The Unpredicted High Affinities of a Large Number of Naturally Occurring Tachykinins for Chimeric NK₁/NK₃ Receptors Suggest a Role for an Inhibitory Domain in Determining Receptor Specificity*

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Three chimeric receptors were constructed by exchanging exon sequences between human NK₁ and NK₃ receptor genes. The resulting chimeric receptors not only retained high affinities for their natural ligands but also exhibited surprisingly high affinities for other naturally occurring tachykinins including neurokinin A, neuropeptide K, neuropeptide Y, edledoisin, kassinin, physalaemin, and phylomedusin. In contrast, these chimeric receptors displayed a wide range of variability in their affinities for non-naturally occurring ligands including selective agonists and antagonists of NK₁, NK₂, and NK₃ receptors. Since the only common feature among these naturally occurring neurokinin peptides is the conserved C-terminal sequences, our data suggest that these conserved sequences must play the major role in conferring high affinity binding to the chimeric receptors. To explain the apparently “improved” affinities of these naturally occurring ligands for the chimeric receptors as compared with their affinities for the parent NK₁ and NK₃ receptors, we are proposing that certain inhibitory domains that are present in the NK₁ and/or NK₃ receptors are compromised in these chimeric receptors. Upon disruption of these inhibitory domains during the formation of chimeras, the naturally occurring ligands can interact more favorably with chimeric receptors through their conserved C-terminal sequences. Based on this hypothesis, the binding affinities of natural tachykinin ligands may be largely determined by their conserved C-terminal sequences, whereas receptor selectivities of these ligands are influenced more by the presence or absence of inhibitory domains rather than specific binding domains on their target receptors.

Substance P, neurokinin A (NKA), and neurokinin B (NKB), the major mammalian neurokinin peptides, belong to the tachykinin family and share a common C-terminal sequence of -Phe-Xaa-Gly-Leu-Met-NH₂. There are at least three neurokinin receptor subtypes, NK₁, NK₂, and NK₉, that have been proposed to mediate the biological functions of these neurokinin peptides. Substance P has higher affinity to the NK₁ receptor than to the other two neurokinin receptor subtypes and is believed to act as an important neurotransmitter (1). The human NK₁ receptor is widely distributed in both central and peripheral nervous systems and has been proposed to be involved in many physiological and pathological conditions such as nociceptive stimuli, neurogenic inflammation, emesis, intestinal motility, vasodilation, smooth muscle contraction, salivary and airway secretion as well as immune response (1–3). The NK₂ receptor is widely distributed in the peripheral nervous system such as in the smooth muscles of the respiratory, gastrointestinal, and urinary tract. Among three tachykinins, NKA has the highest affinity to the NK₁ receptor. Activation of this receptor subtype results in facilitation of transmitter release, neuronal excitation, and stimulation of certain immune cells (3). NK₂ receptors have also been detected in the central nervous system. However, the exact function of this receptor in the central nervous system has yet to be delineated. Compared with the NK₁ and NK₂ receptors, much less is known about the biological function of the NK₉ receptor. NK₉ receptors are mainly distributed in the central nervous system (4), and NKB has the highest affinity to this receptor subtype. With the recent development of selective NK₃ antagonists (5, 6), it is expected that the biological function of the NK₃ receptor will be elucidated in the near future.

All three neurokinin receptor genes have been cloned (7–9). Based on the deduced protein sequences, neurokinin receptors belong to the G-protein-coupled receptor superfamily with the structural characteristics of seven transmembrane helices (TM). All three neurokinin receptors share a high degree of homology (10, 11). Careful examination of the gene structures of the neurokinin receptors has revealed that all three neurokinin receptors are encoded by a five-exon gene structure (7–9, 11). Furthermore, the exon/intron junction sites among all three neurokinin receptor genes are also fully conserved (9, 12). It is conceivable that all of the neurokinin receptor genes may have evolved from a common ancestor gene, and the protein sequences encoded by individual exons may serve as functional motifs for these receptors. If these assumptions are correct, then by shuffling exons among different receptor genes, it should be possible to create “new” tachykinin receptors that might have different pharmacological profiles than the existing tachykinin receptors. Based on this hypothesis, we constructed three chimeric receptors by shuffling exons between human NK₁ and NK₃ receptor genes (Fig. 1). The choice for using NK₁ and NK₃ receptors is based on the fact that these two tachykinin receptor subtypes have the most different pharmacological profiles among the three tachykinin receptors. In the present study, substance P, neurokinin A (NKA), NKB, as well as several nonmammalian tachykinin peptides (Fig. 2) were employed to examine their interactions with three chimeric recep-
tors constructed by exon shuffling. Furthermore, the binding characteristics of some highly selective peptide agonists and non-peptide antagonists that belong to each of three tachykinin receptor categories were also examined. Based on findings from these studies, a hypothesis involving inhibitory domains has been proposed to explain the unique binding properties observed in these chimeric receptors.

**EXPERIMENTAL PROCEDURES**

Materials—

- $^{125}$I-Bolton-Hunter-labeled substance P ($^{125}$I-BHSP), $^{125}$I-NKA, $^{125}$I-eledoisin, and $^{125}$I-MePhe$^7$NKB were purchased from DuPont NEN.
- Substance P, octa-substance P (SP 4–11), hexa-substance P (SP 6–11), penta-substance P (SP 7–11), NKA, NKB, eledoisin, neuropeptide K, neuropeptide-g, kassinin, physalaemin, phyllomedusin, [Sar$^9$,Met(O$_2$)$^{11}$]SP, SPOMe, sepiptide, GR64349, kensitide, [Pro$^7$]NKB, and [MePhe$^7$]NKB were purchased from Peninsula Laboratories, Inc. (Belmont, CA).
- SR140,333, SR142,801, and SR48,968 were kindly provided by Dr. X. Emonds-Alt of Sanofi Recherche (France).
- [Ala$^b$]NKA$^4–10$, L-703,606, and L-659877 were purchased from RBI (Natick, MA).
- Myo-$[^{3}$H$]$inositol was purchased from Amersham Corp.

Cloning of the Human NK1 and NK3 Receptor Genes and Construction of NK1/NK3 Chimeric Receptors—Individual exons of human NK$_1$ or NK$_3$ receptor genes were amplified directly from the human genomic DNA (purchased from Clontech) using polymerase chain reaction. Oligonucleotides, 24–30-mers in length, corresponding to 5' or 3' ends of individual exons, were used as primers in the amplification reaction. Full-length human NK$_1$, or NK$_3$ receptor genes were assembled by connecting individual exons in a recombinant polymerase chain reaction using primers containing overlapping sequences of adjacent exons (13). Chimeric receptors, EX(I), EX(I–II), and EX(I–III), were constructed by shuffling exons between human NK$_1$ and NK$_3$ receptors. As depicted in Fig. 1, the N-terminal protein sequences of the constructed EX(I), EX(I–II), and EX(I–III) chimeric receptors were derived from corresponding exons of the human NK$_1$ receptor gene (13). The authenticity of entire gene sequences was confirmed by dideoxy sequencing. The full-length human NK$_1$, NK$_3$, or recombinant chimeric receptor DNA sequences were inserted into an expression vector pRC/CMV. The recombinant plasmids were transfected into CHO cells by electroporation, and permanent cell lines were selected in the presence of G418 as described previously (14).

Receptor Binding Assay—CHO cells expressing either human NK$_1$, NK$_3$ receptors, or various chimeras were cultured in Ham’s F-12 nutrient mixture supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Cells were seeded to 96-well Wallac (Gaithersburg, MD) rigid cross-talk corrected cell culture plate 1 day before the experiment.

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**FIG. 1.** Proposed structure of the human NK$_3$ receptor and schematic representations of the human NK$_1$, NK$_3$, and three NK$_1$/NK$_3$ chimeric receptors. Upper panel, the proposed structure of the human NK$_3$ receptor. Solid circles with white letters indicate amino acid residues conserved between the human NK$_1$, and NK$_3$ receptors. Open circles with black letters indicate amino acid residues specific for the human NK$_3$ receptor. J unctions between adjacent exons are indicated by arrows and are numbered in order. Lower panel, schematic representations of the human NK$_1$, NK$_3$, and three chimeric receptors. Seven transmembrane helices are presented as rectangular boxes with numbers corresponding to the TMI to TMVII. Filled blocks and bars represent human NK$_1$, sequences, and open blocks and bars represent NK$_3$, sequences. Arrows indicate junctions between adjacent exons.
High Affinity Binding of Natural Ligands to Tachykinin NK\textsubscript{1}/ NK\textsubscript{3}

**Mammalian Tachykinins**

| Peptide | Sequence |
|---------|----------|
| SP      | Arg-Pro-Lys-Pro-Glu-Gln-Phe-Phe-Gly-Leu-Met-NH\textsubscript{2} |
| NKA     | His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH\textsubscript{2} |
| NKB     | Asp-Met-His-Asp-Phe-Val-Gly-Leu-Met-NH\textsubscript{2} |

**Non-mammalian Tachykinins**

| Peptide   | Sequence |
|-----------|----------|
| Eledoisin | pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH\textsubscript{2} |
| Kassinin  | Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH\textsubscript{2} |
| Phaslylemin | pGlu-Ala-Asp-Pro-Ala-Lys-Phe-Tyr-Gly-Leu-Met-NH\textsubscript{2} |
| Phyllomedusin | pGlu-Pro-Ala-Ala-Lys-Phe-Ile-Gly-Leu-Met-NH\textsubscript{2} |

**RESULTS**

Binding Characteristics of the Human NK\textsubscript{1} and NK\textsubscript{3} Receptors—Human NK\textsubscript{1} and NK\textsubscript{3} receptors were expressed in CHO cells at high levels as indicated by their B\textsubscript{max} values listed in Table I. Substance P and [MePhe\textsuperscript{7}]NKB exhibited high affinity to human NK\textsubscript{1} and NK\textsubscript{3} receptors (Table I), respectively, as have been previously reported (3, 14, 16, 17). Chimeric receptors, i.e. EX(I–III), EX(I–II) and EX(I), were also expressed at high levels in CHO cells. All three chimeric receptors exhibited high affinities for substance P with K\textsubscript{d} values in the nanomolar range (Table I). Subsequently, [\textsuperscript{125}I]-BHSP was used as the primary radioligand to examine binding affinities of other tachykinin peptides to these chimeric receptors. The high affinities of substance P to EX(I–III), EX(I–II) and EX(I) chimeric receptors also suggest that the structural integrity has been largely maintained in these chimeric receptors constructed by exon shuffling.

Binding of Naturally Occurring Tachykinin Peptides to NK\textsubscript{1}/NK\textsubscript{3} Chimeric Receptors—In addition to substance P, NKA and NKB also demonstrated high potencies in displacing [\textsuperscript{125}I]-BHSP binding to EX(I–III), EX(I–II), and EX(I) chimeric receptors (Fig. 3). In contrast, NKA and NKB were relatively weak in displacing [\textsuperscript{125}I]-BHSP binding to human NK\textsubscript{3} receptors, whereas substance P and NKB were relatively weak in displacing [MePhe\textsuperscript{7}]NKB binding to human NK\textsubscript{3} receptors (Fig. 3). Since all three chimeric receptors contain sequences encoded by exon 1 of the NK\textsubscript{1} receptor gene as well as exon IV-V of the NK\textsubscript{3} receptor gene, the high affinity binding of substance P and NKB to these chimeras could be attributed to the conservation of these sequences in the chimeras. However, the high potencies of NKA in displacing [\textsuperscript{125}I]-BHSP binding to these chimeric receptors were unexpected. Since these chimeric receptors were constructed using only human NK\textsubscript{1} and NK\textsubscript{3} receptor sequences, the high affinities of NKA to these receptors suggest that some residues conserved among NK\textsubscript{1}, NK\textsubscript{2}, and NK\textsubscript{3} receptors are probably involved. NKA shares a highly conserved C-terminal pentapeptide sequence with substance P and NKB (Fig. 2), and it is possible that the C-terminal sequence of NKA plays a major role in determining its binding affinities to these chimeric receptors. To further study this possibility, several other nonmammalian tachykinin peptides with variable N-terminal sequences were examined for their affinities to EX(I–III), EX(I–II), and EX(I) chimeric receptors. As shown in Table II, all naturally occurring tachykinins examined displayed high affinities to these chimeric receptors. Phaslylemin and phyllomedusin, which have high affinities for the human NK\textsubscript{1} receptor and low affinities to the human NK\textsubscript{3} receptor, displayed high affinities for all three chimeric receptors with IC\textsubscript{50} values in the nanomolar range (Table II). Eledoisin, kassinin, neuropeptide K, and neuropeptide γ, which have relatively low affinities for either human...
NK₁ or NK₃ receptors (11, 18), also demonstrated high affinities for these chimeric receptors (IC₅₀ values in the nano- or subnanomolar range, Table II). Similar results were obtained using either ¹²⁵I-NKA or ¹²⁵I-eledoisin as radioligands (data not shown). Since all eight naturally occurring tachykinin peptides demonstrated similar high affinities for EX(I–III), EX(I–II), and EX(I) chimeric receptors but contain very different N-terminal sequences, it seems to suggest that the C-terminal common sequences largely determine the binding affinities of these tachykinin peptides to the chimeras.

To confirm the high affinities of these peptides for chimeric receptors, direct binding assay using radiolabeled NKA and eledoisin was carried out. Kᵦ and B_max values of ¹²⁵I-NKA and ¹²⁵I-eledoisin binding to all three chimeric receptors are listed in Table III. Data derived from direct binding experiments are consistent with data derived from the competition experiments using ¹²⁵I-BHSP (Table II).

**Fig. 3.** Competition binding isotherms of substance P, neurokinin A, and neurokinin B to human NK₁, NK₂, and three chimeric receptors. ¹²⁵I-BHSP (0.2 nM) was used to label human NK₁ and chimeric receptors, and [¹²⁵I-MePhe⁷]NKB was used to label human NK₃ receptors as described under “Experimental Procedures.” Data were presented as a percent inhibition of the specific binding of 0.2 nM ¹²⁵I-BHSP or [¹²⁵I-MePhe⁷]NKB. The results were representative of three to five independent experiments. Each point represents the mean of triplicate experiments and vertical bar the standard error of the mean.

### Table II

IC₅₀ values of some natural tachykinin peptides on ¹²⁵I-BHSP binding to human NK₁, chimeric EX(I–III), EX(I–II), and EX(I) receptors and on [¹²⁵I-MePhe⁷]NKB binding to human NK₃ receptors

| Receptor | ¹²⁵I-NKA IC₅₀ (nM) | ¹²⁵I-Eledoisin IC₅₀ (nM) |
|----------|--------------------|------------------------|
| Substance P | 0.80 ± 0.01 | 0.04 ± 0.01 |
| NKA | 84.3 ± 11.1 | 1000 |
| NKB | 111 ± 10 | 1000 |
| Eledoisin | 25.0 ± 4.1 | 1000 |
| Kassinin | 170 ± 85 | 1000 |
| NP-α | 25.2 ± 3.0 | 1000 |
| NP-K | 331 ± 35 | 1000 |
| Physalaemin | 0.75 ± 0.06 | 1000 |
| Phyllomedusin | 3.62 ± 0.50 | 1000 |

**Fig. 4.** Phosphatidylinositol turnover stimulated by substance P in CHO cells expressing human NK₁ receptors (Fig. 4) is also potent (EC₅₀ 3.8 ± 0.5 nM) than substance P or NKA (EC₅₀ 219 ± 42 and 87 ± 12 nM, respectively) in stimulating phosphatidylinositol turnover in cells expressing human NK₃ receptors (Fig. 4), which was also consistent with the binding data and the notion that NKB is the preferred ligand for this receptor subtype. Consistent with the binding data, substance P, NKA, and NKB demonstrated high capacities in stimulating phosphatidylinositol turnover in cells expressing EX(I–III), EX(I–II), and EX(I) chimeric receptors as well as NK₁ and NK₃ receptors examined. As has been reported, substance P was very potent (EC₅₀ 0.66 ± 0.11 nM) in stimulation of phosphatidylinositol turnover in CHO cells expressing human NK₁ receptors (Fig. 4). NKA and NKB were also potent in stimulating human NK₁ receptors (EC₅₀ 4.4 ± 0.6 and 7.8 ± 1.0 nM, respectively), although not as potent as substance P. On the other hand, NKB was more potent (EC₅₀ 219 ± 42 and 87 ± 12 nM, respectively) in stimulating phosphatidylinositol turnover in cells expressing human NK₃ receptors (Fig. 4), which was also consistent with the binding data and the notion that NKB is the preferred ligand for this receptor subtype.
highly selective agonists to each subtype of tachykinin receptors were studied using the best of choice of radioligand for these chimeric receptors. Three highly selective NK₁ agonists, [Sar⁹,Met(O₂)¹¹]SP, SPOMe, and septide, were characterized for their affinities for chimeric receptors using ¹²⁵I-BHSP. The inhibition of ¹²⁵I-BHSP binding to chimeric receptors by these agonists indicated very different interaction patterns (Table IV). The potency of [Sar⁹,Met(O₂)¹¹]SP for inhibition of ¹²⁵I-BHSP binding remained relatively high for EX(I–III) and EX(I–II) but was very low for EX(I) chimeric receptors (Table IV), suggesting that the N-terminal NK₁ receptor sequence, especially sequences that include TMV₁, may play an important role in the interaction of this highly selective NK₁ agonist with the human NK₁ receptor. SPOMe, a carboxyl-terminal-modified substance P derivative, showed little affinity for all three chimeric receptors (Table IV), indicating that the interaction sites for this selective substance P derivative are quite different from that for substance P. It seems to suggest that some nonconserved amino acids in the C terminus (TMV₁ and TMVII) of the human NK₁ receptor may contribute to the specific interaction of SPOMe with the human NK₁ receptor, since replacement of this sequence with its counterpart of the human NK₃ receptor resulted in a total loss of binding for SPOMe. Due to its high potency in functional assays and low potency in displacing radiolabeled SP, peptide appears to interact with NK₁ receptors differently from that of substance P (22). As shown in Table IV, sequential addition of sequences encoded by exon 2 and exon 3 of the NK₁ receptor (as in EX(I–II) and EX(I–III)) actually reduced instead of increased their abilities to interact with peptide, indicating a complex interaction profile for this NK₁ agonist. SR₁40,333 and L703,606 are highly selective non-peptide NK₁ antagonists. Both antagonists demonstrated no binding to all three chimeric receptors (Table IV), suggesting that the major interaction sites for these selective non-peptide NK₁ antagonists may not be present in these chimeric receptors. Direct binding assay using ¹²⁵I-L703,606 or [³⁵S]SR140,333 also confirmed the lack of binding for these two NK₁ antagonists to chimeric EX(I–III), EX(I–II), and EX(II) receptors (data not shown).

The binding affinities of selective NK₃ antagonists ([MePhe⁷]NK₃, senktide, and Pro7-NKB) and selective antagonists (PD157, 672, and SR142, 801) to chimeric receptors were also studied using ¹²⁵I-eledoisin. Although all of the NK₃ agonists examined exhibited high affinities for EX(I) chimeric receptors, [MePhe⁷]NK₃, senktide, and Pro⁷-NKB demonstrated lower affinity to the other two chimeric receptors (Table IV). In contrast, NKB and eledoisin, the two natural

 ordinal assays suggest that all three chimeric receptors not only can interact with tachykinin ligands but also are capable of coupling to the phospholipase C signal transduction pathway. Binding of [MePhe⁷]NK₃ to Chimeric Receptors—[MePhe⁷]NK₃ is a potent and highly selective agonist for the NK₃ receptor and has been widely used in radiolabeling this receptor subtype (16, 21). Competition binding assays using [MePhe⁷]NK₃ as the tracer to study ligand-receptor interaction with EX(I–III), EX(I–II), and EX(I) chimeric receptors show poor labeling (data not shown). Direct comparison of [MePhe⁷]NK₃ binding to the wild-type human NK₃ and to EX(I–II) receptors was carried out to examine the binding characteristics of [¹²⁵I]MePhe⁷NK₃ for these receptors. [¹²⁵I]MePhe⁷NK₃ displayed high affinity binding to human NK₃ receptors with Kᵟ of 2.8 ± 0.2 nM (Fig. 5). Functionally, [MePhe⁷]NK₃ was potent in stimulating phosphatidylinositol turnover in cells expressing the human NK₃ receptor. On the other hand, the natural ligand NK₃ displayed relatively low potency in displacing [¹²⁵I]MePhe⁷NK₃ binding to the human NK₃ receptor (IC₅₀, 230 ± 2.4 nM; Fig. 5) and in stimulating phosphatidylinositol turnover in cells expressing the human NK₃ receptor (Fig. 4; EC₅₀, 18.5 ± 1.9 nM). [MePhe⁷]NK₃, therefore, displayed an order of magnitude higher affinity for the human NK₃ receptor than that found in NK₃. Examination of the binding of [MePhe⁷]NK₃ and NK₃ to EX(I–II) chimeric receptors revealed that the relative potencies of two compounds are completely reversed in the chimeric receptor. For instance, NK₃ displaced ¹²⁵I-BHSP binding to EX(I–II) receptors with an IC₅₀ value of 6.3 ± 0.8 nM, whereas [MePhe⁷]NK₃ has an IC₅₀ value of 165 ± 22 nM. Similarly, NK₃ displayed a potency of 7.2 ± 1.6 nM (EC₅₀) in stimulating phosphatidylinositol turnover in cells expressing EX(I–II) receptors, and in the same experiment [MePhe⁷]NK₃ had an EC₅₀ of 71.7 ± 25 nM. Based on these studies, ¹²⁵I-eledoisin instead of [¹²⁵I]MePhe⁷NK₃ was used as the radioligand to characterize the binding of NK₃ ligands to chimeric receptors.

Receptor Binding Characteristics of Selective Agonists and Antagonists—Although ¹²⁵I-BHSP, ¹²⁵I-NKA, and ¹²⁵I-eledoisin have been widely used as radioligands to characterize NK₁, NK₂, and NK₃ receptors, respectively, it has not been possible to characterize the same receptor using all three radioligands as a result of their differences in affinities for different receptor subtypes. Since all three radioligands displayed high affinities to these chimeric receptors, it was possible to characterize a chimeric receptor using any of the three radioligands. In the following experiments, the binding characteristics of some highly selective agonists and antagonists to each subtype of
occurring tachykinin peptides, exhibited high potencies in displacing $^{125}$I-eledoisin binding to all three chimeric receptors (Table IV), which is consistent with the previous results obtained using $^{125}$I-BHSP as the radioligands (Table II). The discrepancy in binding affinities between natural and non-natural tachykinin peptides may be explain by the fact that unlike NKB and eledoisin, $[^{125}$I-MePhe$]$NKB, senktide, and Pro$^7$-NKB all have modified C-terminal pentapeptide sequences. Although they have high affinities to the wild-type human NK$_3$ receptor and demonstrated high selectivity, their interaction sites with the NK$_3$ receptor may be different from that of the natural tachykinin peptides. Additional interaction sites for these modified tachykinin agonists may have been lost in EX(I–II) and EX(I–III) chimeric receptors. PD157,672, a highly selective and potent antagonist, also failed to bind to any chimeric receptors. Since antagonist may not necessarily share the same interaction sites with agonist and PD157,672 is a relatively small molecule as compared with that of the natural ligands, the binding site(s) of PD157,672 could be located in a more restricted region that may have been lost in these chimeric receptors. Another highly selective and potent NK3 antagonist SR142,801(23), on the other hand, retained high af-
In this study, three chimeric receptors were constructed based on the highly conserved structural arrangement of mammalian neurokinin receptor genes. An unexpected finding was that of nine naturally occurring tachykinin peptides examined, i.e., substance P, NKA, NKB, edoainsin, kassinin, neuropeptide K, neuropeptide γ, physalaemin, and phylomedusin, all demonstrated high affinity binding to these chimeric receptors with K<sub>i</sub> values in the nano- or subnanomolar range. Most of these natural tachykinin peptides displayed higher affinities for the chimeric receptors than for their parent human NK<sub>1</sub> or NK<sub>3</sub> receptors. For chimeras constructed between rat NK<sub>1</sub> and NK<sub>3</sub> receptors, Gether et al. (24) have also observed a similar increase in affinities of NKA and edoainsin (24).

Based on the "message and address" hypothesis (25, 26), it has been proposed that the highly conserved C-terminal sequence -Phe-Xaa-Gly-Leu-Met-NH<sub>2</sub> of all neurokinin peptides may serve as the "message," which can relay the signal to the receptor and result in receptor-mediated intracellular signal transduction. On the other hand, the N-terminal highly variable sequences in each neurokinin peptide may serve as the "address," with which the selectivity of individual neurokinin peptides is determined (25, 26). Based on this hypothesis, extensive receptor mutagenesis studies have been conducted in search of specific receptor sequences that may interact directly and specifically with the address portion of naturally occurring neurokinin peptides (27). However, most of the receptor sequences identified to be important for its natural ligand binding are sequences conserved among different receptor subtypes (28, 29), indicating that these receptor sequences may be interacting with the message instead of the address portion of the neurokinin peptides.

To explain our data with the message and address theory, we would have to assume that several new high affinity binding sites specific for various address sequences were created simultaneously in these chimeric receptors. It seems very unlikely that these high affinity binding sites can be created randomly by combining two receptor sequences that contain only low affinity binding sites for these ligands. An alternative interpretation of our data from an evolutionary point of view seems attractive. Based on the highly conserved gene structure of neurokinin receptors, it may be hypothesized that these receptors have evolved from the same ancestral gene. The primate neurokinin receptor binds to the primate peptide by interacting with its C-terminal pentapeptide sequence. During the course of evolution, different neurokinin receptor subtypes as well as different neurokinin peptides may have diverged from their common ancestor; however, the common binding sites for the primate peptide to different subtypes of neurokinin receptors might have been preserved during the course of evolution. Selectivity for individual receptor subtypes for their preferred peptide ligand may have evolved not by creating additional binding sites for each specific ligand but by acquiring steric hindrances (most likely located in the extracellular loops) that will allow only the preferred ligands to interact with the common binding site. These steric hindrances or inhibitory domains could result from the collective effect of many or just a few amino acid residues that can form spatial obstacles to prevent other peptides from reaching the common interaction site. Based on this hypothesis, the high affinity binding of these naturally occurring tachykinin peptides to the chimeric receptors can be simply explained by the disruption of the inhibitory domains in these chimeras, which then allows other natural ligands containing the conserved C-terminal sequence to interact favorably with the common binding site. This hypothesis may also explain the difficulties in trying to find selective natural ligand interaction sites in each receptor subtype. Mutagenesis results indicate that amino acid residues critical for neurokinin binding to NK<sub>1</sub> or NK<sub>2</sub> receptors are mostly conserved among the three neurokinin receptors and are located in the transmembrane domains (28, 29). Consistent with our hypothesis, the existence of distinct negative determinants that restrict the ligand-receptor interaction has also been reported in receptors for the luteinizing hormone and follicle-stimulating hormone (30).
tion of chimeric receptors by exon shuffling may have reversed the evolutionary process by removing these inhibitory domains. As a result, not only substance P and NKB but also six other tachykinin peptides all displayed high and similar affinities to these chimeric receptors. According to this hypothesis, it may be further postulated that the inhibitory domains for substance P and NKA would be located in the N-terminal region encoded by the exon 1 of the human NK3 receptor gene. This conclusion is supported by the fact that replacement of these sequences with their corresponding sequences of the human NK1 receptor resulted in an increase in binding affinities of substance P to these chimeric receptors. Using the same argument, the inhibitory domains for NKB may also be postulated to be located in the C-terminal region encoded by exon 4–5 of the human NK1 receptor gene. Since exon 5 only encodes the C-terminal sequence that is believed to be located intracellularly (Fig. 1), the inhibitory domains for NKB can be further confined to the region encoded by exon 4 of the human NK1 receptor gene. For other naturally occurring tachykinin peptides, various inhibitory domains located in the N-terminal region of NK3 and/or C-terminal region of NK1 receptor(s) may be involved in preventing these peptides from interacting optimally with the common binding site.

The results from studying the binding characteristics of several non-natural selective agonists and antagonists in these chimeric receptors are also quite informative. All of the selective agonists with modified C terminus have lower affinities for these chimeric receptors than that of their natural counterparts, indicating the importance of having the intact conserved C terminus to be fully recognized by the common binding site (Table IV). With the exception of SR 48,968 and SR 142,801, all antagonists examined show little or no binding to these chimeric receptors (Table IV). The lack of binding of antagonists may indicate that these inhibitory domains are likely the favorable targets for antagonist binding. This observation may also have important pharmaceutical implications in the future design and development of new tachykinin antagonists. Binding studies involving substance P fragments also confirmed the importance of the conserved C-terminal sequence in determining binding affinities to the chimeric receptors (Table V). The N-terminal sequence of substance P, although it may not be directly involved in receptor binding, is probably very important in maintaining the proper conformation of the C-terminal peptide as evident by the poor binding of SP 7–11 to the chimeric receptors (Table V).

In summary, functional chimeric receptors were constructed by shuffling exons between the human NK1 and NK3 receptor genes. High affinity binding of nine naturally occurring tachykinin peptides to these chimeric receptors suggests that there may be a common binding pocket for all neuropeptides. The following hypothesis was proposed to explain our data. The binding affinities of natural ligands are largely determined by the interaction of their C-terminal conserved sequences with a common binding site in these receptors, whereas the selectivities of natural ligands are mostly influenced by the presence or absence of inhibitory domains that serve as negative determinants to prevent ligand-receptor interaction.

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