Structure-Function Relationships in the Neuropeptide S Receptor

MOLECULAR CONSEQUENCES OF THE ASTHMA-ASSOCIATED MUTATION N107I

Received for publication, April 17, 2006, and in revised form, June 20, 2006. Published, JBC Papers in Press, June 20, 2006. DOI 10.1074/jbc.M603691200

Virginie Bernier1 2, Rino Stocco3, Michael J. Bogusky4, Joseph G. Joyce5, Christine Parachoniak6, Karl Grenier6, Michael Arget7, Marie-Claude Mathieu8, Gary P. O’Neill9, Deborah Slapetz2, Michael A. Crackower10, Christopher M. Tan11, and Alex G. Therien12

From the Departments of 9 Biochemistry and Molecular Biology and 1 Pharmacology, Merck Frosst Center for Therapeutic Research, Kirkland, Quebec H9H 3L1, Canada and the Departments of 4 Medicinal Chemistry and 9 Vaccine and Biologics Research, Merck & Co., West Point, Pennsylvania 19486

Neuropeptide S (NPS) and its receptor (NPSR) are thought to have a role in asthma pathogenesis: a number of single nucleotide polymorphisms within NPSR have been shown to be associated with an increased prevalence of asthma. One such single nucleotide polymorphism leads to the missense mutation N107I, which results in an increase in the potency of NPS for NPSR. To gain insight into structure-function relationships within NPS and NPSR, we first carried out a limited structural characterization of NPS and subjected the peptide to extensive mutagenesis studies. Our results show that the NH2-terminal third of NPS, in particular residues Phe-2, Arg-3, Asn-4, and Val-6, are necessary and sufficient for activation of NPSR. Furthermore, part of a nascent helix within the peptide, spanning residues 5 through 13, acts as a regulatory region that inhibits receptor activation. Notably, this inhibition is absent in the asthma-linked N107I variant of NPSR, suggesting that residue 107 interacts with the aforementioned regulatory region of NPS. Whereas this interaction may be at the root of the increase in potency associated with the N107I variant, we show here that the mutation also causes an increase in cell-surface expression of the mutant receptor, leading to a concomitant increase in the maximal efficacy (Emax) of NPS. Our results identify the key residues of NPS involved in NPSR activation and suggest a molecular basis for the functional effects of the N107I mutation and for its putative pathophysiological link with asthma.

Asthma is a multifactorial disease with both genetic and environmental components that is characterized by an exaggerated immune response induced upon exposure to antigens. The disease has become a major public health concern as its incidence has increased dramatically in recent years, particularly in developed countries (for a recent review, see Ref. 1). Studies in animal models have identified several genes and proteins that contribute to the asthmatic phenotype, although a complete understanding of the interplay between these factors, and their role in human disease, remains elusive (2).

A number of genetic-linkage studies have recently been carried out to identify possible therapeutic targets for asthma that are relevant in man. One approach, involving whole genome scanning followed by refined genetic mapping, has led to the recent identification of four specific candidate genes (3–6). One of these, GPR154, encoding neuropeptide S receptor (NPSR)4 (4, 7), also known as G protein receptor for asthma susceptibility (GPRA) (4), GPR154 (8), and vasopressin receptor-related receptor 1 (VRR1) (9), has been confirmed as an asthma-linked gene in five distinct Caucasian populations (4, 10, 11). The gene for NPSR encodes at least two splice variants that differ only in their C termini (4, 12) and are referred to herein as NPSR-A and NPSR-B, corresponding to GPRA-A and GPRA-B (4), respectively. A number of single nucleotide polymorphisms in the NPSR sequence are associated with asthma, elevated serum IgE levels, and bronchial hyperresponsiveness (4, 10, 11). One of these single nucleotide polymorphisms is found in the coding region of the gene and results in mutation of residue 107 from Asn to Ile (4).

Very little is known regarding the biochemical and physiological roles of NPSR. Its ligand is a 20-amino acid peptide known as neuropeptide S (NPS) because of its expression in the brain and its putative role in centrally mediated responses (as outlined below) (7). Activation of NPSR by NPS results in increased intracellular Ca2+ mobilization, leading to the conclusion that it is coupled to Gs and Gq proteins (7, 9). Reinforcing the genetic evidence that NPSR is involved in asthma, it has been reported that expression of the NPSR-B isoform, but not the NPSR-A isoform, is increased in bronchial smooth muscle and epithelial cells of asthmatics compared with healthy controls (4, 12). It has also been shown that lung NPSR is up-regulated in a murine model of allergic asthma (4). Recently, it was reported that the B isoform of NPSR is expressed in a wide variety of tissues and cells in addition to

8 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 Both authors contributed equally to this work.
2 Recipient of an Industrial Research Fellowship from the National Sciences and Engineering Research Council.
3 To whom correspondence should be addressed: 16711 Trans Canada Hwy., Kirkland, Quebec H9H 3L1, Canada. Tel.: 514-428-3937; Fax: 514-428-8615; E-mail: alex_therien@merck.com.
4 The abbreviations used are: NPS, neuropeptide S receptor; CHO, Chinese hamster ovary; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; NPS, neuropeptide S; TOCSY, total correlated spectroscopy; WT, wild type; HEK, human embryonic kidney; ELISA, enzyme-linked immunosorbent assay.
the lung (12), although other studies have shown that expression of the receptor is restricted to the brain, in particular the hypothalamus, and the retina (7, 9). Consistent with this latter pattern of expression, NPSR has been shown to be involved in sleep, anxiety (7), and feeding (13) in rodents.

The structural determinants of NPSR activation by NPS are for the most part unknown. A recent report localized the pharmacophore to the NH₂-terminal half of the peptide and demonstrated that the N107I variant of NPSR is a gain-of-function mutant (14). We sought to better characterize structure-activity relationships within the receptor and its ligand as well as to define the molecular basis for the altered functional behavior of the N107I variant. We identify the key residues in NPS involved in activation of NPSR as well as demonstrate that the N107I mutation increases both intrinsic efficacy as well as intracellular trafficking of the receptor to the cell membrane.

**EXPERIMENTAL PROCEDURES**

**Generation of NPSR Vector Constructs**—The NPSR-A isoform was amplified from a hypothalamus Marathon-ready cDNA library (BD Biosciences) by PCR using the Accuprime Pfx DNA polymerase (Invitrogen) following manufacturer’s instructions and using the following primers: “sense 1,” 5′-GTAGGGTAGCCACATGCCGCCACACTTTACAGAGGGCAGCTTCG-3′; “antisense 1,” 5′-TGCTGGATGCCGCCATGCTCTGATGATGACGCCCCAG-3′ and “antisense 2,” 5′-GGCTGGCCAGTGGCTCTGGGAC-3′. These primers introduce a KpnI recognition sequence and a Kozak sequence at the 5′ end, and a NotI cleavage site at the 3′ end. The NPSR-B isoform was generated from the NPSR-A construct by Splicing Overlap Extension PCR. Briefly, in a first round of PCR, the 1051-bp sequence corresponding to the common region of the two NPSR isoforms was amplified by PCR using the NPSR-A isoform sequence as template, and a 108-bp NPSR-B-specific sequence was obtained by annealing and amplification of the following overlapping primers: “sense 2,” 5′-GTAGGGTAGCCACATGCCGCCACACTTTACAGAGGGCAGCTTCG-3′ and “antisense 2,” 5′-GCTGGATGCCGCCAGTGGCTCTGGGAC-3′. These 2 PCR fragments were then spliced together in a second round of PCR using the primers “sense 1” and “antisense 2” (above) to generate the full-length NPSR-B isoform. The NPSR-A-N107I and NPSR-B-N107I constructs were generated from the above isoform-specific sequences by site-directed mutagenesis using the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions and using the following primers: “sense 3,” 5′-GACAGATATTATTTGGCGATTTACTTG-3′ and “antisense 3,” 5′-CCATGTAATCGCGCCGGCCGGAGG-3′. FLAG-tagged versions of the NPSR variants (see above) were plated (40,000 or 100,000 cells/well for CHO and HEK 293 T cells, respectively) in 96-well black-walled, clear-bottom plates. After incubation overnight and washing with Hanks’ balanced salt solution, 105 μl/well of “no wash” calcium dye (Molecular Devices, Sunnyvale, CA) was loaded onto the cells and incubated for 45 min at 37 °C in 5% CO₂. Using the built-in sample addition feature of the FlexStation II instrument, 15 μl of agonist diluted in Hank’s balanced salt solution (containing 20 mM Hepes and 0.24% bovine serum albumin) was added to the cells and fluorescence emission (λex = 485 nm; λem = 525 nm) was measured at 3-s intervals before, during, and after agonist addition for a total of 2 min. Data are reported as the difference between the maximal fluorescence and the baseline fluorescence. EC₅₀ values, representing the concentration of agonist at which 50% activity is obtained, are reported as mean ± S.D. of multiple determinations.

**Calcium Mobilization Assay**—The calcium-mobilization assay was carried out as described previously (18), with the following modifications. CHO or HEK 293 T cells expressing the NPSR variants (see above) were plated (40,000 or 100,000 cells/well for CHO and HEK 293 T cells, respectively) in 96-well black-walled, clear-bottom plates. After incubation overnight and washing with Hanks’ balanced salt solution, 105 μl/well of “no wash” calcium dye (Molecular Devices, Sunnyvale, CA) was loaded onto the cells and incubated for 45 min at 37 °C in 5% CO₂. Using the built-in sample addition feature of the FlexStation II instrument, 15 μl of agonist diluted in Hank’s balanced salt solution (containing 20 mM Hepes and 0.24% bovine serum albumin) was added to the cells and fluorescence emission (λex = 485 nm; λem = 525 nm) was measured at 3-s intervals before, during, and after agonist addition for a total of 2 min. Data are reported as the difference between the maximal fluorescence and the baseline fluorescence. EC₅₀ values, representing the concentration of agonist at which 50% activity is obtained, are reported as mean ± S.D. of multiple determinations.

**NMR Spectroscopy**—Nuclear magnetic resonance (NMR) spectra were acquired at a sample temperature of 25 °C on a Varian Inova 600 MHz spectrometer equipped with a 5-mm triple resonance Z-axis pulsed field gradient probe. NPS sam-
Effects of the N107I Mutation of NPSR

ples were analyzed at 3.1 mg/ml (1.4 mM) in 50 mM sodium phosphate buffer in 90% H2O, 10% D2O, pH 5.0. ¹H chemical shifts were referenced internally to sodium 3-(trimethylsilyl) propionate-2,2,3,3-d4 at 0.00 ppm. The water resonance in all experiments was suppressed by low power saturation using an attenuated transmitter pulse during the relaxation delay. Sequence-specific chemical shift assignments were obtained with the combination of two-dimensional total correlated spectroscopy (TOCSY) (19, 20) and nuclear Overhauser effect spectroscopy (NOESY) (21) experiments using standard methodologies (22). All two-dimensional experiments were acquired in the hypercomplex mode for phase-sensitive presentation (23). Clean TOCSY (19, 24) spectra were recorded with 1 K complex points in t2 and 512 points in t1 consisting of 16–32 transients per increment. Spin locking was achieved with an MLEV-17 (24) mixing sequence for a duration of 65 ms preceded by a G-90-G magnetization randomization sequence using a 2.0-ms gradient pulse prior to the relaxation delay. Data sets were multiplied by a shifted Gaussian apodization function and zero-filled to 2 K by 1 K complex points prior to Fourier transformation. NOESY spectra were acquired using a range of mixing times. Solvent suppression was achieved by selective saturation of the water resonance during the 1-s recycle delay, t1 period, and mixing period. Data sets were acquired with 1 K complex points in t2 and 512 points in t1 with 96 transients per increment. The NOESY data were processed as described above.

CD Spectroscopy—Circular dichroism (CD) measurements were performed on a J-810 spectropolarimeter equipped with a Peltier temperature controller set at 25 °C (JASCO, Easton, MD). Spectra were collected from 180 to 300 nm at a scan speed of 200 nm/min for 5 accumulations/sample with a data interval of 0.2 nm. Spectra were corrected for solvent background, and the peptide concentration was quantified by amino acid analysis. The raw CD signal was converted to molar ellipticity, [θ], by [θ] = Ω_0/10H, where MRW is the mean residue weight (molecular mass divided by number of peptide bonds), l is path length in cm, and c is concentration in mg/ml. Spectral data were input into the program SSE, version 1.00.00 (JASCO), which is based upon the least squares method of Chang–Wu–Yang (25, 26) for estimation of secondary structure characteristics.

Binding Assay—HEK 293 T cells were plated (600,000 cells/well) in poly-D-lysine-coated 6-well plates, incubated for 24 h, and transfected with 0.5 µg of the various NPSR constructs as described above. 48 h following transfection, cells were washed with 2% glucose, 1% bovine serum albumin in phosphate-buffered saline and incubated with various concentrations of 125I-NPS (Phoenix Pharmaceuticals, Belmont, CA) in binding buffer (1 mM tyrosine, 1 mM phenylalanine, 1 mg/ml glucose, 10 mg/ml bovine serum albumin in phosphate-buffered saline) and in the presence or absence of unlabeled NPS (at 100 times the concentration of 125I-NPS) for 2 h at 4 °C with mild agitation. Cells were then washed twice and lysed in the presence of 0.1 M NaOH for 30 min at 37 °C. Bound radioligand was measured by scintillation counting on a 1450 Microbeta counter (PerkinElmer Life Sciences).

Immunofluorescence Microscopy and ELISA—HEK 293 T cells were plated in poly-D-lysine-coated 8-well slides or 24-well plates (at 50,000 or 150,000 cells per well for immunofluorescence microscopy and ELISAs, respectively), incubated for 24 h, and transfected with the various NPSR constructs as described above. Analyses were carried out 48 h following transfection, as follows. For analysis of non-permeabilized cells, cells were incubated in blocking buffer (0.5% bovine serum albumin in phosphate-buffered saline) for 15 min and incubated with anti-FLAG antibody (1:500 in blocking buffer) for 30 min. Cells were then washed, fixed in the presence of 3% paraformaldehyde (15 min, 4 °C), and permeabilized with 0.2% Triton in blocking buffer. For immunofluorescence, cells were then incubated with Alexa Fluor 488-conjugated anti-mouse antibody (1:500, Invitrogen), coverslips were mounted on the slides, and images were acquired on an Axioplan2 imaging microscope (Zeiss, Thornwood, NY). For ELISAs, cells were incubated with hors eradish peroxidase-conjugated anti-mouse antibody (1:500; Roche Applied Science, Laval, Canada) and cleavage of the horseradish peroxidase substrate o-phenylenediamine dihydrochloride (Sigma) was monitored as per the manufacturer’s instructions by measuring spectral absorbance at 492 nm. For analysis of permeabilized cells, cells were fixed and permeabilized prior to incubation with anti-FLAG antibody.

RESULTS

Functional Properties of NPSR Variants—It has previously been demonstrated that a single nucleotide polymorphism leading to mutation of residue Asn-107 to Ile in NPSR is associated with an increased risk for asthma (4). More recently, it was shown that this mutation causes an increase in the potency of NPS for NPSR (14). To confirm the latter finding, we assessed NPS-dependent intracellular Ca2+-mobilization of the wild type (WT) and N107I variants of both NPSR-A and NPSR-B splice isoforms in multiple clones of CHO-NPSR cells. As shown in Table 1, NPS activates NPSR-A-N107I with a ~10-fold higher potency than its WT counterpart NPSR-A. This pattern is also observed for NPSR-B-N107I and NPSR-B and was confirmed in transiently transfected HEK 293 T cells (as described below). A comparison of the two splice variants themselves, however, reveals no significant difference in their potency for NPS, regardless of the nature of residue 107 (Table 1).

Secondary Structure of NPS—To study the molecular basis for the functional difference between the WT and N107I variants, we first sought to investigate structure-function relation-

---

### TABLE 1

| NPSR variant             | CHO-NPSR cells | HEK-NPSR cells |
|--------------------------|----------------|---------------|
| NPSR-A                   | 8.3 ± 5.1      | 26.1 ± 17.3   |
| NPSR-B                   | 9.5 ± 7.6      | 48.3 ± 11.0   |
| NPSR-A-N107I             | 1.4 ± 1.0*     | 2.1 ± 1.3*    |
| NPSR-B-N107I             | 1.0 ± 0.5*     | 3.9 ± 1.0*    |

* p < 0.05 compared to corresponding WT receptor (NPSR-A or NPSR-B) using Student’s t test.
ships in the WT receptor and its ligand. As an initial approach, we assessed the secondary structure of the peptide by NMR spectroscopy. Sequence-specific $^1$H resonance assignments were obtained for the peptide with the combined use of TOCSY and NOESY spectra (22). Qualitative secondary structure characterization was accomplished primarily by two-dimensional NOE studies. As shown in Fig. 1A, the amide proton region of the NOESY spectrum displays a contiguous stretch of moderately intense amide NH to NH NOEs from Gly-5 to Thr-13 (with the exception of NOEs between Lys-11 and Lys-12, which cannot be resolved because of the close proximity of the chemical shifts of these two residues), consistent with helical secondary structure in the region spanning these residues. However, the long range proton-proton interactions, $d_\alpha\alpha(i, i+3)$ and $d_\beta\beta(i, i+3)$, which are characteristic of a defined $\alpha$-helix were not observed. In addition, CD spectroscopy analysis (not shown) was consistent with approximately one-third of the peptide adopting a $\beta$-turn-like, rather than $\alpha$-helical, conformation. Taken together, the NMR and CD data indicate the presence of a nascent helix from residue Gly-5 to Thr-13, which may adopt a true $\alpha$-helical conformation upon receptor binding (see “Discussion” and Ref. 27). The putative three-dimensional conformation of NPS containing such a helix is shown in Fig. 1B.

**Functional Roles of Residues in NPS**—To investigate the role of specific amino acid residues within NPS, we carried out mutagenesis of the peptide (see Fig. 2) and assessed the effect of these mutations on NPSR activation in the CHO-NPSR cells using the Ca$^{2+}$-mobilization assay. Examples of concentration-response curves for NPS and selected truncated mutant peptides are shown in Fig. 3, A and B, whereas a summary of the EC$_{50}$ values obtained for all truncated mutants is shown in Fig. 3C and Table 2. The results demonstrate that residues in the NH$_2$-terminal third of the peptide are necessary and sufficient for receptor activation. Indeed, removal of the C-terminal 14 residues (peptide 1-6) has limited effect on the potency of the peptide, whereas removing Ser-1 (peptide 2-20) is detrimental to function and removing the first two (peptide 3-20) or three (peptide 4-20) NH$_2$-terminal residues results in largely inactive peptides. Interestingly, peptides 1-7, 1-8, and 1-10 are significantly less potent compared with both full-length NPS and peptide 1-6, indicating that residues in this region, although not critical for function, can affect receptor activation.

To further probe the role of residues in the NH$_2$-terminal half of NPS, we individually substituted residues 1 to 10 with Ala and assessed the ability of the resulting peptides to activate NPSR-A. As shown in Fig. 4 and Table 2, substitution of residues 2, 3, and 4 with Ala results in substantial to complete inactivation of the peptide, as does the G7A mutation. Taken together with the findings obtained with the truncated NPS mutants (above) these results indicate that the first 6 residues, in particular residues Phe-2, Arg-3, Asn-4, and Val-6, are necessary for receptor activation, whereas residue Gly-7 is critically located and can modulate the inherent activity of the peptide.

**Interaction between NPSR Residue Asn-107 and Residues in NPS**—Having identified regions of NPS that are critical for NPSR activity, we wished to assess the possible role of residue...
Effects of the N107I Mutation of NPSR

Asn-107 (in the receptor) on ligand binding and activation. We hypothesized that we might see an altered behavior of the mutant peptides on NPSR-A-N107I activation if this residue is involved in receptor activation and/or ligand binding. We therefore assessed the NH2-terminal truncated and Ala scan mutants on the N107I variant of NPSR-A. As shown in Fig. 5 (with EC50 values shown in Table 2), the pattern of activation with the NH2-terminal truncated mutants is significantly altered with the N107I variant compared with its WT counterpart. Whereas peptides 1-10, 1-8, and 1-7 have EC50 values for NPSR-A that are 21-, 17-, and 116-fold higher than WT NPS, respectively, these same peptides are equipotent to the WT peptide (with perhaps a modest 2-fold increase in EC50 for peptide 1-10) on the NPSR-A-N107I variant. In addition, although substitutions of residues 2–4 with Ala results in partial to complete inactivation on both receptor variants, confirming their importance in receptor activation, the G7A mutation is considerably more detrimental to NPSR-A activity compared with NPSR-A-N107I. Taken together, these results suggest that Asn-107 plays a key role in modulating receptor activation and that its interaction with Gly-7 of NPS plays a regulatory role in NPSR, but not in NPSR-A-N107I.

Functional Effect of the N107I Mutation in a Transient Expression System—Whereas the results described above strongly suggest that the N107I mutation directly affects the intrinsic activation/binding of NPSR, we, nevertheless, wished to assess whether it might have indirect effects on function by altering receptor density at the cell surface. In the absence of suitable NPSR antibodies for such studies, we opted to tag the receptor with a FLAG epitope on the NH2-terminal end of the receptor. In addition, to circumvent the relatively low cell surface expression of NPSR in CHO cells, which precludes functional determinations in transiently transfected cells (data not shown and see Ref. 14), we tested a number of alternate cell
lines for these studies. Based on comparative immunofluorescent microscopy analyses with various cell types (not shown), we identified HEK 293 T cells as being the most conducive to high cell surface expression of NPSR. Consistent with this, HEK 293 T cells show a concentration-dependent increase in Ca\(^{2+}\)-mobilization in the presence of NPS following transfection with FLAG-NPSR-A (Fig. 6A). To investigate the effect of the N107I mutation on cell surface expression, we first confirmed the gain-of-function phenotype of this variant in both the NPSR-A and NPSR-B backgrounds in this system (Fig. 6A and Table 1). Interestingly, in addition to the ~12-fold difference in EC\(_{50}\) values between the WT and N107I variants, a 70 ± 23% increase in maximal activation (E\(_{\text{max}}\)) is also observed for the mutant variants compared with the corresponding WT variants. Despite the important shift in EC\(_{50}\) values, there was no significant difference in the binding affinity (K\(_d\) = 5.7 ± 3.6 and 3.3 ± 1.1 nM for NPSR-A and NPSR-A-N107I, respectively) of NPS for the two receptor variants (Fig. 6B), consistent with what has been previously demonstrated (14). On the other hand, we did observe a significant difference in maximal 125I-NPS binding (not shown) between the two variants, suggesting that the N107I mutation affects cell surface expression of the receptor.

**Cell Surface Expression of WT and N107I Variants of NPSR and Impact on Function**—We explored the question of cell surface expression of the two NPSR variants using this transient expression system and taking advantage of the availability of anti-FLAG antibodies. As shown in Fig. 7A, immunofluorescence analysis of cells transfected with either variant of NPSR-A shows a higher level of N107I versus WT receptor expressed at the cell surface (non-permeabilized cells) despite similar levels of total receptor expression (permeabilized cells). Peptides for which the –fold increase in EC\(_{50}\) is higher than 200 are shown as having a 200-fold increase. NPSR-A, filled bars; NPSR-A-N107I, empty bars. A, summary of –fold increases in EC\(_{50}\) values for C-terminal-truncated peptides. B, summary of –fold increases in EC\(_{50}\) values for alanine point mutant peptides.
with varying amounts of pcDEF3(FLAG-NPSR-A-N107I) DNA to alter receptor expression, and measured the impact of this change on the potency (EC$_{50}$) of NPS and its maximal efficacy ($E_{\text{max}}$) on NPSR in the Ca$^{2+}$-mobilization assay. To ensure that only receptor expression per cell was affected in this experiment (and not total number of cells transfected), we kept the total amount of DNA constant for each transfection by supplementing with "empty" pcDEF3 vector (see "Experimental Procedures").

**DISCUSSION**

The data presented herein provide information on structure-function relationships within NPSR and its ligand, and propose a molecular basis for the functional differences observed between the WT receptor and the asthma-linked mutant N107I. The gain-of-function phenotype of this variant of NPSR is associated with an increase in both the intrinsic efficacy of its ligand NPS (associated with a decrease in EC$_{50}$) and in its cell surface expression (leading to an increase in $E_{\text{max}}$). Taken together with the finding that the N107I mutation is associated with an increase in asthma susceptibility (4, 10, 11), our data suggest that NPSR antagonism may be a suitable therapeutic approach for the treatment of this disease.

A first objective of this work was to better characterize structure-function relationships within NPSR and its ligand. Limited structural characterization of the NPS peptide by NMR (see Fig. 1) and CD spectroscopy identified a putative nascent helix between residues Gly-5 and Thr-13. The speculative $\alpha$-helical conformation for this region of NPS shown in Fig. 1B is based on the observation that nascent helices are expected to stabilize into $\alpha$-helices in constrained environments, such as might occur in the context of receptor binding (27). Whatever its true nature, this region of defined structure seems to have a role in modulating receptor activity (as discussed below), but is not required for receptor activation per se. Thus, a peptide in which most of the structured region of NPS is removed (peptide 1-6) is largely equipotent with the full-length WT peptide on the NPSR-A and NPSR-A-N107I receptors (see Figs. 3 and 5 and Table 2), indicating that the residues necessary and sufficient for receptor activation are located in the NH$_2$-terminal third of the peptide. Combined with the Ala scan mutagenesis studies...
striking difference in activation of the two NPSR variants by peptide 1-7 is that there is electrostatic repulsion between the two residues in the WT variant of NPSR, which becomes more prevalent when Gly-7 is mutated to the more hydrophobic Ala. The N107I variant, on the other hand, might be predicted to interact favorably with Gly-7 due to the hydrophobic nature of Ile, with only a slight steric disruption of the interaction in the presence of the moderately larger side chain of an Ala residue in the G7A mutant peptide.

As first demonstrated by Reinscheid and co-workers (14) and confirmed herein (Fig. 6, Table 1), mutation of residue Asn-107 of NPSR to Ile leads to an increase in the potency of NPS for the receptor. The use of a transient expression system has allowed us to demonstrate that in addition to this increase in potency, the maximal efficacy (E_max) of NPS is also increased on the N107I variant compared with the WT variant. We also show that this increase in E_max is likely secondary to a significantly higher level of cell surface receptor density of the mutant compared with the WT receptor. Thus, as demonstrated by immunohistochemistry and ELISA (Fig. 7), the cell surface expression of NPSR-A-N107I is significantly higher than that of NPSR-A. In addition, artificially altering receptor expression by transfecting cells with different amounts of NPSR vector DNA leads to changes in E_max (Fig. 8). On the other hand, the decrease in E_max observed with the N107I variant is likely related not to cell surface expression of NPSR, but rather to differences in the intrinsic potency of NPS on its receptor, because increasing the expression of NPSR as described above does not significantly affect E_max values (Fig. 8). In addition, the increase in potency is not associated with a change in the affinity of NPS for NPSR (Fig. 6B; see also Ref. 14), but appears to be due to a difference in the intrinsic efficacy of NPS, which is its ability to activate the receptor once it is bound. Mechanistically, it is tempting to speculate that this is related to the interaction between residue 107 of the receptor and the helical regulatory region of NPS, in particular residue Gly-7, as described above.

In conclusion, we have identified key residues in the NPSR receptor and NPS ligand involved in receptor activation. These results provide a foundation for future structural and functional studies on novel receptor and define the molecular basis for the increased asthma susceptibility associated with the N107I variant of NPSR.

REFERENCES

1. Lilly, C. M. (2005) J. Allergy Clin. Immunol. 115, S526–S531
2. Epstein, M. M. (2004) Int. Arch. Allergy Immunol. 133, 84–100
3. Allen, M., Heinzmann, A., Noguchi, E., Abecasis, G., Broxholme, J., Pon-ting, C. P., Bhattacharyya, S., Tinsley, J., Zhang, Y., Holt, R., Jones, E. Y., Lech, N., Carey, A., Jones, H., Dickens, N. J., Dimon, C., Nicholls, R., Baker, C., Xue, L., Townsend, E., Kabesch, M., Weiland, S. K., Berg, J., von Mutius, E., Adcock, I. M., Barnes, P. J., Lathrop, G. M., Edwards, M., Moffatt, M. F., and Cookson, W. O. (2003) Nat. Genet. 35, 258–263
4. Laitinen, T., Polvi, A., Rydman, P., Vendelin, J., Pullkkinen, V., Salmikan-gas, P., Makela, S., Rehn, M., Pirskanen, A., Rautanen, A., Zucchelli, M., Gullsten, H., Leino, M., Alenius, H., Petays, T., Haatham, T., Laitinen, A., Laprise, C., Hudson, T. J., Laitinen, L. A., and Kere, J. (2004) Science 304, 300–304
5. Van Eerdewegh, P., Little, R. D., Dupuis, J., Del Mastro, R. G., Falls, K., Simon, J., Torrey, D., Pandit, S., McKenny, J., Braunschweiger, K., Walsh, A., Liu, Z., Hayward, B., Folz, C., Manning, S. P., Bawa, A., Saracino, L.
Effects of the N107I Mutation of NPSR

Thackston, M., Benchekroun, Y., Capparell, N., Wang, M., Adair, R., Feng, Y., Dubois, J., FitzGerald, M. G., Huang, H., Gibson, R., Allen, K. M., Pedan, A., Danzig, M. R., Umland, S. P., Egan, R. W., Cuss, F. M., Rorke, S., Clough, J. B., Holloway, J. W., Holgate, S. T., and Keith, T. P. (2002) Nature 418, 426–430

6. Zhang, Y., Leaves, N. I., Anderson, G. G., Ponting, C. P., Broxholme, J., Holt, R., Edser, P., Bhattacharyya, S., Dunham, A., Adcock, I. M., Pulleyn, L., Barnes, P. J., Harper, J. I., Abecasis, G., Cardon, L., White, M., Burton, J., Matthews, L., Mott, R., Ross, M., Cox, R., Moffatt, M. F., and Cookson, W. O. (2003) Nat. Genet. 34, 181–186

7. Xu, Y. L., Reinscheid, R. K., Huittion-Resendiz, S., Clark, S. D., Wang, Z., Lin, S. H., Brucher, F. A., Zeng, J., Ly, N. K., Henriksen, S. J., de Lecea, L., and Civelli, O. (2004) Neuron 43, 487–497

8. Gloriam, D. E., Schioth, H. B., and Fredriksson, R. (2005) Biochim. Biophys. Acta 1722, 235–246

9. Gupte, J., Cutler, G., Chen, J. L., and Tian, H. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 1508–1513

10. Kormann, M. S., Carr, D., Klopp, N., Illig, T., Leupold, W., Fritzsch, C., Weiland, S. K., von Mutius, E., and Kabesch, M. (2005) Am. J. Respir. Crit. Care Med. 171, 1358–1362

11. Melnyk, R. A., Partridge, A. W., and Deber, C. M. (2002) J. Mol. Biol. 315, 63–72

12. Melen, E., Bruce, S., Doekes, G., Kabesch, M., Laitinen, T., Lauener, R., Lindgren, C. M., Riedler, J., Scheunius, A., Hage-Hamsten, M., Kere, J., Pershagen, G., Wickman, M., and Nyberg, F. (2005) Am. J. Respir. Crit. Care Med. 171, 1089–1095

13. Beck, B., Fernette, B., and Stricker-Krongrad, A. (2005) Biochem. Biophys. Res. Commun. 332, 859–865

14. Reinscheid, R. K., Xu, Y. L., Okamura, N., Zeng, J., Chung, S., Pai, R., Wang, Z., and Civelli, O. (2005) J. Pharmacol. Exp. Ther. 315, 1338–1345

15. Guan, X. M., Kobilka, T. S., and Kobilka, B. K. (1992) J. Biol. Chem. 267, 21995–21998

16. Goldman, L. A., Cutrone, E. C., Kotenko, S. V., Krause, C. D., and Langer, J. A. (1996) BioTechniques 21, 1013–1015

17. Melnyk, R. A., Partridge, A. W., and Deber, C. M. (2002) J. Mol. Biol. 315, 63–72

18. Mathieu, M. C., Sawyer, N., Greig, G. M., Hamel, M., Kargman, S., Duccharme, Y., Lau, C. K., Friesen, R. W., O’Neill, G. P., Gervais, F. G., and Therien, A. G. (2005) Immunol. Lett. 100, 139–145

19. Bax, A., and Davis, D. G. (1985) J. Magn. Reson. 65, 355–360

20. Wuthrich, K. (2005) NMR of Proteins and Nucleic Acids, Wiley, New York

21. States, D. J., Haberkorn, R. A., and Ruben, D. J. (1982) J. Magn. Reson. 48, 286–292

22. Levitt, M. H., Freeman, R., and Frenkel, T. (1982) J. Magn. Reson. 47, 328–330

23. Chang, C. T., Wu, C. S., and Yang, J. T. (1978) Anal. Biochem. 91, 13–31

24. Yang, J. T., Wu, C. S., and Martinez, H. M. (1986) Methods Enzymol. 130, 208–269

25. Dyson, H. J., Rance, M., Houghten, R. A., Wright, P. E., and Lerner, R. A. (1988) J. Mol. Biol. 201, 201–217

26. Hoyer, D., and Boddeke, H. W. (1993) Trends Pharmacol. Sci. 14, 270–275