q21 were strongly associated with childhood onset asthma in both panels, with a combined P value of $<10^{-12}$. The positive association with childhood asthma was replicated in two other cohorts, German children and the British 1958 Birth Cohort.

Further analysis of global gene expression in B-cell -derived EBV- transformed lymphoblastoid cell lines from children in the asthma family panel, found a strong association ($p<10^{-22}$) between SNPs associated to childhood asthma and transcript levels of the *ORMDL3* gene on chromosome 17q21. *ORMDL3* is a member of a gene family, which encodes transmembrane proteins anchored in the endoplasmic reticulum (Moffatt et al., 2007). However, its exact functional properties and effects in asthma remain to be elucidated. Interestingly, chromosome 17q21 is a novel locus related to asthma, as previous genome-wide linkage studies have failed to identify it. On the other hand, the genome-wide association study by Moffatt et al. (2007) failed to detect an association to any of the previously identified asthma susceptibility loci, including chromosome 7p14.3. However, in a large-scale genome-wide association study of seven common diseases by the Welcome Trust Case Control Consortium (2007), many previously identified associations were replicated in addition to novel findings. For example, an association was replicated with P value $< 5x10^{-7}$ on chromosome 3p21, 5q33, 10q24 and 18p11 for Crohn's disease, in addition to 5 novel association signals.

In genome-wide linkage studies, trait-based (e.g. bronchial hyperresponsivenesss, blood eosinophil counts, total and serum specific serum IgE levels) approaches have been commonly used. In genome-wide association studies, quantitative trait components may not be determined. In the case of other complex diseases, such as migraine, determining qualitative trait components may have an enormous effect in dissecting the genetic susceptibility. Anttila et al., (2006) identified a linkage between several migraine traits and markers in chromosome 4q24. The pulsation trait identified a locus on 17p13 and the age of onset trait revealed a locus on 4q28. In

addition, a trait combination phenotype identified a locus on 18q12. Therefore, a genome-wide linkage study may still be a valid method to find novel susceptibility loci in the future.

3. Expression of NPSR1

NPSR1 is expressed ubiquitously in various endogenous cell lines. These include the lung epithelial cell lines, NCI-H358 and BEAS-2B. It is noteworthy that NPSR1 is expressed in the lung epithelium and smooth muscle in human, but not nearly so strongly in mouse. We used polyclonal NPSR1 antibodies in expression profiling. Thus, an important issue is antibody specificity. In the present work, the specificities of the NPSR1-A and NPSR1-B antibodies were carefully determined, for example using several parallel antibodies and the appropriate negative controls, and by carrying out peptide blocking experiments. In addition, expression profiling was carried out by multiple techniques giving consistent results, such as RT-PCR and *in situ* hybridization to define expression at RNA level, and immunohistochemistry and western blotting to define expression at protein level. Our results from RT-PCR suggest an overall expression level being relatively low in many cell lines and in the lung. This is consistent with other reports (Xu et al., 2004).

NPSR1 may have significance in regulating diseased states as we observed increased level of NPSR1-B expression in the smooth muscle of asthmatic bronchi compared to normal subjects. In the experimental mouse model, whereby the lung inflammation was induced by combining OVA and *Stachybotrys chartarum* mould sensitizations, the expression level of *Npsr1* (mouse lacks *NPSR1-B* isoform) was increased. However, it is possible that OVA sensitization/challenging alone may not be enough in all cases to induce changes in *Npsr1* expression. Allen et al. (2006) could not show expression of *Npsr1* in the lungs of mice with allergic disease induced by OVA challenging. This result is in line with our unpublished data on mouse tissues and Npsr1 expression studies. Human and mouse differ considerably from each other with respect to the physiological roles of the NPS-NPSR1/Nps-Npsr1 pathways.

In case of NPSR1, the use of endogenous cell lines in experiments is limited due to the overall low expression levels of the receptor. Thus, an overexpressing NPSR1 stable cell line is a model of choice. Benefits of this model include good sample availability, for example in gene expression studies.

4. The NPS-NPSR1 pathway regulating other allergic and respiratory disorders

Our results from Affymetrix gene expression analyses found candidate genes for respiratory diseases among the significantly up-regulated genes. These differentially

expressed genes included *tenascin C* (*TNC*) and *prostaglandin-endoperoxide synthase 2* (*PTGS2*). In addition, *MMP10* (matrix metallopeptidase 10) was among the significantly up-regulated genes. Our further studies show that MMP10 is strongly expressed and co-localized with NPSR1-A in the pulmonary epithelium, and also in macrophages and eosinophils of human sputum samples.

In the Affymetrix study, immune responses such as 'defense responses to pest, pathogens and parasites' as well as 'wound healing' and 'chemotaxis' were among the most enriched GO pathways. Shared genes in these groups are *CXCL2* [chemokine (C-X-C motif) ligand 2; previously named macrophage inflammatory protein 2-alpha], *FOSL1* and *IL8.* The results also support our previous findings in a macrophage cell. In order to study NPSR1 functions in immune cells, we showed that NPS induces phagocytosis of unopsonized bacteria as well as both directed (chemotaxis) and random cell migration (wound healing) (Pulkkinen et al., 2006b). Thus, it is feasible to think that one of the possible functions of NPSR1 is to regulate innate immunity reactions. NPSR1 is expressed in tissues that have contact with environmental substances, i.e. lung, colon, gastrointestinal tract and skin, as well as in macrophages and T lymphocytes, both cells of the first line of defence, further suggesting that NPSR1 may have relevance in regulating innate immunity responses.

Our results from microarray gene expression analyses indicate that activation of NPSR1 with NPS may lead to up-regulation of the genes, which have been earlier reported as candidate genes for COPD. These genes include *SERPINE2*, which was found to be up-regulated around 4-fold upon NPS–NPSR1-A signalling. This gene is located in a region on chromosome 2q that has shown overlapping linkage to both COPD and asthma-related traits. Furthermore, we found *EGR1, FOS, CTGF, CYR61* to be up-regulated by NPS. These genes were among differentially expressed genes in the study between two groups of smokers (Ning et al., 2006). It is now of great interest to study a putative role of NPSR1 in regulating COPD.

Recently, an association of *NPSR1* to respiratory distress syndrome (RDS) was reported among Finnish preterm infants (Pulkkinen et al., 2006a). The haplotype H4/H5 was associated with an increased risk. In preterm infants, RDS is the major risk factor for bronchopulmonary dysplasia (BPD). RDS is a multifactorial developmental disease caused by lung immaturity and presenting as high-permeability lung edema. It is characterized by a transient deficiency of alveolar surfactant during the first week of life Surfactant proteins (SPs) have earlier shown to associate with an increased risk of RDS. Surfactant proteins facilitate microbial aggregation, and phagocytosis and killing of micro-organisms by alveolar macrophages (Hallman and Haataja, 2007). Interestingly, we have detected relatively high expression levels (compared to many other cell lines) of *NPSR1* in the lung epithelial cell line NCI-H358. This cell line has cytoplasmic characteristics of Clara cells and is known to

produce surfactant associated protein SP-A. Furthermore, it has been shown in many epidemiological studies that preterm children with RDS have an increased risk for asthma or wheezing disease (Schaubel et al., 1996; Evans et al., 1998; Smith, 2003).

The common genetic background of the allergic diseases is suggested by the studies, whereby a susceptible child has transient or persistent disease stages that begins with atopic dermatitis in the young infant and continues with the development of asthma and allergic rhinitis later in life (Strachan et al., 1996; Gustafsson et al., 2000;).

Thus, an association of *NPSR1* to atopic dermatitis has been studied, but so far there is a lack of association (Soderhall et al., 2005; Veal et al., 2005).

5. The NPS-NPSR1 pathway as a neurogenic regulator

Neuropeptides regulate a variety of different physiological and psychological functions. They may contribute to inflammation of the lungs via axon reflexes meaning that their main source is neuronal axon terminals of the airways. They have an effect on target cells, such as mast cells, macrophages and vascular smooth muscle cells causing neurogenic inflammation (De Swert and Joos, 2006). As an example, neuropeptide Y (NPY) regulates vasoconstriction, energy balance and feeding, anxiety, depression and neuroendocrine secretion (Wahlestedt et al., 1985; Morris and Pavia, 1998; Kalra et al., 1999; Kask et al., 2002; Redrobe et al., 2002;).

The NPS-NPSR1 pathway has also been implicated as a novel neuropeptide system regulating many physiolocigal processes such as anxiety and arousal (Xu et al., 2004). There is evidence that NPS might regulate energy balance and feeding (Smith et al., 2006) as well as anxiety (Xu et al., 2004). Reinscheid et al. (2005) determined the distribution of Npsr1 and Nps precursor mRNAs in rat brain. They found the highest expression levels of Npsr1 mRNA in the cortex, thalamus, hypothalamus and amygdale, while the NPS precursor was mainly expressed in brainstem nuclei.

Interestingly, we could determine many neuronal genes among the target genes of NPSR1 by our Affymetrix based gene expression analysis. These genes are *SV2C (synaptic vesicle glycoprotein 2C), GLIPR1 (GLI pathogenesis related, glioma), NPTX2 (neuronal pentraxin II), METRNL (meteorin, glial cell differentiation regulator –like), SDC4 (syndecan 4), NRG1 (neuregulin 1), NTS (neurotensin) and NRP1 (neuropilin 1).* It should be noted, that we detected expression of these neuronal genes in the stimulated NPSR1 overexpressing stable cells of kidney epithelial origin. However, interactions between these genes and *NPSR1* need to be confirmed by other methods before further conclusions can be made.

Neuropeptides are nowadays known to be expressed beside the brain, in the lung and inflammatory cells (De Swert and Joos, 2006). We have reported the expression of

NPSR1 in alveolar macrophages, blood eosinophils, monocytes and T-lymphocytes (Pulkkinen et al., 2006b). Furthermore, we detected a low expression of NPS in the lung epithelium. Exact expression patterns of NPS in immune cells remains to be elucidated. It is possible that the nervous system, for example afferent nerves in the airways, could be a major source of NPS, as they are in the case of neuropeptide Y.

CONCLUSIONS AND FUTURE PROSPECTS

In this study we have identified, by positional cloning, a novel susceptibility gene for asthma, *NPSR1*, on chromosome 7p14.3. In part of this work, both main transcripts, *NPSR1-A* and *–B*, were cloned, and the polymorphisms increasing the risk for asthma were determined. Some functional characterization of NPSR1 was done using molecular biology methods. In particular, the expression pattern of NPSR1 in a variety of tissues and cell lines, was examined in detail. In addition, NPSR1-mediated downstream target genes and the related pathways were investigated by microarray expression and GO pathway analyses.

We initially replicated our genetic linkage results in two cohorts: Finnish allergy families and Canadian asthma families. Thereafter, a positive association of *NPSR1* with asthma related phenotypes has been replicated in five independent studies that together strengthen the significance of *NPSR1* as a novel asthma susceptibility gene.

Our data demonstrate that NPSR1 is expressed in the lung epithelium, and appears in the smooth muscle in asthmatic cases. Our data strongly suggest that the NPS-NPSR1 pathway regulates cell proliferation, as well as immune responses. However, the most abundant NPSR1 expression is detected in the brain. Therefore, it would be worthwhile to study whether the NPS-NPSR1 pathway also contributes to neurogenic inflammation and whether, by that means, it contributes to some asthma related phenotypes. One of the experimental models of choice for such a study would be a NPSR1 knock out mouse model.

Our Affymetrix based gene expression analyses were carried out using NPSR1 overexpressing cell lines. We detected many immunological as well as neuronal genes among target genes of NPSR1. One potential novel pathway that we identified is NPS induced up-regulation of MMP10 that may have importance in tissue remodeling. We detected co-localized expression of NPSR1 and MMP10 in the lung epithelium, smooth muscle, and in eosinophils and macrophages of human sputum samples. Replication of these results in a selected endogenous cell line might give more confidence to these interactions, but so far sensitivity of the assay due to low *NPSR1* expression levels has been an obstacle.

It has been recently reported in a cross-national study (Scott et al., 2007) that a range of common mental disorders occurs with greater frequency among persons with asthma. In this diagnostic interview based study, pooled estimates of age-adjusted and sex-adjusted odds of mental disorders, among persons with asthma relative to those without asthma were 1.5 (95% Confidence interval=1.4, 1.7) for anxiety disorders. Therefore, it would be of interest to study whether the NPS-NPSR1 pathway would contribute to both asthma and anxiety.

In conclusion, the NPS-NPSR1 pathway appears to provide a rich subject for future research.

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