Identification of Potential Tyrosine-containing Endocytic Motifs in the Carboxyl-tail and Seventh Transmembrane Domain of the Neurokinin 1 Receptor*

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Although agonist-induced endocytosis of G-protein-coupled receptors is critical for receptor desensitization and resensitization, receptor motifs that interact with the endocytic apparatus have not been adequately characterized. We examined the effects of mutating the rat neurokinin-1 receptor on endocytosis using 125I-substance P, fluorescent substance P, and receptor antibodies. Substance P induced rapid internalization of wild-type receptors that were targeted to perinuclear endosomes. Truncation of the C-tail at residues 324, 342, and 354 reduced internalization up to 80% and caused retention of receptors at the cell surface and in superficial endosomes. Mutation of Tyr-341 and Tyr-349 in potential tyrosine-containing endocytic motifs of the C-tail also impaired internalization. A Y305A mutant within the putative NPXY endocytic motif of the seventh transmembrane domain showed impaired signaling and was minimally expressed at the plasma membrane but was found in cytoplasmic vesicles. In contrast, a Y305F mutant signaled normally and was normally expressed at the plasma membrane but showed impaired internalization. Thus, endocytosis of the neurokinin 1 receptor relies on several tyrosine-containing sequences in the C-tail and seventh transmembrane domain.

Receptor endocytosis is a fundamental process with many diverse functions. Endocytosis of some receptors, such as the transferrin receptor, is required for the uptake of nutrients by many cell types. These receptors internalize constitutively, without the need to bind ligand. In contrast, agonist binding is required for internalization of receptors for many hormones, neurotransmitters, and growth factors (1). Endocytosis of these receptors contributes to receptor desensitization, resensitization, and down-regulation, critical processes that regulate cellular responses.

The main pathway for constitutive and agonist-induced endocytosis is by clathrin-coated pits, although alternative pathways involve non-coated vesicles or caveolae (2). Clathrin-mediated endocytosis probably requires interaction of specific receptor motifs with components of the endocytic machinery (3). Tyrosine-containing motifs of six residues forming a tight β-turn are common, and interchangeable endocytic signals are found in the intracellular domains of constitutively internalized and tyrosine kinase receptors (1, 4). In constitutively internalized receptors, they may be continuously exposed to the endocytic machinery, whereas agonist-induced internalization of the signaling receptors may require receptor modification to expose a cryptic endocytic motif. Thus, a point mutation that inactivates the tyrosine kinase activity of the epidermal growth factor receptor abolishes agonist-induced internalization, probably by preventing exposure of an endocytic motif (5). In contrast, deletion of the entire tyrosine kinase domain abolishes the endocytic switch and results in constitutive endocytosis, which is probably due to continuous exposure of endocytic motifs.

Although regions in the C-tail and TMD1 VII have been implicated as important for endocytosis of some G-protein-coupled receptors (6–12), a common endocytic motif has not been identified, and the nature of the molecular switch for agonist-induced internalization is unknown. Agonist-induced internalization may require activation of signaling mechanisms or receptor phosphorylation. Arguing against the first possibility are observations with receptor mutants that either signal normally and show impaired internalization or exhibit impaired coupling to G-proteins and normal internalization (13, 14). This suggests that different domains are involved in internalization and G-protein coupling. Agonist binding induces phosphorylation of Ser and Thr residues of many G-protein-coupled receptors, and Ser/Thr-rich regions are critical for internalization (6, 8–10, 15). However, phosphorylation of the β2-AR is not required for internalization (16–18).

Recently, there has been considerable interest in the mechanism and function of endocytosis of the NK1R, a G-protein-coupled receptor for the neuropeptide SP (19–22). SP is a neurotransmitter in the central and peripheral nervous systems, stimulates smooth muscle contraction and exocrine gland secretion, and induces neurogenic inflammation, actions that are mediated in part by the NK1R (23). SP stimulates clathrin-mediated endocytosis and recycling of the NK1R in multiple cell types (19–22). It is important to identify endocytic motifs of the NK1R since receptor endocytosis and recycling determine the ability of a cell to respond to SP (24). Although much is known about the extracellular and transmembrane domains of the NK1R that interact with receptor agonists and antagonists

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(25, 26), there is little information about NKIR domains that are necessary for endocytosis. Thus, the aim of this investigation was to identify motifs for SP-induced endocytosis of the NKIR. Based on our knowledge of endocytic motifs of single TMD receptors and on the limited information on G-protein-coupled receptors, we mutated the rat NKIR and investigated the effects of the mutations on agonist-induced endocytosis. We identified Tyr-containing motifs in the C-tail and TMD VII of the NKIR that are important for endocytosis and which resemble motifs of the single TMD receptors.

**EXPERIMENTAL PROCEDURES**

*Reagents—SP* was labeled with Bis-functional cyanine 3.18 as described (27). A rabbit polyclonal antiserum (number 11884-5) was raised to a 15-residue peptide (K**^9^**TMTESSPSYNMLA**^34^**), corresponding to the intracellular C terminus of the rat NKIR (28). A mouse monoclonal antibody (M2) to the Flag peptide (DYKDDDDK) was from International Biotechnologies, Inc. (New Haven, CT). A mouse monoclonal antibody (X22) to the heavy chain of clathrin was a gift from Dr. Francis Brodsky (University of California, San Francisco).

*Mutagenesis of Rat NKIR—*Mutants were generated by PCR using the rat NKIR cDNA with an N-terminal Flag-peptide in pcDNA3/neo as a template (28). PCR of Flag epitope allowed detection of NKIR with the Flag antibody as well as the antibody to the C terminus of the receptor. The Flag does not alter the signaling or trafficking of the NKIR (19, 28).

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**Expression Vectors and Cell Lines—**We stably expressed receptors in KNKR cells, which do not express endogenous NKIR, using Lipofectin (28). Clones were selected in medium containing 800 KNRK cells, which do not express endogenous NK1R, using Lipofectin. All mutated receptors were cloned into the pCMV phage vector (29). The fidelity of PCR amplification was verified by sequencing all constructs.

**Results**—To identify regions of the NKIR that are important for endocytosis, we generated a series of mutants (Fig. 1). C-terminal Truncations—First, we progressively truncated the C-tail (designating receptors as “Δ” followed by number of the last residue). The intracellular C-tail of the rat NKIR extends 99 residues from the plasma membrane (309–407). Tyr, Ser, and Thr residues, usually 30–50 residues from the plasma membrane, have been implicated in endocytosis of several G-protein-coupled receptors (8, 29–32). The C-tail of the NKIR contains three Tyr residues (Tyr-331, Tyr-341, and Tyr-349) that are conserved between species and which may be important for endocytosis. Tyr residues 341 and 349 are surrounded by six Ser and Thr residues. We designed four truncated receptors to remove varying portions of the C-tail (Fig. 1): Δ311, Δ324, Δ311, Δ324, and Δ354 containing a stop codon and a NoI restriction site. The fidelily of PCR amplification was verified by sequencing all constructs in both directions. All mutated receptors were cloned into HindIII/NoI sites of pcDNA3.

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NK1R-342 and NK1R-324 cells, since they showed the largest defects in endocytosis. The initial rate of internalization was $2.4 \pm 0.5/\text{min (}r^2 = 0.999\text{)}$ for NK1R-342 cells and $3.0 \pm 0.6/\text{min (}r^2 = 0.971\text{)}$ for NK1R-324 cells, significantly slower than for NK1R-wt cells ($p < 0.05$ compared with NK1R-wt, Fig. 2A).

We used cy3-SP, immunofluorescence, and confocal microscopy to determine the effects of the mutations on the subcellular distribution of NK1R. We have shown that cy3-SP and NK1R-342 and NK1R-324 cells, since they showed the largest defects in endocytosis. The initial rate of internalization was $2.4 \pm 0.5/\text{min (}r^2 = 0.999\text{)}$ for NK1R-342 cells and $3.0 \pm 0.6/\text{min (}r^2 = 0.971\text{)}$ for NK1R-324 cells, significantly slower than for NK1R-wt cells ($p < 0.05$ compared with NK1R-wt, Fig. 2A).

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**Fig. 1.** Mutations of the C-tail and TMD VII of the rat NK1R. Mutations were made by truncating the C-tail (indicated by arrows) or by replacing individual residues (indicated by black circles and white letters).

**Fig. 2.** Internalization of 125I-SP by wild-type and mutated NK1R. Cells were incubated with 125I-SP for 60 min at 4 °C for equilibrium binding. They were either acid-washed immediately or were incubated at 37 °C for 1, 2, 4, or 8 min and then washed with acid to separate acid-labile (cell surface) from acid-resistant (internalized) label. Results are expressed as the percentage of the specifically bound counts in the internalized fractions and are the mean ± standard error of triplicate observations from $n = 3$ experiments. The inset bar graphs show the rate of internalization calculated from the initial 2 min. *, $<0.05$ compared with NK1R-wt cells.

Endocytic Domains of the NK1 Receptor

NK1R are co-localized in the same endosomes up to 30 min after internalization in KNRK cells expressing NK1R-wt (19–21). Thus, cy3-SP can be used to localize the NK1R at these times. When NK1R-wt cells were incubated with cy3-SP at 4 °C and immediately fixed, cy3-SP was confined to the plasma membrane (Fig. 3). After 2 min at 37 °C, there was minimal detectable surface labeling, and cy3-SP was found in superficial endosomes. After 5 min, cy3-SP was mostly present in perinuclear endosomes (Fig. 3), and the distribution was similar at 10 and 30 min. In NK1R-354, -342, and -324 cells at 4 °C, cy3-SP was confined to the plasma membrane (Fig. 3). In NK1R-354 cells, a substantial amount of cy3-SP remained at the plasma membrane after warming, and endosomes containing cy3-SP remained in a superficial location and did not proceed to a perinuclear region (Fig. 3). The largest defect in internalization was observed in NK1R-342 cells, in which cy3-SP was retained at the plasma membrane and was rarely detected in endosomes even after 30 min (Fig. 3). In NK1R-324 cells, cy3-SP was also detected at the plasma membrane at all time points and there was minimal internalization (Fig. 3).

We assessed the function of the NK1R-wt and the mutated receptors by measuring SP-induced changes in $[\text{Ca}^{2+}]_i$ and determining the EC$_{50}$ and maximal response. In NK1R-wt cells, SP induced a prompt increase in $[\text{Ca}^{2+}]_i$ with an EC$_{50}$ of 0.55 nM (Table I). SP also increased $[\text{Ca}^{2+}]_i$ in NK1R-354, -342, and -324 cells, but SP was more potent, and the dose-response curves were shifted to the left between 3- and almost 7-fold (Fig. 4A, Table I). Maximal responses were similar for NK1R-wt and the truncated receptors. Those receptors with the largest defects in internalization were further characterized by determining the $K_D$ and $B_{\text{max}}$ of the receptors. The $K_D$ for 125I-SP binding was 7.4 ± 1.6 nM and the $B_{\text{max}}$ was 58.4 ± 4.9 fmol/10$^5$ cells for NK1R-wt (Table I). The $K_D$ and $B_{\text{max}}$ were similar in NK1R-342 and -324 cells, thus, although we do not know the reason for increased sensitivity of truncated receptors to SP, their impaired ability to internalize cannot be explained by defective signaling, and defects in ligand binding cannot explain the impaired endocytosis of these truncated receptors.

**Tyrosine Mutations in the C-tail—**Tyr-containing endocytic motifs, in which the Tyr is of critical importance, have been
identified for many of the single TMD proteins (1, 32). We investigated potential Tyr-containing internalization motifs in the C-tail of the NKIR by mutating conserved residues. Individual mutation of the conserved tyrosines (Tyr-331, Tyr-341, and Tyr-349) in the C-tail to Ala significantly reduced the extent of internalization of 125I-SP but to different degrees. Thus, after 5 min at 37°C, 50.5 ± 2.3% of specific counts were internalized by NKIR-Y331A, 48.1 ± 3.8% by NKIR-Y341A cells, and 40.6 ± 3.9% by NKIR-Y349A cells, compared with 57.2 ± 2.0% for NKIR-wt cells (p < 0.05 compared with NKIR-wt, Table I). The greatest effect, for the Y349A mutant, represents a reduction in the extent of internalization of 30%. A double Tyr-341 and Tyr-349 mutation did not have an additive effect on attenuation of endocytosis. The initial internalization rates were 6.3 ± 0.8%/min (r² = 0.980) for NKIR-Y341A and 4.0 ± 0.6%/min (r² = 0.981) for NKIR-Y349A cells and, therefore, significantly slower than for NKIR-wt cells (9.9 ± 0.9%/min) (p < 0.05 compared with NKIR-wt, Fig. 2B).

At 4°C, cy3-SP was confined to the plasma membrane in all mutants involving Tyr residues in the C-tail (NKIR-Y341A and NKIR-Y349A, Fig. 5; NKIR-Y331A and NKIR-Y341A/Y349A not shown). After 5 min at 37°C, cy3-SP was retained at the plasma membrane and found in superficial and perinuclear endosomes in cells expressing NKIR-Y331A (not shown) and NKIR-Y341A (Fig. 5). In NKIR-Y349A cells, cy3-SP was mostly retained at the plasma membrane and in superficial endosomes after 5 min, although there were a few perinuclear vesicles containing cy3-SP (Fig. 5). This is in contrast to NKIR-wt cells, in which cy3-SP was mostly found in many perinuclear endosomes after 5 min (Fig. 3).

EC50 values for SP-induced Ca2+ mobilization ranged from 0.037 to 0.156 nM for NKIR-Y331A, -Y341A, and -Y349A cells compared with 0.55 nM for the NKIR-wt (Fig. 4B, Table I). Maximal Ca2+ responses were similar. The Ka and Bmax were similar to NKIR-wt except for the Y341 mutant, where the Ka was ~4-fold lower. Thus, defects in endocytosis are unlikely to be the result of markedly abnormal binding and signaling.

Tyrosine Mutations in TMD VII—A Tyr-containing motif (NPX2-3-Y) in TMD VII is highly conserved within G-protein-coupled receptors and may be a common endocytic motif (7). We examined this possibility for the NKIR by mutating Tyr-305 in TMD VII to Phe or Ala. Phe can replace Tyr in many, but not all, proteins without a significant effect on internalization, whereas Ala substitutions usually abolish the signal (1).

Mutation of Tyr-305 to Phe significantly reduced the extent of endocytosis of NKIR, as measured by internalization of 125I-SP. Thus, after 5 min at 37°C, only 40.3 ± 4.7% of specific counts were internalized by NKIR-Y305F cells, which represents a reduction of internalization by 30% compared with NKIR-wt cells (p < 0.05 compared with NKIR-wt, Table I). Mutation of Tyr-305 to Ala resulted in increased internalization at 4°C, although there was low specific binding compared with the other mutants. After 60 min at 4°C, 29.4 ± 7.1% of specific counts were internalized in NKIR-Y305A cells, compared with 13.8 ± 1.7% in NKIR-wt cells (Table I). However, after 5 min at 37°C, the proportion of internalized 125I-SP was similar for NKIR-Y305A and NKIR-wt cells. The initial internalization rates were 7.9 ± 1.1%/min (r² = 0.980) for NKIR-Y305F cells and 7.1 ± 0.6%/min (r² = 0.981) for NKIR-Y305A cells (Fig. 2C). These rates were slower than for NKIR-wt cells but not statistically significant different.

At 4°C, cy3-SP was confined to the plasma membrane of NKIR-Y305F cells (Fig. 6). In contrast, there was only very weak surface binding of cy3-SP in NKIR-Y305A cells, and cy3-SP was also detected in vesicles at 4°C (Fig. 6). In NKIR-Y305F cells after 5 min at 37°C, cy3-SP remained at the cell surface and in superficial endosomes, indicating diminished internalization of NKIR-Y305F compared with NKIR-wt (Fig. 6). However, in NKIR-Y305A cells, cy3-SP was found in perinuclear endosomes after 5 min (Fig. 6).

The low surface binding and apparent internalization of 125I-SP and cy3-SP by NKIR-Y305A cells at 4°C prompted us to directly localize the NKIR by immunofluorescence with an antibody to the C-tail of the receptor. When NKIR-wt and NKIR-Y305F cells were incubated with SP at 4°C and immediately fixed, receptor immunoreactivity was mostly confined.
to the plasma membrane with very few immunoreactive endosomes (Fig. 7). In sharp contrast, in NK1R-Y305A cells at 4 °C, receptor immunoreactivity was mostly found in perinuclear vesicles with minimal surface labeling (Fig. 7). After 10 min at 37 °C, receptor immunoreactivity was detected in larger perinuclear vesicles in NK1R-wt cells and retained at the plasma membrane or superficial endosomes in NK1R-3054, NK1R-342, or NK1R-324 cells. Scale bar = 10 μm.

In NK1R-305F cells, the EC₅₀ and efficacy of SP-induced Ca²⁺ mobilization were similar to NK1R-wt cells (Fig. 4C, Table I). However, SP was over 4-fold less potent and 7-fold less efficacious in NK1R-305A than in NK1R-wt cells (Fig. 8, Table I). The Kᵢ values for NK1R-305A and NK1R-305A were similar to NK1R-wt, but the Bₘₐₓ for NK1R-305A cells was markedly lower. Thus, the Y305F mutation does not affect SP-induced Ca²⁺ mobilization, whereas the Y305A mutation interferes with Ca²⁺ mobilization.

Serine/Threonine-Mutations and a Mutation of the EMKST Motif in the C-tail—Ser/Thr-rich regions are critical for internalization of several G-protein-coupled receptors (6, 8–10, 15). Based on our results with the truncated receptors and on studies suggesting that Ser and Thr residues in the vicinity of Tyr residues may be a more common determinant of internalization signals than their possible location in a consensus site for phosphorylation (6, 12), we mutated Ser and Thr residues in the C-tail of the NK1R. We also mutated Lys-337 because it is situated in a EMKST motif of the yeast α-pheromone receptor (35). Mutation of the lysine in this motif prevents endocytosis.

The time course of internalization of the wild-type NK1R was compared to the NK1R-Y341A and NK1R-Y349A cells (Table I). Mutation of all Ser and Thr residues between 338 and 352 (NK1R-DST338-352) slightly enhanced internalization after 5 min at 37 °C but not after 10 min (Table I). The initial internalization rate for NK1R-DST338-352 cells was 13.9 ± 1.4%/min (r² = 0.998), higher than in NK1R-wt cells, but the difference did not reach statistical significance (Fig. 2D).

Cy3-SP was confined to the plasma membrane at 4 °C for all
mutants (Fig. 9). Surprisingly, after 5 min at 37 °C cells expressing NK1R-DST338-352 demonstrated a halo-like retention of cy3-SP in small peripheral vesicles, seemingly in contrast to the data obtained by using 125I-SP. To a lesser extent, this phenomenon was also detectable in cells expressing NK1R-S338G/T339A, NK1R-T339A/S347A, and NK1R-DST338-352 cells. Cells were incubated with cy3-SP at 4 °C and either fixed immediately or washed and incubated at 37 °C for 5 min and then fixed. At 4 °C, cy3-SP was confined to the plasma membrane in all cell lines. After 5 min at 37 °C, cy3-SP was detected in small superficial vesicles immediately beneath the plasma membrane in NK1R-S338G/T339A and NK1R-DST338-352 cells (arrows) and in perinuclear endosomes (arrowheads). Scale bar = 10 μm.

Effects of Mutations on the Mechanism of Endocytosis—To
determine if the mutated receptors with abnormal rates of endocytosis were internalized similarly to NK1R-wt at sites of clathrin (21), we colocalized cy3-SP and clathrin. Cells were incubated with cy3-SP for 60 min at 4 °C, washed, incubated at 37 °C for 2 or 5 min, and fixed, and clathrin was localized by immunofluorescence. In cells expressing NK1R-wt, most superficial vesicles containing cy3-SP were initially stained with the clathrin antibody, as indicated by superimposition of confocal images, where yellow denotes colocalization (Fig. 10). In contrast, cy3-SP was mostly retained at the cell surface of cells expressing truncated receptors such as NK1R-8342, and there was no clathrin colocalization (Fig. 10). Although cy3-SP was internalized more slowly in cells expressing NK1R-Y349A, NK1R-Y331A, or NK1R-Y305F, superficial vesicles containing cy3-SP were still stained with the clathrin antibody (Fig. 10). In cells expressing NK1R-Y305A, there were few superficial cy3-SP were still stained with the clathrin antibody (Fig. 10). Although cy3-SP was internalized more slowly in cells expressing NK1R-Y349A, NK1R-Y331A, or NK1R-Y305F, superficial vesicles containing cy3-SP were still stained with the clathrin antibody (Fig. 10). In cells expressing NK1R-Y305A, there were few superficial vesicles detected containing cy3-SP, although these were occasionally stained with the clathrin antibody (Fig. 10). In cells expressing NK1R-Y305A, there were few superficial vesicles containing cy3-SP were still stained with the clathrin antibody (Fig. 10). In cells expressing NK1R-Y305A, there were few superficial vesicles detected containing cy3-SP, although these were occasionally stained with the clathrin antibody (Fig. 10). In cells expressing NK1R-Y305A, there were few superficial vesicles containing cy3-SP were still stained with the clathrin antibody (Fig. 10). In cells expressing NK1R-Y305A, there were few superficial vesicles containing cy3-SP were still stained with the clathrin antibody (Fig. 10). In cells expressing NK1R-Y305A, there were few superficial vesicles containing cy3-SP were still stained with the clathrin antibody (Fig. 10). In cells expressing NK1R-Y305A, there were few superficial vesicles containing cy3-SP were still stained with the clathrin antibody (Fig. 10). In cells expressing NK1R-Y305A, there were few superficial vesicles containing cy3-SP were still stained with the clathrin antibody (Fig. 10). In cells expressing NK1R-Y305A, there were few superficial vesicles containing cy3-SP were still stained with the clathrin antibody (Fig. 10).

DISCUSSION

The combination of receptor mutagenesis, analyses using radiolabeled and fluorescently labeled SP, and use of antibodies to the NK1R and clathrin permitted detailed investigation of endocytic motifs of the NK1R. The results show that there are several sequences within the C-tail and TMD VII of the rat NK1R that are important for SP-induced endocytosis. We compared SP-induced endocytosis of mutant and wt NK1R stably expressed in KNRK cells. We have previously examined trafficking of the NK1R in KNRK cells in detail and found that it behaves like the NK1R in neurons and endothelial cells (19–21, 36).

The C-Tail of the NK1R Contains Endocytic Motifs—Truncation of the NK1R at residue 355 had a relatively small effect on internalization, indicating that either residues 355 to 407 are not critical for endocytosis or this region contains positive and negative endocytic signals whose influences are offset when both are removed. Further deletion at residue 342 markedly slowed the rate of endocytosis and trafficking to a perinuclear region, indicating that more proximal regions of the C-tail are critical for internalization. The dramatically reduced rate of internalization of NK1R-8342 compared with NK1R-8354 or NK1R-wt indicates that residues 343–354 include an important endocytic domain. Thus, the NK1R resembles the receptors for gastrin-releasing peptide, thyrotropin-stimulating hormone, angiotensin II, and parathyroid hormone, in which truncation of the C-tail also decreases the rate of internalization (8, 12, 37, 38). In contrast, removal of the C-tail of the luteinizing hormone/chorionic gonadotropin receptor and the avian β2-AR increases the rate of internalization (29, 39, 40). Further deletion of residues 325 to 342 (NK1R-8324) slightly enhanced the degree of endocytosis compared with NKIR-8342. This may be explained by the presence of a negative endocytic signal between residues 325 and 342. Such a signal (EVQ) is found at the membrane-cyttoplasmic interface of TMD VII of the parathyroid hormone receptor (12). Alternatively, incremental shortening of the C-terminal tail may increase the lateral mobility of the receptor in the plasma membrane and, therefore, its probability of becoming trapped in a clathrin-coated pit (41). With the exception of NK1R-8311, all of the truncated receptors were appropriately expressed at the plasma membrane. This lack of surface expression may explain the poor functional responses of the naturally occurring truncated NK1R to SP (33, 42). Similar mistargeting occurs for other receptors truncated N-terminal to the putative palmitoylation site or lacking the entire C-tail (12, 29).

Conserved Tyrosine Residues Contribute to Endocytic Motifs of the C-Tail—TYR-containing endocytic motifs, in which the Tyr is of critical importance, have been identified for many of the single TMD proteins (1, 32). They usually contain six residues forming an exposed β-turn. Positions 1, 3, and 6 are frequently occupied by aromatic or large hydrophobic residues, with positions 2 and 4 more important to the function of the motif than position 1. Either position 3 or 6 must contain an aromatic residue, and residues in positions 2, 4, and 5 tend to be polar and are often found in turns.

All of the conserved Tyr residues in the C-tail of the NK1R contribute to endocytosis, since each mutant showed defective internalization, albeit to different degrees. Mutation of Tyr-349 had the largest effect on internalization. This is located between residues 343 and 354, and removal of these residues in NK1R-8342 resulted in the greatest defect in internalization between the three truncated mutants. Tyr-341 and Tyr-331 are probably part of signals of lesser importance. However, only the residues surrounding Tyr-331 fit the typical consensus sequence for Tyr endocytic motifs, with aromatic or large hydrophobic residues in positions 3 and 6 (G/DxTYEGL). Tyr-349 could be placed in a hexapeptide motif with Tyr-349 in position 1 and Val and Leu in positions 3 and 6 (14DxKVSRL). Tyr-341 is not surrounded by any other aromatic or large hydrophobic residues that fit a 1-3-6 pattern. Tyr-containing motifs in the C-tail of other G-protein-coupled receptors, such as the parathyroid hormone receptor and angiotensin II1a receptor, are also important for internalization (9, 12). However, mutation of surrounding residues and the exchange of motifs between G-protein-coupled and single TMD receptors will be required to determine the extent to which these motifs resemble the Tyr-containing endocytic motifs of single TMD receptors. In contrast, Tyr residues in the C-tails of the β2-AR and m2 muscarinic receptor are not critical for internalization (43, 44).

The NK1R-8338–352 was internalized marginally more quickly than NK1R-wt in response to SP. Thus, in contrast to some other G-protein-coupled receptors, in which Ser/Thr-rich regions are critical for internalization (6, 8–10, 15), the ST338–352 region plays only a subtle role in internalization of the NK1R.

The TMD VII NPX2–3Y Sequence Is Not a General Endocytic Motif—A TYR-containing motif (NPX2–3Y) in TMD VII is highly conserved within the G-protein-coupled receptors. It closely resembles endocytic motifs of the low density lipoprotein and insulin receptors, both of which fit the six-residue consensus sequence for TYR-containing endocytic motifs (1, 32). It has been suggested that this may be a common endocytic motif for G-protein-coupled receptors (7).

We investigated this possibility by mutating this region of the NK1R. The NK1R-Y305F and NK1R-Y305A mutants behaved very differently. The conservative Y305F mutation did not affect the subcellular distribution of the receptor in the basal state after binding SP at 4 °C but caused diminished internalization after incubation at 37 °C. The more drastic Y305A mutation markedly altered the subcellular distribution of the receptor so that it was in intracellular vesicles even in the basal state. This could be due to mistargeting of newly synthesized receptors to the plasma membrane. However, several observations indicate that some receptors reach the plasma membrane. First, there was a low level of detectable binding of 125I-SP and cy3-SP to the cell surface at 4 °C. Second, there was internalization of 125I-SP after warming at a similar rate as for the NK1R-wt. Finally, the prominent intra-
FIG. 10. Confocal photomicrographs showing the distribution of cy3-SP (left panels) and immunoreactive clathrin (center panels) in NK1R-wt, NK1R-D342, NK1R-Y349A, NK1R-Y305F, NK1R-Y305A, and NK1R-ΔST338-352 cells. Cells were incubated with cy3-SP at 4°C, washed, and incubated at 37°C for 2–5 min (similar results were obtained at both times), and clathrin was localized by immunofluorescence using a secondary antiserum coupled to fluorescein isothiocyanate. The right panel is formed by superimposing the left and center panels. The arrowheads on images of NK1R-wt, NK1R-Y349A, NK1R-Y305F, NK1R-Y305A, and NK1R-ΔST338-352 cells indicate vesicles containing cy3-SP that are stained by the clathrin antibody. The arrows on images of NK1R-D342 cells indicate that there is no co-localization of cy3-SP and clathrin at early time points. Scale bar = 5 μm.
cellular pools of cy3-SP observed after incubation at 37 °C must have undergone endocytosis, since SP is hydrophilic and can only bind surface receptors. An alternative explanation for the detection of NK1R-Y305A in intracellular vesicles in the basal state is that this receptor internalizes constitutively. In support of this possibility is the elevated internalization of 125I-SP and cy3-SP at 4 °C. Thus, the Y305A mutation may induce a conformational change in the receptor that permits its interaction with the endocytic apparatus at 4 °C or even without agonist binding. If this is the case, the NPX2-2Y sequence in TMD VII could resemble the switch function of the tyrosine kinase domain for agonist-induced internalization of the epidermal growth factor receptor (5). The tyrosine kinase point mutation and the Y305F mutation may interfere with agonist-induced changes in receptor conformation, thereby preventing exposure of cryptic endocytic motifs. In contrast, deletion of the tyrosine kinase domain and the Y305A mutation may cause the receptors to assume a conformation in which endocytic motifs are constitutively exposed. Further experimentation is required to test this hypothesis.

Mutation of the TMD VII NPX2-2Y domain also affected signaling. Although the Y305F mutation did not affect SP-induced Ca2+ mobilization, both potency and efficacy of SP were reduced in NK1R-Y305A cells as compared with NK1R-wt cells. The impaired signaling of NK1R-Y305A may be explained by its low level of expression at the cell surface. Alternatively, the NPX2-2Y motif may have induced a conformational change in the receptor that alters its ability to interact with mechanisms of signal transduction.

The role of the NPX2-2Y domain in endocytosis and signaling has been examined for other receptors. Mutation of the corresponding Tyr in TMD VII of the β2-AR to Ala (Y326A) abolishes internalization, reduces agonist-induced phosphorylation by G-protein receptor kinase 2, and depresses activation of adenyl cyclase (47, 45, 46). Overexpression of this kinase rescues receptor phosphorylation and internalization. Thus, the β2-AR-Y326A mutation alters the ability of the agonist-occupied receptor to achieve a conformation required for phosphorylation, signaling, and internalization. The more conservative Y326F mutation of the β2-AR reduces internalization by only 25% and also depresses phosphorylation and activation of adenyl cyclase (45). A tyrosine to alanine mutation of the NPX2-2Y sequence in TMD VII of the angiotensin II1a receptor also reduces internalization by 25% (9, 11). In contrast, an equivalent mutation does not affect endocytosis of the receptor for gastrin releasing peptide (47).

These observations and the results of the present study suggest that the NPX2-2Y sequence of TMD VII is not an endocytic motif but may be important for maintaining the appropriate conformation of the receptor for endocytosis or signaling to occur. Therefore, Tyr-305 of the NK1R may be critical for transducing agonist binding at the outer face of the receptor into a conformational change in the C-tail that is necessary to trigger endocytosis or signaling. Recent structural models of G-protein-coupled receptors suggest that the NPX2-2Y motif is in an appropriate location to receive a signal from agonist-induced conformational changes in the ligand-binding region (48).

Mechanisms of SP-Induced Endocytosis of the NK1R—The NK1R internalizes by a clathrin-mediated mechanism (21), although some G-protein-coupled receptors internalize by caveolin-dependent pathways (49, 50) or by mechanisms that are independent of clathrin and caveolin (51). Despite the finding that many of the mutations affected the rate of internalization, or even resulted in retention of the receptor in very small, superficial vesicles, many of these internalized vesicles were colocalized with clathrin at early time points. Thus, the effect of the mutations is to alter the rate of clathrin-mediated endocytosis, rather than to divert the receptor into a different endocytic pathway. Further analyses will be required to identify proteins that interact with potential endocytic domains of the NK1R. However, Tyr-containing motifs in the C-tails of some membrane proteins interact directly with the clathrin-associated protein complex AP-2 (52, 53). The μ2-chain of AP-2 interacts with the SDYQWL motif of the C-tail of the integral membrane protein TGN38, as well as with similar motifs from lamp-1, CD68, H2-Mb, and the transferrin-receptor (54). The importance of the interaction between Tyr-containing endocytic motifs and the AP-2 complex has recently been questioned, since deletion of the high affinity AP-2 binding site in the C-tail of the epidermal growth factor receptor abolishes AP-2 binding to the receptor without affecting internalization (55).

Conclusions—Multiple domains in the intracellular C-tail and TMD VII of the NK1R are important for SP-induced endocytosis. All of the conserved Tyr residues in the C-tail participate in endocytosis although Tyr in positions 341 and 349 are most important. Although mutation of the Tyr residue of the NPX2-2Y sequence in TMD VII alters endocytosis, it is probable that this region is important for maintaining the correct conformation of the receptor and that it is not an endocytic motif. None of the point mutations reproduced the marked inhibition of endocytosis observed with the truncated receptors or abolished internalization, emphasizing that NK1R internalization relies on multiple endocytic motifs.

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Endocytic Domains of the NK1 Receptor

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