Recycling ability of the mouse and the human neurotensin type 2 receptors depends on a single tyrosine residue

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Accepted 15 September 2001
Journal of Cell Science 115, 165-173 (2002)

Summary

Receptor recycling plays a key role in the modulation of cellular responses to extracellular signals. The purpose of this work was to identify residues in G-protein coupled neurotensin receptors that are directly involved in recycling. Both the high affinity receptor-1 (NTR1) and the levocabastine-sensitive NTR2 are internalized after neurotensin binding. Here, we show that only the mouse NTR2 recycled to the plasma membrane, whereas the rat NTR1 and the human NTR2 did not. Using site-directed mutagenesis, we demonstrate that tyrosine 237 in the third intracellular loop is crucial for recycling of the mouse NTR2. We show that the mouse NTR2 is phosphorylated on tyrosine residues by NT. This phosphorylation is essential for receptor recycling since the tyrosine kinase inhibitor genistein blocks this process. The absence of recycling observed with the human NTR2 could be completely explained by the presence of a cysteine instead of a tyrosine in position 237. Indeed, substitution of this cysteine by a tyrosine gave a mutant receptor that has acquired the ability to recycle to the cell surface after neurotensin-induced internalization. This work demonstrates that a single tyrosine residue in the third intracellular loop of a G-protein-coupled receptor is responsible for receptor phosphorylation and represents an essential structural element for receptor recycling.

Key words: Neurotensin, Receptor, Recycling, Internalization, Tyrosine, Phosphorylation

Introduction

Most of the G-protein-coupled receptors (GPCR) are internalized from the cell surface to intracellular compartments in response to agonist stimulation (Koenig and Edwardson, 1997; Lefkowitz, 1998). Some receptors can return to the plasma membrane after sequestration (Botto et al., 1998; Ishii et al., 1998), whereas others are targeted to lysosomes and degraded (Innamorati et al., 1998; Vandenbulcke et al., 2000). The cellular trafficking of agonist-activated receptors subserves various physiological functions including receptor desensitization-resensitization and regulation of gene expression (Koenig and Edwardson, 1997). Although, several GPCR have been shown to be recycled following their sequestration, few data are available about sorting motifs involved in this process. For the V2 vasopressin receptor, the presence of a serine cluster in the C-terminal tail of the protein has been shown to prevent recycling of the receptor (Innamorati et al., 1998). More recently, the different intracellular sorting following internalization between protease-activated receptor-1 and substance P receptors has been shown to be the consequence of distinct cytoplasmic tails of these receptors (Trejo and Coughlin, 1999).

The neuropeptide neurotensin (NT) exerts important neuroleptic and non-neuroleptic effects in the central nervous system and is involved in various neuroendocrine regulations (Rostène and Alexander, 1997). Three neurotensin receptors (NTRs) have been identified and cloned from rat, mouse and human (Tanaka et al., 1990; Chalon et al., 1996; Mazella et al., 1996; Mazella et al., 1998; Vita et al., 1998). Two of them (NTR1 and NTR2) belong to the GPCR superfamily. The high affinity rat NTR1 (rNTR1) (Tanaka et al., 1990), couples predominantly to Gq and activates phospholipase C (Hermans et al., 1992; Chabry et al., 1994), whereas the signaling pathway of the levocabastine-sensitive NTR2 is still controversial, depending upon the receptor species and the cell system used to measure signaling (Vincent et al., 1999). Concerning their cellular trafficking, both NTRs are internalized following interaction with NT (Chabry et al., 1994; Botto et al., 1998) but, whereas the mouse NTR2 returns to the plasma membrane by a monensin-sensitive mechanism, no recycling is observed with the rNTR1 (Botto et al., 1998, Vandenbulcke et al., 2000).

We have previously shown that Thr422 and Tyr424 located at the end of the cytoplasmic tail of the rNTR1 were critical for internalization (Chabry et al., 1995), whereas the third intracellular loop (i3) of the rNTR1 has been shown to be involved in the coupling to phospholipase C (Yamada et al., 1994). The different behavior of the recycling mNTR2 and of the non-recycling rNTR1 led us to search for sorting motifs involved in the recycling of the mNTR2. The present study demonstrates that Tyr237, located in i3 of the mNTR2, although not directly involved in NT internalization, is a critical motif for recycling and NT-induced phosphorylation of the receptor. As expected from these results, the human NTR2

(hNTR2) in which a cysteine residue naturally replaces tyrosine in position 237 failed to recycle to the plasma membrane following internalization. However, the single substitution of cysteine 237 by a tyrosine gave a hNTR2 mutant which can recycle as efficiently as the mNTR2.

Materials and Methods

Materials

Neurotensin was purchased from Peninsula Laboratories. ^{125}I-Tyr$_3$-NT was prepared and purified as described previously (Sadoul et al., 1994). [^{33}P]H_3PO_4 (4000 Ci/mmol) was purchased from ICN Biomedicals Inc. The pcDNA3 expression vector was from Invitrogen and the pEGFP-N1 expression vector from Clontech. Taq polymerase and reagents for PCR were from Appligen, oligonucleotides from Eurogentec, Dulbecco's modified Eagle's medium from Life Technologies Inc., gentamycin, 1-10-phenanthroline, phenylarsine oxide (PheAsO), monensin, sucrose, genistein, filipin, nystatin, mowiol, paraformaldehyde, protease and phosphatase inhibitors cocktails from Sigma France and fetal calf serum from Roche diagnostics. The mouse monoclonal anti-phosphotyrosine antibody, the rabbit polyclonal anti-GFP antibody and the anti-hemagglutinin (HA) monoclonal antibody conjugated with protein-A agarose or with HRP were from Santa Cruz Biotechnology Inc. and Cy^{TM}-5 donkey anti-mouse antibody from Jackson Immunoresearch Laboratory Inc. The pcDNA3 plasmid containing the human NTR2 cDNA was a generous gift from P. Kitabgi (IPMC, Valbonne).

Mutants NTR construction

All studies were performed using the mouse or human NTR2 and the rat NTR1 cDNAs as templates. Point mutated receptors were constructed by four-primer PCR mutagenesis. The first-round PCR used primer T7 and an intramolecular antisense primer bearing the deletion or primer Sp6 and a sense primer (the exact complement of the antisense) with pcDNA3-NTR2 (mouse or human) as a template. The second round reaction combined the first round products with primers T7 and Sp6. The final product was cut with *Hin*dIII and *Apa*I, purified and ligated into the *Hin*dIII-*Apa*I digested pcDNA3 vector.

Chimeras between the mNTR2 and the rNTR1 (containing parts of the third intracellular loop of each other) (Fig. 1) were constructed by the six-primer PCR mutagenesis with internal NTRs primers (36-mer) overlapping both receptor cDNAs (Fang et al., 1999). The sequence of the entire region of each mutant cDNA generated by PCR was verified by dideoxynucleotide sequencing.

Mutant NTR expression

Transient transfections were performed with 1-5 μg of recombinant pcDNA3 or pEGFP-N1 expression vector by the DEAE-dextran precipitation method (Cullen, 1987). Binding, internalization and recycling assays were performed on cells plated in 12 mm cell culture dishes approximately 60 hours after transfection as previously described (Chabry et al., 1995). Membranes from nontransfected COS-7 cells were totally devoid of specific ^{125}I-Tyr$_3$-NT binding.

Binding studies on cell homogenates

Binding experiments were carried out on freshly prepared cell membrane homogenates as previously described (Chabry et al., 1995). Cell membranes (10-50 μg) were incubated in 250 μl of 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin (binding buffer) with 0.4 nM ^{125}I-Tyr$_3$-NT (2000 Ci/mmol) and various concentrations of unlabeled neurotensin. After 20 minutes 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 and recycling studies on whole transfected cells

Internalization experiments

Internalization experiments were performed on cells plated in 12 mm well culture dishes as previously described (Botto et al., 1998). Briefly, cells were preincubated at 37°C in the absence of drug or with PheAsO (10 μM), monensin (25 μM), sucrose (0.45 M), genistein (100 μM) or Brefeldin A (10 μg/ml) in an Earle's Tris-Hepes buffer for 30 minutes. Cells were then incubated in the presence or in the absence of drugs with 0.1 to 0.3 nM ^{125}I-Tyr$_3$-NT for various times and washed twice with 0.5 ml of equilibration buffer (neutral wash) or with 0.5 ml of the same buffer containing 0.5 M NaCl (pH 4) for 3 minutes to remove nonsequestrated radioactivity (acid-NaCl wash). Cells were harvested with 1 ml of 0.1 N NaOH and counted in a γ-counter. Nonspecific binding was determined in the presence of 1 μM unlabeled NT.

Recycling experiments

Cells were first preincubated with drugs as described above and then incubated with 100 nM unlabeled NT for 15 minutes at 37°C to induce internalization. The peptide remaining on the cell surface was removed by a series of ice cold washes: three with Earle's Tris-Hepes buffer, two with 150 mM NaCl, 5 mM acetic acid, and three with Earle's Tris-Hepes buffer. Fresh Earle's Tris-Hepes buffer was added and cells were incubated at 37°C for various times. The amount of cell surface receptor was then measured in binding assays carried out with 0.5-2 nM ^{125}I-Tyr$_3$-NT for 30 minutes either at 37°C in the presence of the internalization blocker PheAsO or at 4°C without PheAsO. The nonspecific binding was determined in the presence of 1 μM unlabeled NT. There was no significant increase in cell surface binding sites for the rNTR1, the hNTR2 and for the Y237A-mNTR2 mutant after 30 minutes, indicating that de novo synthesis was negligible during the time course of the experiment.

Phosphorylation experiments

Transfected cells plated in 100 mm dishes were either homogenized to determine the binding capacities as described above or metabolically labeled in phosphate and serum free medium with [^{33}P] orthophosphate (250 μCi/ml) for 3 hours and then incubated in the presence of 100 nM NT for various times. Cells were washed twice with ice-cold PBS and lysed in 1 ml of extraction buffer (25 mM Hepes, pH 7.4, 50 mM NaCl, 1% Triton X-100, 1 mM $CaCl_2$) in the presence of mammalian protease (Sigma P8340) and phosphatase (Sigma P5726) inhibitors cocktails. Lysates were clarified by centrifugation (15,000 ***g***, 10 minutes) at 4°C and the protein concentration was determined using the Bradford procedure. Identical amounts of lysate proteins were immunoprecipitated by incubation with a monoclonal agarose coupled anti-HA antibody (10 μg/ml) overnight at 4°C. Immunoprecipitates were washed four times with the extraction buffer in the absence of inhibitors, and solubilized in a SDS-PAGE loading buffer containing 6 M urea. Proteins were separated by SDS-PAGE and either autoradiographied or transfered onto nitrocellulose for tagged-receptor quantification revealed by western blotting. The labeling of the 45 kDa band was quantified using the NIH Image 1.62 software and expressed in arbitrary unit per pmol of receptor.

Cells transfected with mNTR2-GFP or Y237A-mNTR2-GFP were first preincubated for 30 minutes at 37°C in the presence or in the absence of the tyrosine kinase inhibitor genistein (100 μM) and then incubated in the same buffer without or with 100 nM NT for various times. Proteins were extracted as described above in the presence of

protease and phosphatase inhibitors cocktails and identical amounts of lysate proteins (150 μg) were immunoprecipitated using a monoclonal anti-phosphotyrosine antibody (4 μg/ml) overnight at 4°C. Antibodies were then collected with protein A-Sepharose beads and protein complexes were washed three times at 4°C with the lysis buffer. Immunoprecipitates were resuspended in reducing SDS sample buffer and then proteins were separated by SDS-PAGE, transfered onto nitrocellulose and revealed using polyclonal anti-GFP antibodies.

Confocal microscopy experiments

Cells were transfected with the GFP-tagged mNTR2 or Y237A-mNTR2 and grown on 12 mm coverslips. After preincubation for 10 minutes at 37°C in Earle's Tris-Hepes buffer, cells were incubated in the presence or in the absence of 100 nM NT at 37°C for various periods of time. After a rapid washing step, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes at room temperature, washed twice in PBS, then incubated with 50 mM NH_4Cl in PBS for 10 minutes to quench excess of free aldehyde groups. Fixed cells were permeabilized by incubation in PBS containing 10% horse serum and 0.05% Triton X-100 for 20 minutes at room temperature, then incubated with a 1:250 dilution of a mouse monoclonal antiphosphotyrosine antibody in PBS buffer containing 5% horse serum and 0.05% Triton X-100 for 1 hour. Cells were rinsed three times in PBS buffer and incubated with a Cy™-5 conjugated donkey anti-mouse antibody diluted 1:600 in PBS containing 5% horse serum and 0.05% Triton X-100 for 45 minutes. After two washes with PBS and one wash with water, coverslips were mounted on glass slides with mowiol for confocal microscopy examination. Labeled cells were visualized under a Leica laser scanning confocal microscope (TCS-SP) equipped with a DM-IRBE inverted microscope and an argon-krypton laser. Samples were scanned under both 488 nm and 647 nm wavelength excitation; images were acquired as single transcellular optical sections and averaged over 8 scans/frame.

Results

Mutated and chimeric receptors

Preliminary experiments indicated that deletion of the mNTR2 C-terminus did not affect internalization/recycling properties of the mutated receptor (not shown). Subsequently, our investigations targeted the third intracellular loop (i3), a loop already described to be involved in the sequestration of the Hm1 muscarinic cholinergic receptor (Lameh et al., 1992). To identify the residue(s) located in i3 that could be specifically involved in the internalization/recycling behaviour of NT receptors, we made point mutations in the mouse and human NTR2 (75% identity) and chimeras between the rNTR1 and the mNTR2, which share 39% identity (Fig. 1B).

Table 1. Binding parameters and internalization efficiencies of NT receptors

Receptor	K_d (nM)	(*n*)	B_{max} (fmol/mg)	% of internalization
WT-mNTR2	2.92±0.24	10	385±37	42.0±1.5
WT-hNTR2	1.92±0.28	4	650±55	85.0±3.6
WT-rNTR1	0.45±0.09	6	4078±371	57.0±2.1
1/2-i3-Nt-mNTR2	2.94±0.12	3	1829±111	27.0±0.8
1/2-i3-Ct-mNTR2	2.57±0.26	3	512±43	40.1±2.8
Y237A-mNTR2	2.47±0.35	6	1833±156	28.0±1.2
Y237A-mNTR2	2.64±0.28	3	625±68	26.0±1.6
1/2-i3-Nt-rNTR1	0.23±0.07	5	1925±128	42.0±1.5
C237Y-hNTR2	2.47±0.35	4	660±49	81.0±3.1
WT-mNTR2 stably transfected	3.10±0.18	3	402±48	41.0±1.3

Dissociation constant (K_d) and maximal binding capacities (B_{max}) values were calculated as described in Materials and Methods. Internalization properties were determined from experiments performed on whole cells. Data are means±s.e.m. of duplicate determinations from the indicated number of experiments (*n*).

Binding properties of wild-type and mutated NT receptors

Binding parameters (K_d and B_{max} values) of all the receptors used in this study have been established on membrane homogenates prepared from corresponding transiently transfected COS cells (Table 1). The affinity of NT for the wild-type mouse and human NTR2 and for all the mutants or chimeras made with the mNTR2 were similar, with K_d values

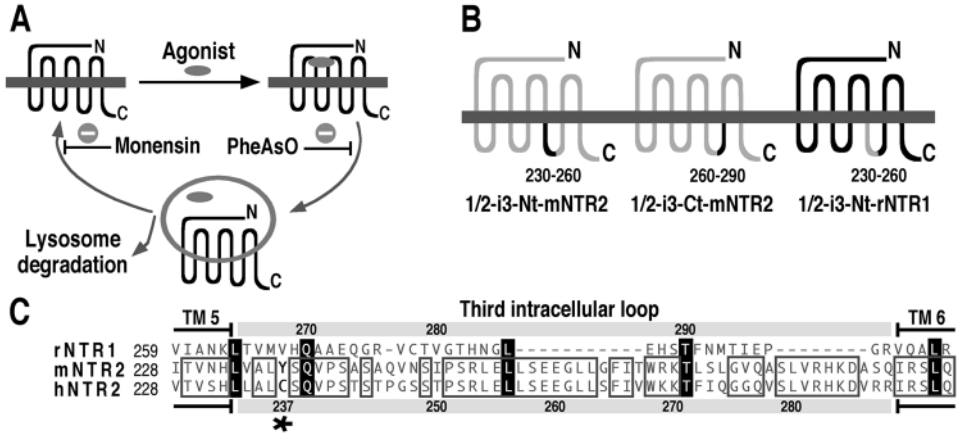

Fig. 1. Chimeric NT receptors and modeling of internalization-recycling processes. (A) Modeling of internalization-recycling processes showing the effects of drugs used in this study. (B) Putative seven-transmembrane topology of chimeric receptors indicating i3 portions of rat NTR1 (black line) and of mouse NTR2 (grey line). (C) Sequence alignment of the third intracellular loop (i3) of the rat NTR1 (rNTR1), the mouse and human NTR2 (mNTR2 and hNTR2, respectively). Invariant residues for all three NT receptors are highlighted in black. Identical amino acids of the two NTR2 are boxed. The location of residue 237 is emphasized by an asterisk. TM, transmembrane domain.

ranging between 2 and 3 nM. The binding capacities of native or mutated mouse and human NTR2 were also comparable (from 385 to 650 fmol/mg) except for mouse mutated receptors in which the tyrosine 237 was absent (1800 fmol/mg). Note that the level of expression was always higher with the rNTR1 (Table 1) as previously described (Botto et al., 1998). When the Y237A-mNTR2 mutant was expressed with a level similar to that of the wild-type mNTR2 (about 600 fmol/mg) or in CHO cells stably transfected with the mNTR2 (B_{max}=400 fmol/mg), a similar amount of ligand internalization was measured (Table 1).

Internalization and recycling of wild-type and mutated NT receptors

All the receptors were tested for their ability to internalize and/or to recycle upon NT activation (Table 1). The pharmacology of both processes is schematized in Fig. 1A. Internalization is quantitatively defined as the amount of ^{125}I-Tyr$_3$-NT remaining bound to the cells after acid-NaCl-wash and expressed as the percent of total ^{125}I-Tyr$_3$-NT bound at 37°C. The internalization process was completely blocked either in the presence of 10 μM PheAsO or by working at 4°C since the binding obtained under these conditions was totally removed by the acid-wash step.

As expected from our previous results (Chabry et al., 1994; Botto et al., 1998), we observed that part of the mNTR2 recycled to the plasma membrane after internalization whereas the rNTR1 did not (Fig. 2A). Monensin efficiently blocked recycling of the mNTR2 as well as brefeldin A (BFA), the inhibitor of vesicular transport or genistein, a tyrosine kinase inhibitor (Fig. 2A,B). This result will allow us to use monensin, brefeldin A and genistein in order to measure NT internalization in conditions where receptor recycling should be blocked (see below).

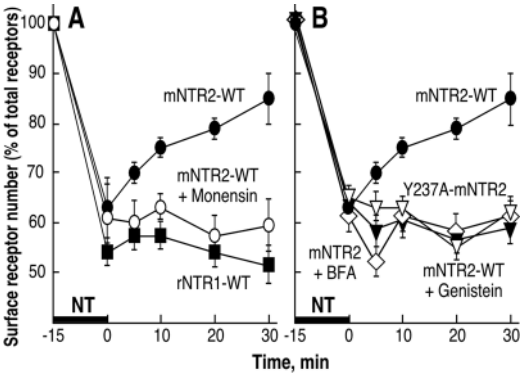

Fig. 2. Time course of receptor recycling. After induction of receptor sequestration for 15 minutes with 100 nM NT, the peptide was removed by acid washes and the amount of cell surface receptor was determined at indicated times of incubation at 37°C as described in Materials and Methods. (A) Recycling of the wild-type (WT) rNTR1 (*n*=4) (closed squares), mNTR2-WT (*n*=6) (closed circles) and effect of the recycling inhibitor monensin on the mNTR2-WT (*n*=3) (open circles). (B) Recycling of the mNTR2-WT in the absence of drug (closed circles) (*n*=6) or in the presence of genistein (closed inverted triangles) (*n*=2) or brefeldin A (BFA) (open diamonds) (*n*=2), and of the Y237A-mNTR2 mutant (inverse open triangles) (*n*=4).

Since the C-terminal tail of the mouse NTR2 was not involved in its cellular trafficking, our investigations focused on the i3 loop. Modifications in i3 of the mNTR2 were made by construction of chimeric receptors in which either the N-terminal or the C-terminal half of this loop was substituted by corresponding sequences of the rNTR1 (Fig. 1B), a region not involved in its sequestration process (Chabry et al., 1995). Interestingly, substitution of the i3 N-terminal half of the mNTR2 by the corresponding sequence of the rNTR1 (1/2-i3-Nt-mNTR2) decreased the efficiency of internalization of the mutant receptor from 42 to 27% (Table 1), and totally inhibited its recycling process. By contrast, substitution of the i3 C-terminal half of the mNTR2 by the rNTR1 sequence (1/2-i3–Ct-mNTR2) affected neither the internalization nor the recycling properties of the receptor (Table 1).

To test the involvement in receptor recycling of Ser, Thr or Tyr residues in the N-terminal half of i3, three mutants, T229A, Y237A and S242A, were constructed. Substitution of either Thr229 or Ser242 by an alanine residue was without effect on the expression, internalization or recycling processes of the mNTR2 (not shown). However, the Y237A-mNTR2 mutant showed a decrease in the amount of internalization independently of its level of expression (Table 1) and an absence of recycling following NT-induced sequestration as shown in Fig. 2B.

Properties of the Y237A-mNTR2 mutant

The substitution of the single Tyr237 by an alanine residue resulted in the modification of several cellular parameters of the mNTR2. First, the membrane expression of the Y237A-mNTR2 mutant was enhanced to 1833±156 fmol/mg of proteins (*n*=6) as measured by saturation experiments performed on membrane homogenates, which was five to six times greater than the expression of the wild-type mNTR2 (Table 1). However, the level of mRNA expression was identical for the mutant and the wild-type receptor (not shown).

Measurement of the reappearance of cell surface receptor following NT-induced sequestration clearly shows that, unlike the wild-type mNTR2 (Fig. 2A), the Y237A-mNTR2 mutant failed to recycle (Fig. 2B). Moreover, the percentage of bound ^{125}I-Tyr$_3$-NT internalized by the Y237A-mNTR2 mutant was decreased to 28±1.2% (*n*=6), a reduction of approximately a factor of 2 compared with the wild-type mouse NTR2 (42±1.5%) (Table 1).

In order to know whether the loss of sequestration efficiency of the Y237A-mNTR2 was the consequence of either the loss of recycling or of a simple defect in internalization, we studied the influence of monensin, brefeldin A and genistein on the amount of sequestrated ligand. The percentage of bound ^{125}I-Tyr$_3$-NT resistant to hypertonic acid washes (internalized ligand) was measured in kinetic experiments performed both on the wild-type and mutant mouse receptors (Fig. 3). For the wild-type mNTR2, the amount of sequestrated ^{125}I-Tyr$_3$-NT (42±1.5%) was reduced in the presence of monensin to 29±0.9% (Fig. 3A), suggesting that a fraction of the internalized receptor recycled to the plasma membrane where it could internalize a new molecule of iodinated NT. According to this interpretation, the monensin-insensitive proportion of sequestrated ligand corresponds to receptors remaining inside the cells. For the Y237A-mNTR2 mutant, the same percentage

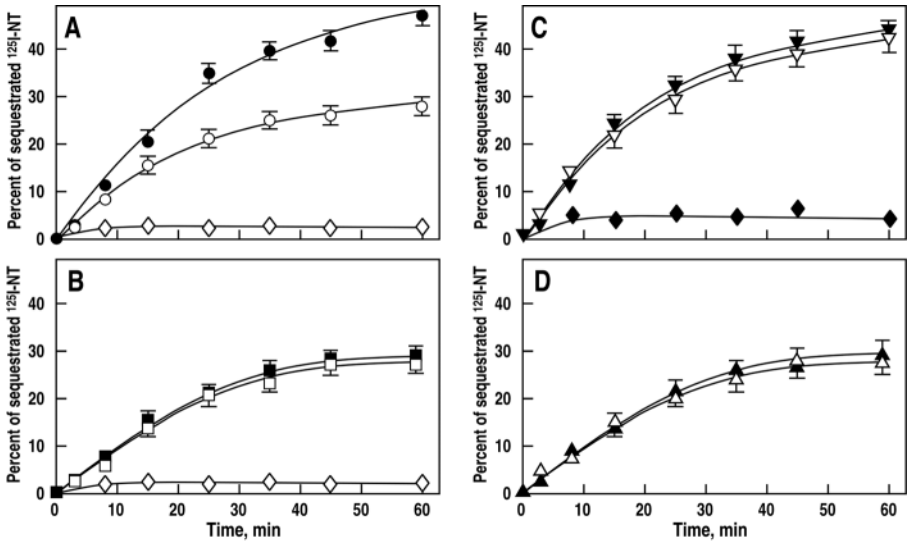

Fig. 3. Effect of various drugs on the amount of internalized ^{125}I-Tyr$_3$-NT measured by kinetics experiments performed in COS-7 cells transfected with the wild-type mNTR2 (A,C) or the Y237A-mNTR2 mutant (B,D). In cells expressing the wild-type mNTR2 (A,C), the amount of internalized ^{125}I-Tyr$_3$-NT was measured in the absence of drug (closed circles) or in the presence of monensin (open circles), brefeldin A (closed inverted triangles), genistein (open inverted triangles) after acid-NaCl wash and expressed as the percent of total cell-associated ligand at each time. The residual amount of sequestrated-NT was determined in the presence of PheAsO (open diamonds) or sucrose (closed diamonds). In cells expressing the Y237A-mNTR2 mutant (B,D), the internalization of ^{125}I-Tyr$_3$-NT was measured in the absence of drug (closed squares) or in the presence of monensin (open squares), brefeldin A (closed triangles), genistein (open triangles). Each point represents the mean±s.e.m. of at least three different experiments determined in duplicate.

(about 28%) of ^{125}I-Tyr$_3$-NT bound to the cells was resistant to the acid wash step in the absence or in the presence of monensin (Fig. 3B), suggesting that, in this case, all the internalized receptor molecules remained trapped into the cells and did not recycle to the membrane. These data demonstrated that the amount of receptor (about 29%) remaining sequestrated into the cells in the presence of monensin was identical both for the wild-type and the mutated receptors. The difference in internalization efficiencies (Fig. 3A,B) between the wild-type and the mutated mNTR2 could be attributed to the loss of recycling ability consecutive to the Y237A mutation. However, in the presence of brefeldin A or genistein, no recycling was observed for the wild-type mNTR2 (Fig. 2B) and the amount of internalized receptor was identical for the wild-type and the mutant receptor, respectively, both in the absence or in the presence of the drug (Fig. 3C,D). This was in contradiction with results obtained with monensin and then the most appropriate interpretation for the difference in the amount of internalization between the wild-type and the Y237A mutant receptors should be that the loss of the tyrosine 237 leads to a receptor bearing an additional defect in endocytosis. Note that in the presence of PheAsO or in hypertonic sucrose, the internalization was completely abolished since acidic washes totally removed ^{125}I-Tyr$_3$-NT

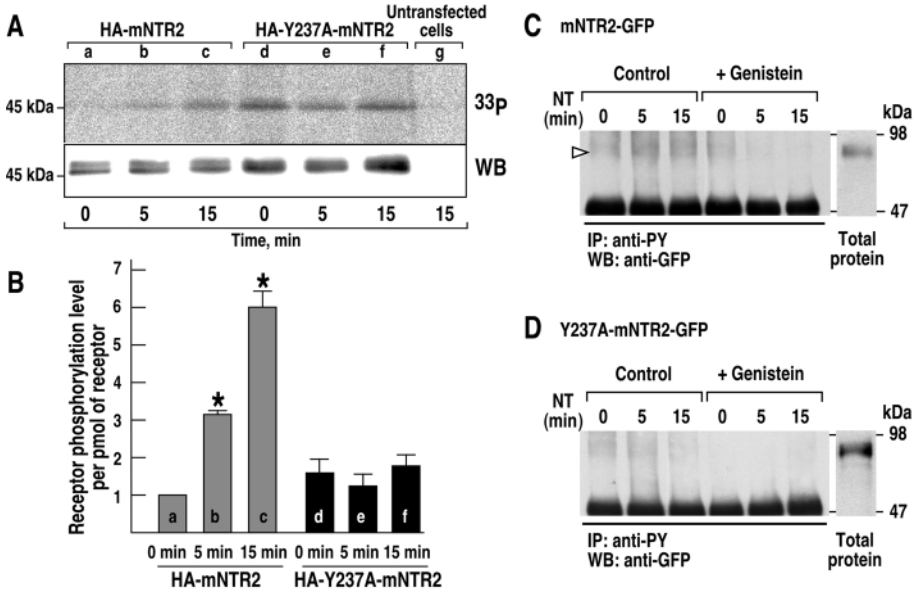

Fig. 4. Time course of receptor phosphorylation. (A and B) The amount of ^{33}P incorporated into proteins in response to various incubation times with 100 nM NT was quantified after immunoprecipitation of the WT HA-tagged-mNTR2 and the HA-Y237A-mNTR2 mutant. (A) Autoradiography and western Blot (WB) of phosphorylated WT HA-mNTR2 (lanes a-c) and HA-Y237A-mNTR2 mutant (lanes d-f) receptors from a representative experiment. (B) Summary of the quantification of receptor phosphorylation. Values for each receptor were expressed in arbitrary unit (basal intensity of phosphorylation in the absence of NT per pmol of receptor) and are means±s.e.m. of three independent experiments. *P<0.05. (C,D) The tyrosine phosphorylation of the WT GFP-tagged-mNTR2 and the GFP-Y237A-mNTR2 mutant upon NT treatment was assessed by phosphotyrosine immunoprecipitation. Cells transfected with the WT GFP-tagged-mNTR2 (C) or the GFP-Y237A-mNTR2 mutant (D) were incubated with 100 nM NT for indicated times in the absence or in the presence of 100 μM genistein, a tyrosine kinase inhibitor. Immunoprecipitation (IP) was performed on total cell extracts using mouse anti-phophotyrosine antibodies. Western blot (WB) were probed with rabbit anti-GFP antibodies. The western blot shown is representative of two independent experiments.

(Fig. 3A,C). We also tested the effect of inhibitors of caveolae-dependent internalization nystatin and filipin. In both case, ^{125}I-Tyr$_3$-NT internalization on cells expressing either the wild-type or mutant mNTR2 was not modified (not shown).

Receptor phosphorylation

To investigate the importance of Tyr237 in the level of phosphorylation of the receptor, we measured and compared the incorporation of radioactive phosphate in both the wild-type hemagglutinin (HA) tagged mouse NTR2 (HA-mNTR2) and the HA tagged Y237A-mNTR2 (HA-Y237A-mNTR2) mutant. As illustrated in Fig. 4A,B, incubation with NT increased the phosphorylation state of the mouse HA-mNTR2 by six times (Fig. 4A,B, lanes a-c) but did not modify the basal labeling of the HA-Y237A-mNTR2 mutant (Fig. 4A,B, lanes d-f). The labeling of the immunoprecipitated 45 kDa band was specific since no labeling was observed in immunoprecipitates from untransfected cells (Fig. 4A, lane g). When the labeling obtained from three different experiments was normalized in pmol of expressed receptor (Fig. 4B), the basal phosphorylation level of the Y237A-mNTR2 appeared identical to that of the wild-type mNTR2. Moreover, phosphorylation of the mutated receptor remained unchanged upon NT stimulation.

To confirm that tyrosine residues were indeed phosphorylated, cells expressing GFP-tagged mNTR2 and Y237A-mNTR2 were treated or not with NT. Then, lysed proteins were submitted to immunoprecipitation using monoclonal anti-phosphotyrosine antibodies and revealed after blotting with a polyclonal anti-GFP antibody. As shown in Fig. 4C, NT induced the phosphorylation of tyrosine residue(s) in the mNTR2-GFP, the specificity of the tyrosine phosphorylation was demonstrated by the absence of NT effect in the presence of the tyrosine kinase inhibitor genistein (Fig. 4C). In cells expressing the Y237A-mNTR2-GFP, NT did not induce tyrosine phosphorylation (Fig. 4D).

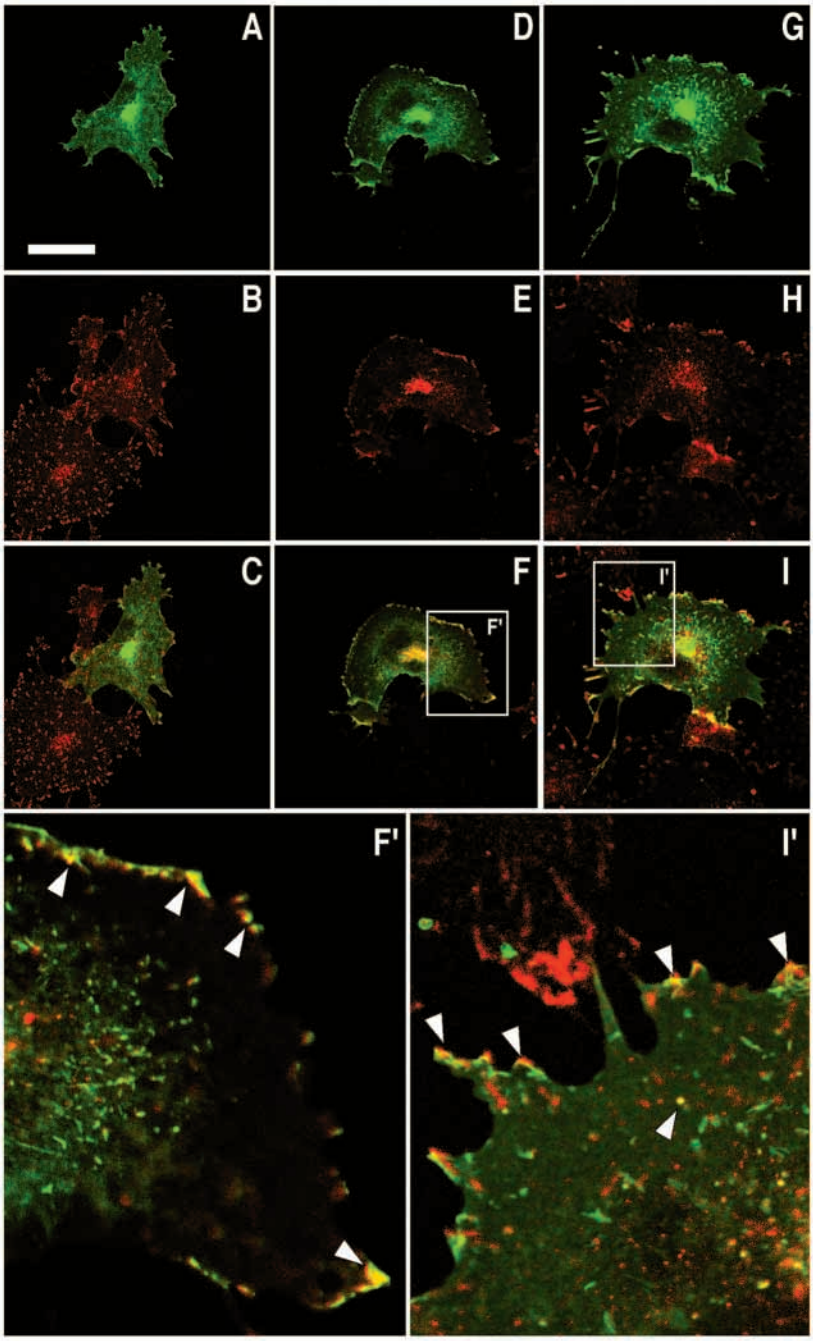

Fig. 5. GFP-tagged mNTR2 and phosphotyrosine staining in transfected COS-7 cells. Transiently transfected COS-7 cells were either untreated (A-C) or treated with 100 nM NT at 37°C for 5 minutes (D-F) or 15 minutes (G-I) prior to fixation. GFP receptor fluorescence (A,D,G) and antiphosphotyrosine labeling revealed with Cy-5 conjugated donkey anti-mouse antibody (B,E,H) are visualized as described in Materials and Methods. Overlays of GFP-mNTR2 and phosphotyrosine fluorescent labeling are shown in C, F and I. The yellow color indicates colabeling. F′ and I′ are enlargements of boxes in F and I images. Arrowheads show clusters of colabeling. Bar, 10 μm.

Confocal microscopy study

To confirm the phosphorylation of tyrosine residue upon NT stimulation in the wild-type mNTR2, we performed immunocytochemical colocalisation experiments both with the wild-type and mutant GFP-tagged receptors and with phosphotyrosine proteins (Fig. 5; Fig. 6). In cells transfected with the wild-type GFP-tagged mNTR2, incubation of NT for 5 or 15 minutes at 37°C induced clustering of receptors to the membrane (Fig. 5D,G). Concurrently, NT increased the labeling obtained with the antiphosphotyrosine antibody at the plasma membrane (Fig. 5E,H). After 5 minutes of NT incubation, many of the receptors clustered at the plasma membrane were also stained with the antiphosphotyrosine antibody (Fig. 5F,F′). After 15 minutes, the colabeling remained intense both at the cell surface and in some intracellular vesicles (Fig. 5I,I′).

In cells transfected with the GFP-tagged Y237A-mNTR2, incubation with NT for 5 or 15 minutes also led to membrane clustering of receptors (Fig. 6D,G). However, at 15 minutes, large intracellular vesicles containing the GFP-tagged mutant receptor were observed under the plasma membrane (Fig. 6G,I,I′). In contrast to cells transfected with the wild-type mNTR2, the labeling obtained with antiphosphotyrosine antibody remained unchanged upon NT stimulation (Fig. 6E,H). Moreover, superimposed images clearly revealed the absence of colabeling between mutated receptors and phosphotyrosine residues (Fig. 6 F′,I′), showing that the GFP-tagged Y237A-mNTR2 is not phosphorylated on tyrosine residues upon incubation with NT. Taken together, results obtained in phosphorylation experiments (western blot and confocal imaging) strongly suggest that NT induced the incorporation of a phosphate group on the Tyr237 of the wild-type mNTR2.

Critical role of the presence of Tyr237 in NTRs

To confirm the importance of the Tyr237 as a pivotal motif in the recycling process of the mNTR2, we incorporated its N-terminal half i3 region, bearing the Tyr237, in place of the corresponding sequence in the rNTR1 (1/2-i3Nt-rNTR1 in Fig. 1B). Although the wild-type form is unable to recycle, the chimeric rNTR1 can recycle to the cell surface as efficiently as the mNTR2 (Fig. 7A,B). These data demonstrate that incorporation of an mNTR2 amino acid sequence bearing the Tyr237 into the rNTR1 was sufficient to acquire the ability to recycle.

The essential role of the single Tyr237 as a recycling motif for NTRs was definitively proved by experiments performed with the human NTR2 in which a cysteine residue is naturally present at position 237 (Fig. 1C). As expected, the hNTR2 efficiently internalized upon NT incubation (85±3.6%, Table 1) but failed to return to the plasma membrane as did the mNTR2 (Fig. 7A). The capacity of Tyr237 to act as a recycling motif for NT receptors was directly demonstrated by incorporating this residue into the hNTR2 in place of the endogenous Cys237 (Fig. 1C). The mutant receptor (C237Y-hNTR2) kept its ability to internalize NT (81±3.1%, Table 1) and can recycle to the plasma membrane as efficiently as the mNTR2 after NT-induced internalization (Fig. 7). Recycling of the C237Y-hNTR2 at the plasma membrane was completely abolished by the recycling inhibitor monensin (Fig. 7B).

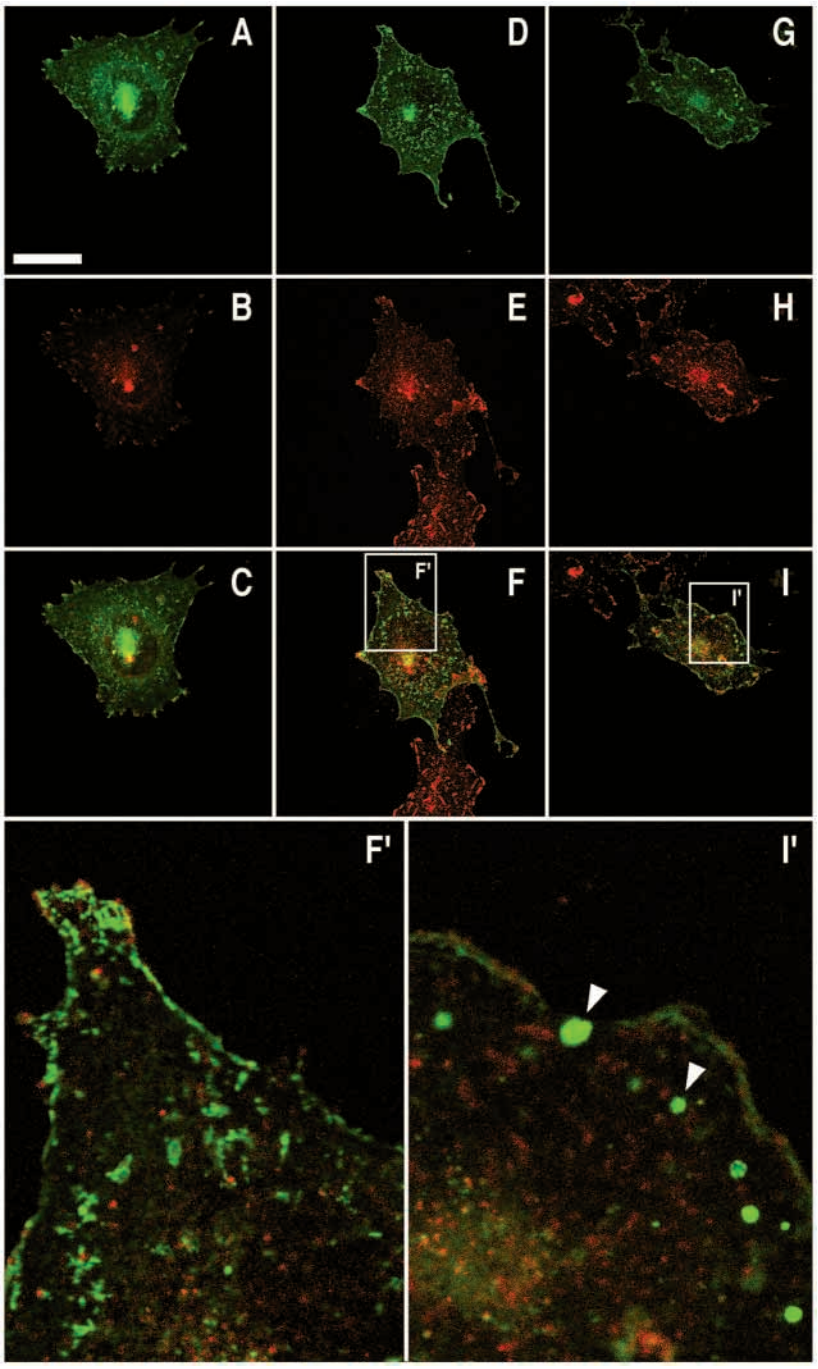

Fig. 6. GFP-tagged Y237A-mNTR2 and phosphotyrosine staining in transfected COS-7 cells. Transiently transfected COS-7 cells were either untreated (A-C) or treated with 100 nM NT at 37°C for 5 minutes (D-F) or 15 minutes (G-I) prior to fixation. GFP receptor fluorescence (A,D,G) and antiphosphotyrosine labeling revealed with Cy-5 conjugated donkey anti-mouse antibody (B,E,H) are visualized as described in Materials and Methods. Overlays of GFP-Y237A-mNTR2 and phosphotyrosine fluorescent labeling are shown in C, F and I. The yellow color indicates colabeling. F′ and I′ are enlargements of boxes in F and I images. Arrowheads show clusters of mutated receptors. Bar, 10 μm.

Discussion

The important finding of the present report is that the single Tyr237 residue located in the i3 loop of the mouse NTR2 is an essential structural element for receptor recycling. We also demonstrated that replacement of this amino acid by an alanine residue abolishes the NT-induced phosphorylation of the receptor.

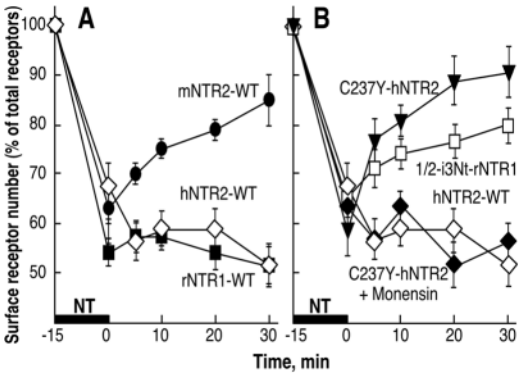

Fig. 7. Time course of receptor recycling. After induction of receptor sequestration for 15 minutes with 100 nM NT, the peptide was removed by acid washes and the amount of cell surface receptor was determined after indicated times of incubation at 37°C as described in Materials and Methods. (A) Recycling of the rNTR1-WT (*n*=4), mNTR2-WT (*n*=6) and hNTR2- WT (*n*=4). (B) Recycling of the hNTR2-WT (*n*=4), 1/2-i3Nt-rNTR1 chimera (*n*=4), the C237Y-hNTR2 mutant (*n*=4) and effect of the recycling inhibitor monensin on the C237Y-hNTR2 (*n*=2).

This is the first observation demonstrating the implication of the third intracellular loop of a GPCR in recycling process. Indeed, to date, motifs that have been identified to be responsible for GPCR recycling are exclusively located in the C-terminal tails of proteins. Trejo and Coughlin demonstrated that when the non-recycling protease-activated receptor-1 bears the C-terminal tail of the recycling substance P receptor, the chimeric receptor acquires the ability to recycle like the substance P receptor itself (Trejo and Coughlin, 1999). These results suggest that the cytoplasmic tail of protease-activated receptor-1 and substance P receptor specify their distinct intracellular sorting patterns following internalization. The role of the C-terminal tail in the recycling properties of GPCR has been demonstrated for the V2 vasopressin receptor in which a serine cluster prevents recycling (Innamorati et al., 1998). More recently, a structural motif has been identified in the C-terminus of the human luteinizing hormone (LH) receptor as being responsible for its routing to the recycling pathway. The incorporation of this motif onto the non-recycling rat LH receptor redirects most of the rat LH receptor to the recycling pathway (Kishi et al., 2001)

For NT receptors, the importance of i3 and more precisely of the Tyr237 residue in the recycling process is clearly established in the present work. Incorporation of an amino acid sequence from mNTR2 bearing the Tyr237 into the non-recycling rNTR1 leads to a chimeric receptor which has acquired the property to recycle following internalization. The most direct evidence comes from the hNTR2 in which the tyrosine residue in position 237 is replaced by a cysteine. Accordingly, this receptor, although efficiently internalized, is unable to return to the plasma membrane. We demonstrate that the single replacement of cysteine 237 by a tyrosine residue confers to the mutant receptor the ability to recycle after sequestration. These results show that the presence of Tyr237 in the i3 loop is a necessary and sufficient condition for recycling of NT receptors from various species.

Although internalization efficiencies of both chimeric rNTR1 and mutated hNTR2 are not modified when compared with their corresponding wild-type receptors (Table 1), replacement of Tyr237 by Ala decreases the amount of sequestrated ligand of the mNTR2. This observation suggests that this mutation also leads to a defect in the endocytosis process of the mNTR2. This lower internalization efficiency of the Y237A-mNTR2 cannot be attributed to the absence of recycling since any difference has been measured in the internalization amount of the wild-type receptor when recycling is blocked by genistein or brefeldin A.

The fact that the internalization of both the wild-type and the Y237A-mNTR2 was insensitive to nystatin and filipin but sensitive to sucrose suggests that the sequestration process of NT receptors undergoes via a clathrin-dependent pathway rather than via caveolae vesicles.

The wild-type recycling mNTR2 is phosphorylated upon NT activation (Fig. 4), whereas the phosphorylation level of the non-recycling Y237A-mNTR2 mutant is insensitive to NT. Note that when the labeled bands obtained in phosphorylation experiments were normalized according to the amount of receptor expression, the basal phosphorylation level appears identical and very low for both the wild-type and mutated receptors. The hypothesis that tyrosine could be the substrate of specific tyrosine kinases has been confirmed using anti-phosphotyrosine antibodies and genistein, a tyrosine kinase inhibitor which totally blocks the tyrosine phosphorylation of the receptor and consequently its recycling. Taking into account these data, we can postulate that the phosphorylation of the tyrosine 237 plays a crucial role in the recycling process of the mNTR2 although not necessary for its sequestration. A possible explanation would be that the first step of the sequestration process leading to internalization of the ligand-receptor complex is a conformational modification of the receptor whereas the phosphorylation step of the receptor is crucial to target the sequestrated ligand-receptor complex into recycling vesicular compartments. Phosphorylation by tyrosine kinases already have been shown to be involved not in recycling but in internalization processes. For example, the sequestration level of the type 1 angiotensin II receptor was affected but not totally inhibited by specific tyrosine kinase inhibitors (Becker et al., 1999). Previous data have shown that the phosphorylated/dephosphorylated state of serine residues is critical for the recycling process of some GPCR (Innamorati et al., 1998; Oakley et al., 1999; Trejo and Coughlin, 1999).

We also show that immunostaining of phosphotyrosine residues is strongly superimposed with the GFP-tagged mNTR2 fluorescence in cells incubated with NT (Fig. 5), whereas no colabeling was observed with the GFP-Y237A-mNTR2 (Fig. 6). These results confirm the crucial role of this tyrosine residue in the NT-induced phosphorylation of the mNTR2. The absence of NT-induced phosphorylation for the Y237A-mNTR2 mutant probably modifies intracellular protein-protein specific interactions between the receptor and cytoplasmic effectors, and consequently changes its intracellular routing following sequestration. According to this interpretation, in cells transfected with the GFP-Y237A-mNTR2, large intracellular receptor vesicles are observed after 15 minutes of NT activation (Fig. 6I′), such intracellular vesicles are not visible in cells transfected with the wild-type GFP-mNTR2 (Fig. 5).

In conclusion, our data clearly demonstrate that a single tyrosine residue in the third intracellular loop is essential for

recycling of NT receptors. We are now trying to assess the possible involvement of well-known trafficking proteins such as arrestins, clathrin and Rab GTPases in the endocytic pathways of the recycling mNTR2 as well as in the sequestration of the non-recycling rNTR1 and hNTR2. The differences in intracellular routing of these homologous NT receptors provide a useful system to further study the mechanisms of internalization and recycling of GPCR.

This work was supported by the Centre National de la Recherche Scientifique (CNRS) and the Ministère de l'Education Nationale, de la Recherche et de la Technologie (MENRT). S.M. is a fellowship recipient of the Association pour la Recherche sur le Cancer (ARC). We thank Franck Aguila for excellent artwork.

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