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Thr-422 and Tyr-424 Residues in the Carboxyl Terminus Are Critical for the Internalization of the Rat Neurotensin Receptor*

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In order to identify the amino acid sequences responsible for the internalization of the cloned rat brain neurotensin receptor, we carried out site-directed mutagenesis of the cDNA encoding the receptor followed by expression of the receptor into mammalian COS 7 cells. In cells transfected with the full-length neurotensin receptor, 56% of iodinated neurotensin specifically bound to the cells after 60 min of incubation at 37 °C was internalized. Deletions made in the third intracellular loop did not affect receptor internalization. By contrast, internalization was reduced to 5% of total in cells in which almost all the carboxyl-terminal tail of the receptor had been deleted (R392stop). In order to determine which part of the tail was responsible for this effect, several Ser and Thr residues were deleted in the carboxyl cytoplasmic sequence of the receptor. Almost all of these receptors were internalized as efficiently as the wild type. Only the form of the neurotensin receptor truncated at Glu-421 (deletion of the last three residues, TLY) produced a significant decrease in the amount of ligand internalized. Finally, point mutations of Thr-422 and Tyr-424 residues to Gly led to an almost complete loss of ligand internalization demonstrating the involvement of these 2 residues in the internalization process. Replacement of the last three amino acids by the cytoplasmic endocytosis signal of the vesicular stomatitis virus did not restore the efficiency of neurotensin receptor internalization. These biochemical results were confirmed by confocal microscopic analysis. Cells transfected with the wild type receptor showed a temperature-dependent intracellular accumulation of a fluorescent analog of neurotensin, whereas cells transfected with a receptor truncated at the carboxyl terminus showed a clustering of the fluorescent peptide at the cell surface.

Plasma membrane neurotensin receptors (NTRs)[1] have been described as being rapidly sequestrated from the cell surface after interaction with their ligand (1, 2). This sequestration process is followed by a rapid internalization of receptor-ligand complexes, which is both time- and temperature-dependent (2, 3). The region of the receptor responsible for the internalization process has not yet been defined. However, studies on the internalization of other G-protein-coupled receptors (GPCRs) have singled out two intracellular domains essential for ligand-induced internalization: the third cytoplasmic loop in the case of the muscarinic cholinergic receptor (4) and the carboxyl terminus in the case of both the thyrotropin-releasing hormone (5) and gastrin-releasing peptide receptors (6). In all three cases, Ser and Thr residues were found to be critical for receptor sequestration suggesting that they may be critical for internalization of all G-protein-coupled receptors. The two putative palmitoylation sites, Cys-*X*-Cys, in the cytoplasmic tail of the thyrotropin-releasing hormone receptor were also shown to be involved in the internalization process (5). More recently, the highly conserved Tyr residue in the NP*X*X*Y* sequence present in the seventh transmembrane domain of almost all GPCRs has been shown to be required for agonist-mediated internalization of the β_2-adrenergic receptor (7) but not for that of the gastrin-releasing peptide receptor (8).

The only rat neurotensin receptor cloned to date (9) possesses all of the amino acid sequences implicated in the internalization of other GPCRs; the third cytoplasmic loop contains several Ser and Thr residues, while the intracellular tail bears four clusters of Ser and Thr and a Cys-*X*-Cys (386–388) sequence. We have therefore studied the influence of these different regions on neurotensin-induced receptor internalization using site-directed mutagenesis and expression of the mutated receptors into mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Neurotensin (NT) was purchased from Peninsula Laboratories. α-^{125}I-BH-NT(2–13) was prepared and purified as described previously (10). Fluo-NT was kindly provided by Advanced Bioconcept Inc., Montreal. The pcDNA I expression vector was purchased from Invitrogen, Dulbecco's modified Eagle's medium from Life Technologies, Inc., gentamycin, 1,10-phenanthroline, and phenylarsine oxide from Sigma, and fetal calf serum and restriction endonucleases from Boehringer Mannheim.

Mutant NTR Construction—A 1.45-kilobase fragment of the NT receptor cDNA (9) corresponding to the total reading frame plus the 5′ end noncoding sequence was obtained by polymerase chain reaction and standard cloning techniques and used as a template for site-directed mutagenesis to make mutant cDNAs. Intramolecular deletions and point mutations were obtained by oligonucleotide-directed mutagenesis from uracyled single strand according to the method of Kunkel (11). Carboxyl-terminal deletions and point mutations (Fig. 1) were made by polymerase chain reaction using oligonucleotides bearing mutations and the restriction site *Not*I. The structure of each mutated NT receptor cDNA was confirmed by dideoxy sequencing using appropriate primers (12).

Mutant NTR Expression—All cDNA constructs were subcloned into the eukaryotic expression vector pcDNA I, which contains the cytomegalovirus promoter. Transient transfections were performed with 1 μg of recombinant pcDNA I plasmid by the DEAE-dextran precipitation method (13) onto semi-confluent COS 7 cells grown in 100-mm cell culture dishes. Binding and internalization assays were performed approximately 60 h after transfection. Membranes from nontransfected

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[1] The abbreviations used are: NTR, neurotensin receptor; GPCRs, G-protein-coupled receptors; NT, neurotensin; BH, Bolton-Hunter; VSV, vesicular stomatitis virus.

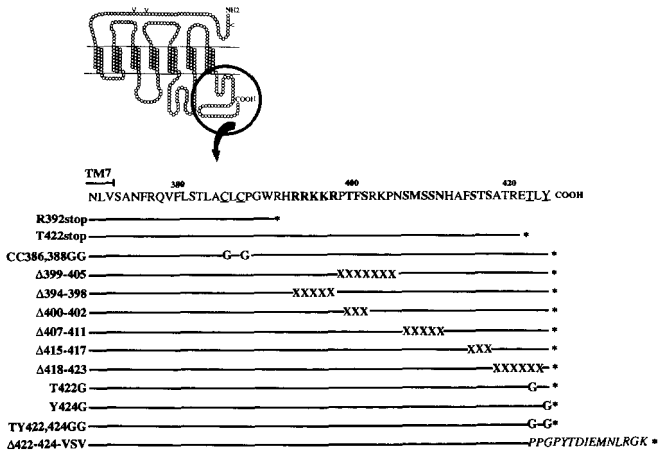

FIG. 1. **Topographical model of the different point mutations and deletions of the carboxyl-terminal region of the neurotensin receptor.** The final 52 amino acids (residues 373–424) of the wild type rat neurotensin receptor are depicted. *TM7* refers to the end of the seventh transmembrane domain, and the *asterisk* indicates the carboxyl terminus. *X* indicates a deleted amino acid. *G* represents a point substitution with a Gly residue. *Italic letters* refer to the tagged sequence added after Glu-421 for the Δ422–424-VSV mutant. All mutants were created by site-directed mutagenesis.

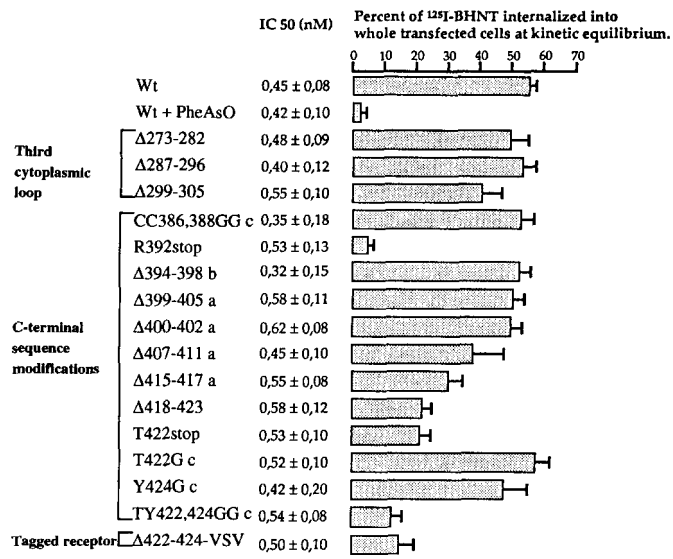

FIG. 2. **Binding affinity and internalization efficiency of wild type and mutated neurotensin receptors expressed in COS 7 cells.** Data are expressed as the mean ± S.E. of at least four separate experiments. The efficiency of internalization is expressed as the ratio between internalized and total cell-associated ligand at equilibrium, *i.e.* after incubation for 30 min at 37 °C. *a*, deletion of a cluster of Ser and Thr residues; *b*, deletion of a cluster of positively charged residues; *c*, point mutation. Binding experiments were carried out on transfected cell membranes for 20 min at 25 °C. IC_{50} (nM) represents the concentration of the peptide that inhibits 50% of the specific binding. The mean B_{max} value obtained for both the wild type (*Wt*) and mutated receptors is 2240 ± 250 fmol/mg protein. *PheAsO*, phenylarsine oxide; *BHNT*, Bolton-Hunter neurotensin.

COS 7 cells were totally devoid of specific α-^{125}I-BH-NT(2–13) binding.

Biochemical Studies—Binding experiments were carried out on cell membrane homogenates freshly prepared as described previously (14). Cell membranes (10 μg) were incubated in 250 μl of 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin (binding buffer) with 0.2 nM α-^{125}I-BH-NT(2–13) (2000 Ci/mmol) and various concentrations of unlabeled neurotensin. After 20 min at 25 °C, incubation media were filtered through cellulose acetate filters (Sartorius). Filters were rinsed twice with 3 ml of ice-cold binding buffer and counted in a Packard γ-counter (counting efficiency, 80%). Nonspecific binding was determined in the presence of 1 μM unlabeled NT and represented less than 5% of the total binding.

Internalization experiments were performed by suspending dissociated cells (0.5 × 10^6/ml) in an Earle-HEPES-Tris 25 mM buffer, pH 7.5, supplemented with 0.1% glucose and 0.1% bovine serum albumin, containing 0.8 mM 1,10-phenanthroline and 0.1 μM *N*-benzyloxycarbonyl-prolylprolinal to prevent ligand degradation. Suspended cells (200 μl) were incubated at 37 °C with 0.2 nM α-^{125}I-BH-NT(2–13) for various periods of time in a total volume of 250 μl in the presence or in the absence of 10 μM phenylarsine oxide, an internalization blocker. At the end of the incubation, 25 μl of a 1 M acetic acid solution containing 5 M NaCl was added in samples for 2 min at 37 °C to dissociate surface-bound ligand molecules. Cells were then filtered through GF/C glass fiber filters (Millipore), and filters were washed twice with 5 ml of ice-cold buffer before counting. In all cases, parallel incubations were conducted in the presence of 1 μM unlabeled NT to determine nonspecific binding. The efficiency of internalization was expressed as the percent of specific α-^{125}I-BH-NT(2–13) binding that was resistant to the acid-NaCl wash (3, 14).

Confocal Microscopic Studies—Dissociated cells (10^5 cells) were incubated for 30 min at 37 °C in 100 μl of Earle's buffer containing 0.8 mM 1,10-phenanthroline with 0.5–1 nM fluo-NT in the presence or in the absence of 1 μM NT. Incubations were terminated by centrifugation at 500 × *g* for 10 min after which the cell pellets were rapidly washed with 500 μl of Earle's buffer, resuspended in 20 μl of fresh buffer, deposited on glass microscope slides, air dried, and coverslipped with Aquamount (15). Confocal microscopic examination was carried out with a Leica inverted microscope equipped with a krypton laser (488 nm) with an output power of 2–50 mV and a VME bus MC 68020/68881 computer system coupled to an optical disc for image storage (Leica, St. Laurent, Canada). Cells were optically sectioned using 1-μm steps, and images were printed out by means of a Focus Imagecorder (Foster City, CA).

RESULTS AND DISCUSSION

As previously reported by others (9), COS cells transiently transfected with the wild type NTR expressed high levels of α-^{125}I-BH-NT(2–13) binding sites, as determined on membrane preparations (B_{max} = 2240 ± 250 fmol/mg protein; Fig. 2). The affinity of α-^{125}I-BH-NT(2–13) for the transfected receptors (K_d = 0.45 nM) and the specificity of the binding for a series of neurotensin analogs (data not shown) were also consistent with previous reports (9, 14). When the binding experiments were carried out on whole cells, a large fraction of the radioactivity specifically bound at 60 min remained resistant to acid washes, indicating that it was internalized inside the cells. Interestingly, no G-protein coupling of the receptor was observed in these cells (data not shown), suggesting that receptor sequestration/internalization still proceeds even if the receptors are functionally uncoupled from the second messenger pathway. Similarly, in the case of the β_2-adrenergic receptor (16), functional coupling to G protein was shown not to be required for agonist-induced internalization of the receptor.

The NTR possesses several Ser and Thr residues within transmembrane domains 5 and 6. Because earlier studies on the Hm1 muscarinic cholinergic receptor had shown that Ser- and Thr-rich domains in the third cytoplasmic loop were required for internalization (4), we first tested the influence of sequences that include these residues on the internalization of the NTR. Deletions of three of these Ser- and Thr-containing sequences (Δ273–282, Δ287–296, and Δ299–305) affected neither the affinity of mutated receptors for iodinated neurotensin nor the efficiency of internalization of the receptor-ligand complexes (Fig. 2).

Subsequently, our investigation targeted the two potential palmitoylation sites Cys-386 and Cys-388. Indeed, homologous residues were previously found to be involved in the internalization of the thyrotropin-releasing hormone receptor (5). Substitution of the Cys-386 and Cys-388 with glycine residues (Fig. 1, *CC386,388GG*) in a full-length mutant produced a protein able to bind and internalize α-^{125}I-BH-NT(2–13) with properties identical to those of the wild type NTR (Fig. 2). We con-

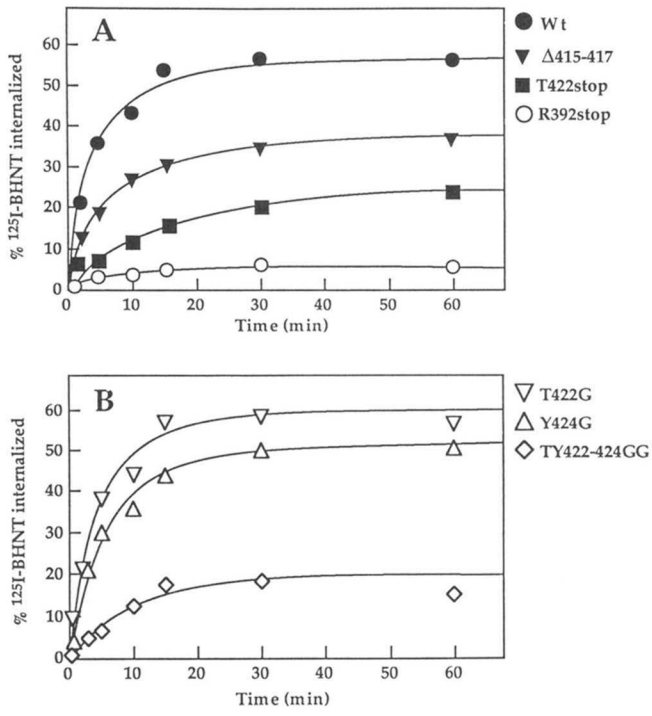

FIG. 3. **Internalization kinetics of wild type and mutated neurotensin receptors expressed in COS 7 cells.** *Panel A*, internalization rates for wild type (*Wt*) and truncated neurotensin receptors. *Panel B*, internalization rates for neurotensin receptors with point mutations on the carboxyl-terminal end. The amount of ligand internalization is expressed as the percent of total cell-associated ligand at each time. For both *panels*, each *point* represents the mean of at least three different experiments, with duplicate determinations. *BHNT*, Bolton-Hunter neurotensin.

clude from these results that if these two Cys residues (386, 388) can be palmitoylated to form bridges with the plasma membrane, they are not implicated in the internalization of the NTR.

In a third series of experiments, we tested the involvement of the carboxyl-terminal tail in the internalization of the NTR by truncating the receptor distal to the Trp-391 residue (Fig. 1, *R392stop*). This mutated receptor, in which a cluster of positive charges RRKKR and a series of Thr and Ser residues were deleted, bound α-^{125}I-BH-NT(2–13) with the same affinity (IC_{50} = 0.53 nM) as the unaltered NTR (Fig. 2). However, in cells transfected with this truncated receptor, the level of ligand-induced internalization was markedly reduced as compared with that observed in cells transfected with the wild type (Fig. 2). This result indicates that, as previously observed for several other GPCRs (5–7), the structural information necessary for internalization of the NTR is contained within the carboxyl terminus of the receptor.

In order to more precisely localize the tail elements critical for the internalization process, we made several deletions in the carboxyl tail (Δ394–398, Δ400–402, Δ407–411, Δ415–417, Δ418–423) of the NTR or truncated it after the Glu-421 residue (Fig. 1, *T422stop*). All of these receptors maintained the same affinity as the unaltered NTR for α-^{125}I-BH-NT(2–13). However, three of these, the Δ415–417, the T422stop, and the Δ418–423 did show a reduction of internalization efficiency; the greatest degree of inhibition was observed in the case of the T422stop NTR (Figs. 2 and 3*A*). These results suggest that only selective Ser and Thr in the carboxyl-terminal tail are involved in the internalization of the NTR and that the last three carboxyl-terminal residues (TLY) are especially critical.

To further identify which residues of the last three carboxyl

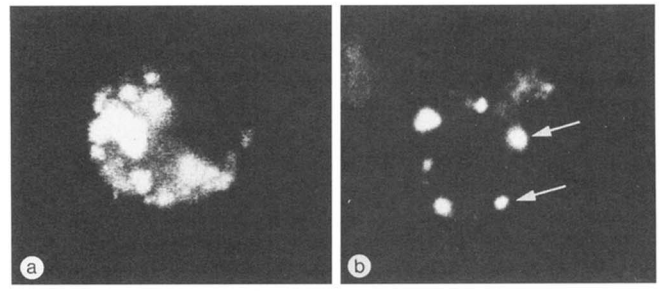

FIG. 4. **Confocal microscopic imaging of COS 7 transfected with either the wild type (*panel A*) or the Δ422–424-VSV mutated form (*panel B*) of the rat neurotensin receptor.** Cells were incubated with 1 nM fluo-NT for 30 min at 37 °C. Optical sections were taken at the cells midheight and averaged over 32 scans/frame. Whereas the ligand is clearly internalized in cells transfected with the wild type receptor, it remains clustered on the cell surface in cells transfected with the mutated form (*arrows*).

termini are implicated in the internalization process, we substituted Thr-422 and Tyr-424 with a Gly, either separately or in combination. Substitution of Thr-422 did not affect the efficiency of neurotensin internalization, whereas substitution of Tyr-424 slightly decreased the amount of radioactive ligand internalized as compared with cells transfected with the native NTR (Fig. 3*B*). Maximal inhibition of the internalization was obtained with the mutant T422G,Y424G, which combined substitutions of Thr and Tyr residues (Figs. 2 and 3*B*). These results demonstrate that although neither Thr-422 nor Tyr-424 is individually critical for neurotensin-induced internalization, the integrity of these two residues should be preserved for maximal efficiency of the internalization process.

In order to determine if the addition of the cytoplasmic internalization signal YTDI (17) to the truncated T422stop NTR could restore the internalization properties of this mutant, we incorporated the peptide sequence from the vesicular stomatitis virus (VSV) containing the YTDI signal into the receptor after the Glu-421 residue (Fig. 1). The corresponding Δ422–424-VSV mutant receptor bound iodinated NT with an affinity identical to that of the wild type NTR but internalized as poorly as its corresponding untagged receptor (Fig. 2). These results indicate that the internalization of the NTR and the vesicular stomatitis virus involves different sequences.

Confocal microscopic analysis of the effect of a tail end NTR mutation on internalization was carried out on COS 7 cells transfected with the Δ422–424-VSV tagged receptor using fluo-NT as a marker. Fluo-NT is a fluorescein isothiocyanate-tagged derivative of neurotensin, which was previously shown to bind with the same affinity and selectivity as native neurotensin to the rat brain NTR (15). Examination of serial optical sections through cells labeled with fluo-NT at 37 °C differed markedly depending upon the type of receptor transfected. No labeling was observed on nontransfected cells (results not shown). In cells transfected with the wild type NTR, the labeling was mainly cytoplasmic and took the form of small, rounded, and intensely fluorescent granules (Fig. 4*a*), whereas in cells transfected with the modified NTR, the labeling remained confined to the cell surface where it formed large "hot spots" (Fig. 4*b*). The intracellular labeling in cells transfected with the wild type NTR conformed to that observed in other cell types endowed with native NTRs (15) and likely reflects internalization of the ligand through the endocytic pathway (18). The fact that this pattern was no longer apparent in cells transfected with the Δ422–424-VSV mutant confirms that the Δ422–424-VSV tagged receptor lost its capacity to be internalized. However, the preservation of "hot spots" on the membrane suggests that the receptor retained its ability to aggregate on the cell surface after binding NT.

In conclusion, we have shown that the carboxyl-terminal cytoplasmic tail of the NTR is essential for internalization. Although no unique residue is absolutely necessary for this process to occur, residues located at the very carboxyl-terminal end are the most important for internalization. Thus, the order of increasing loss of sequestration for our various deletion mutants is $\Delta 394-398 \leq \Delta 399-405 = \Delta 400-402 < \Delta 407-411 < \Delta 415-417 < \Delta 418-423 =$ T422stop. The most striking result is that substitution of both carboxyl-terminal Tyr-424 and of Thr-422 with Gly is sufficient to prevent internalization almost completely, although individual modification of each one of these residues is without noticeable effect. Why both amino acids should be needed for efficient internalization remains a matter for speculation. One possibility is that these residues jointly interact with a single intracellular component, for instance through phosphorylation of their lateral side chains. Removal of a single amino acid would be compensated for by the other, but deletion of the two would abolish the interaction with the intracellular component and, by way of consequence, the internalization. Comparison of our data with those obtained for other G protein-linked receptors shows that no general sequestration motif can be defined in terms of primary structure. Since hydroxylated residues are often important for internalization of this type of receptor, a special tridimensional arrangement of Ser, Thr, and Tyr residues in the sequence could constitute the structural element specifically recognized by the cell for internalization.

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