Crucial Role of Neuron-enriched Endosomal Protein of 21 kDa in Sorting between Degradation and Recycling of Internalized G-protein-coupled Receptors*

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Abstract

Recycling of endocytosed G-protein-coupled receptors involves a series of molecular events through early and recycling endosomes. The purpose of this work was to study the role of neuron-enriched endosomal protein of 21 kDa (NEEP21) in the recycling process of neurotensin receptors-1 and -2. Here we showed that suppression of NEEP21 expression does not modify the internalization rate of both receptors but strongly inhibited the recycling of the neurotensin receptor-2. In contrast, overexpression of NEEP21 changes the behavior of the neurotensin receptor-1 from a non-recycling to a recycling state. Recycling of the neurotensin receptor-2 involves both the phosphatidylinositol 3-kinase and the recycling endosome pathways, whereas recycling of the neurotensin receptor-1 induced by overexpression of NEEP21 only occurs by the phosphatidylinositol 3-kinase-dependent pathway. Taken together, these results confirm the essential role of NEEP21 in the recycling mechanism and show that this protein acts at the level of early endosomes to promote sorting of receptors toward a recycling pathway.

G-protein-coupled receptors (GPCRs) activated by their agonists are sequestered into intracellular compartments as ligand-receptor complexes. Complexes are then routed to early endosomes in which vesicular acidification dissociates the ligand from the receptor. At the level of early endosomes (EEs), receptors and/or ligands are sorted either to the degradation or to the recycling pathways (1, 2). Molecular events leading to the choice of the sorting pathway are still unknown despite their importance in the regulation of cellular responses to various stimuli. The degradation pathway always uses the late endosome network to deliver receptors into lysosomes. By contrast, receptor recycling to the plasma membrane can be achieved either directly from early endosomes or through tubular vesicular recycling endosomes (REs) (3). These two recycling pathways can be distinguished by their recycling rate, by their sensitivity to different drugs, and by the involvement of various small Rab GTPases. Each Rab GTPase interferes with a particular trafficking step (4) and can be used as a specific endosomal marker.

Recently, neuron-enriched endosomal protein of 21 kDa (NEEP21) has been shown to be involved in receptor recycling. NEEP21 is strongly expressed during neuronal maturation (5, 6) and is able to form a complex with the SNARE protein syntaxin 13 (6), which is bound to EE and RE membranes. Syntaxin 13 plays an essential role in the fusion of EE vesicles (7) as well as in transferrin (TF) receptor recycling (8), because antibodies blocking syntaxin 13 inhibited both events. Syntaxin 13, the Rab5 effector protein EEA1, and Rabaptin-5 are part of the multiprotein complex that is necessary for membrane fusion between various vesicles (7). NEEP21, as well as syntaxin 13, is localized in the membrane receptor recycling structures and particularly in the Rab4-containing vesicles (6). Down-regulation of NEEP21 expression leads to an important delay of recycling for both the Tf and AMPA receptors (6).

Although NEEP21 has been shown to be crucial in the recycling of transferrin and glutamate receptors nothing is known about its role on the GPCR internalization/recycling processes. In the present study, we used the two receptors of the neuropeptide neurotensin (NT) belonging to the GPCR family, namely the NTSR1 and NTSR2. These two receptors can be distinguished by their different affinities for NT. Moreover, although both receptors are internalized upon NT activation, only the NTSR2 is able to act as a second messenger (9, 10), with the NTSR1 being targeted to lysosomes (11) (for review see Ref. 12). NTSR1 and NTSR2 constitute good models to measure the effects of variation of NEEP21 expression on the different intracellular behaviors of receptors following ligand-induced sequestration. We showed here that overexpression of NEEP21 had no influence on the internalization rates of both receptors but differentially regulated their recycling properties. Indeed, NEEP21 overexpression not only slightly accelerated NTSR2 recycling but also transformed the non-recycling NTSR1 into a recycling receptor. We also demonstrated that down-regulation of NEEP21 strongly attenuated NTSR2 recycling. Finally, we showed that endogenous NEEP21-dependent NTSR2 recycling and NEEP21-forced NTSR1 recycling take different intracellular pathways.

EXPERIMENTAL PROCEDURES

Materials—Neurotensin was purchased from Peninsula Laboratories. [125I-Tyr3]NT was prepared and purified as described previously (13). The pcDNA3 expression vector was from Invitrogen. Dulbecco’s modified Eagle’s medium was from Invitrogen. Gentamycin, 1–10-phenanthroline, brefeldin A, Mowiol, and paraformaldehyde were from Sigma, France, and fetal calf serum was from Roche Diagnostics. Lyso-tracker DS Red was from Molecular Probes. The polyclonal antibody...
directed against NEEP21 and monoclonal anti-EE antibody have been characterized previously (6, 14). Anti-NTR1, mouse monoclonal anti-actin, and the rabbit polyclonal anti-green fluorescent protein (GFP) antibodies were from Santa Cruz Biotechnology, Inc.

NTR Expression—Transient transfections were performed with 1–5 µg of recombinant pcDNA3-NEEP21-EE, pcDNA3-GFP-antisense-NEEP21 (6) or pcDNA3-NTR1, and pcDNA3-NTR2 expression vector by the DEAE-dextran precipitation method (15). Binding, internalization and recycling assays were performed on cells plated in 12-mm cell culture dishes – 60 h after transfection as described previously (16). Membranes from non-transfected COS-7 cells were totally devoid of specific [125I-Tyr3]-NT binding.

Binding Studies—Binding experiments were carried out on freshly prepared cell membrane homogenates as described previously (15). Cell membranes (10–50 µg of protein) 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 [125I-Tyr3]-NT (2000 Ci/mmol) and various concentrations of unlabeled NT. 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 and Recycling Studies on Whole Transfected Cells, Internalization Experiments—Internalization experiments were performed on cells plated in 12-mm well culture dishes. Briefly, cells were preincubated at 37 °C in an Earle’s buffer (25 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 3.6 mM MgCl2) for 30 min. Cells were then incubated with 0.1–0.3 nM [125I-Tyr3]-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 (ph 4) containing 0.5 mM NaCl for 3 min to remove non-equilibrated radioactivity (acid-NaCl wash). Cells were harvested with 1 ml of 0.1 N NaOH and counted in a γ-counter. Nonspecific internalization was determined in the presence of 1 µM unlabeled NT.

Recycling Experiments—Cells were first preincubated for 30 min at 37 °C in the absence of drug or with 1 µM wortmannin or 10 µg brefeldin A in the Earle’s buffer. Then the cells were incubated in the presence or in the absence of drug with 100 nM unlabeled NT for 15 min at 37 °C to induce internalization. The peptide remaining on the cell surface was removed by a series of ice-cold washes, three with 150 mM NaCl, 5 mM acetic acid, and three with the Earle’s buffer. Fresh Earle’s buffer was added, and cells were incubated at 37 °C for various times in the presence or in the absence of drug. The amount of cell-surface receptor was then measured in binding assays carried out with 0.5–2 nM [125I-Tyr3]-NT for 30 min at 37 °C in the presence of the internalization blocker Brefeldin A. 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 NTR1 or the NTR2 after 30 min, indicating that de novo synthesis was negligible during the time course of the experiment.

The total number of NTR1 binding sites at the cell surface, upon persistent NT stimulation, was measured as described for the human β1-adrenergic receptor down-regulation (17). Briefly, cells transfected with NTR1 alone, with NTR1 plus NEEP21, or with NTR1 plus antisense (AS)-NEEP21 were plated in 24-well plates and exposed at 37 °C to 100 nM NT for increasing times up to 24 h. The plates were rapidly washed several times with the Earle’s buffer and incubated with 0.5–2 nM [125I-Tyr3]-NT for 30 min at 37 °C in the presence of the internalization blocker Brefeldin A. Radioactivity remaining that was associated with cells was counted as described above.

Western Blot Experiments—Proteins from cells transfected with various constructs were denatured by boiling at 95 °C for 3 min using 2× or 6× Laemmli sample buffer, resolved using 10% acrylamide gels, and subsequently electrophoresed onto nitrocellulose membranes. Membranes were blocked and incubated with primary antibody either overnight at 4 °C or for 2–4 h at room temperature. The bound antibody was visualized using a horseradish peroxidase-conjugated goat anti-rabbit or a horseradish peroxidase-conjugated goat anti-mouse followed by chemiluminescence reagents.

Confocal Microscopy Experiments—Cells were transfected with plasmids coding either for NTR1 alone or for NTR1 and NEEP21 and grown on 12-mm cover slips. After preincubation for 10 min at 37 °C in Earle’s buffer, cells were incubated with 100 nM NT at 37 °C for 40 min in the presence of 500 nM Lysotracker red DND-99. After a rapid washing step, cells were fixed with 4.5% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, washed twice in PBS, then incubated with 50 mM NH4Cl in PBS for 10 min to quench excess free aldehyde groups. Fixed cells were permeabilized by incubation in PBS containing 10% horse serum and 0.05% Triton X-100 for 20 min at room temperature, then incubated with a 1:250 dilution of a rabbit polyclonal anti-NTR1 antibody in PBS buffer containing 5% horse serum and 0.05% Triton X-100 for 1 h. Cells were rinsed three times in PBS buffer and incubated with fluorescein isothiocyanate-conjugated donkey anti-rabbit antibody diluted 1600 in PBS containing 5% horse serum and 0.05% Triton X-100 for 45 min. After two washes with PBS and one wash with water, cover slips were mounted on glass slides with Mowiol for confocal microscopy examination. Labeled cells were visualized under a Bio-Rad laser scanning confocal microscope (MCR 1024) equipped with a DM-IRBE inverted microscope and an argon-krypton laser. Samples were scanned under both 488 and 647 nm wavelength excitation. Images were acquired as single transcellular optical sections by the accumulation of photons. The degree of NTR1 localized into the lysosomal compartment was calculated from seven cells for each condition using the Lasersharp 3.2 processing software. The values obtained corresponded to the proportion of NTR1-positive vesicles contained with the lysosomal marker Lysotracker.

RESULTS

NEEP21 Is Expressed in COS-7 Cells—We first assessed the endogenous expression of NEEP21 in COS-7 cells. Western blot analysis of cell extracts using the anti-NEEP21 antibody reveals a detectable but weak expression of a protein of 21 kDa (Fig. 1A). This protein corresponds to NEEP21, because cell extracts from COS-7 cells transfected with the pcDNA3-antisense-NEEP21 plasmid (AS-NEEP21) showed no expression (Fig. 1A). Then we tested the expression of exogenous NEEP21 and AS-NEEP21 following transient transfection into COS-7 cells. As shown in Fig. 1B, homogenates from cells transfected with pcDNA3-NEEP21-EE vector express a single protein of 21 kDa revealed by the monoclonal anti-EE antibodies. Western blot analysis using anti-GFP antibodies detected a protein band of 29 kDa only in homogenates from cells transfected with the GFP-AS-NEEP21 expression vector (Fig. 1C).

Therefore, we could use these different experimental conditions to measure the influence of NEEP21 at the maximal binding capacity (Bmax) and slightly mod-
TABLE I

|       | NTR1 | NTR1 + NEEP21 | NTR2 | NTR2 + NEEP21 |
|-------|------|---------------|------|--------------|
| $K_d$ (nM) | 0.3 ± 0.17 | 0.5 ± 0.2 | 3.5 ± 0.1 | 4.5 ± 0.3 |
| $B_{max}$ (fmol/mg) | 1020 ± 45 | 993 ± 65 | 83 ± 17 | 90 ± 11 |

Fig. 2. Effect of overexpression or suppression of NEEP21 on the amount of internalized [125I-Tyr3]NT in COS-7 cells transfected with the NTR1 (A) or the NTR2 (B). The amount of cell-associated [125I-Tyr3]NT was measured without (closed circles for NTR1 and closed squares for NTR2) or with (open circles for NTR1 and open squares for NTR2) the overexpression of NEEP21 after the acid-NaCl wash. Identical experiments were carried out with the suppression of NEEP21 (open circles for NTR1 and triangles for NTR2). Results are expressed as the percent of total cell-associated [125I-Tyr3]NT.

Fig. 3. Time course of receptor recycling. After induction of receptor sequestration for 15 min with 100 nM NT, the unlabeled peptide was removed by acid washes, and the amount of cell surface receptor was determined at the indicated times of incubation at 37 °C with [125I-Tyr3]NT as described under “Experimental Procedures.” A, recycling of the NTR1 in the absence (closed circles) or in the presence (open circles) of cotransfected NEEP21. B, recycling of the NTR2 alone (closed squares) or cotransfected with NEEP21 (open squares) or with AS-NEEP21 (open triangles). Each point represents the means ± S.E. of at least three different experiments determined in triplicate.

Fig. 4. Colocalization of internalized NTR1 and of lysosomes in COS-7 cells transfected with NTR1 alone (A–F) or with NTR1 and NEEP21 (G–L) and incubated with 100 nM NT at 37 °C. After 40 min of incubation, NTR1 (A and D) is colocalized with lysosomes (B and E) as shown in merge (arrows in C and F). When NEEP21 is overexpressed, NTR1 (G and J) is no longer localized in lysosomes (H and I, K, and L). Bar, 10 μm.

Effect of the Variation of NEEP21 Expression on NTR1 and NTR2 Internalization/Recycling Properties—The effect of overexpression or suppression of NEEP21 on the ability of NTR1 and NTR2 to internalize NT was studied using the cotransfection of plasmids expressing NT receptors and NEEP21, or GFP-AS-NEEP21. Neither suppression nor overexpression of NEEP21 had any effect on the internalization properties of both receptors (Fig. 2, A and B), confirming previous results analyzing glutamate receptor internalization (6). The internalization rates were comparable with control levels, with about 60% for the NTR1 and 35–40% for the NTR2.

Using identical experimental conditions, we observed that although overexpression of NEEP21 does not modify the recycling rate of the NTR2, suppression of NEEP21 expression prevents NTR2 recycling to the cell surface (Fig. 3B). Interestingly, overexpression of NEEP21 completely changes the intracellular behavior of the NTR1, which became able to recycle after NT-induced sequestration (Fig. 3A).

Overexpression of NEEP21 Changes the Cellular Location of the Sequestered NTR1—The ability of the NTR1 to recycle when NEEP21 is overexpressed allowed us to study its cellular location after NT-induced internalization. We demonstrated previously by confocal microscopy that after 40 min of incubation at 37 °C with NT, the internalized NTR1 was recovered in a Lamp1-positive lysosomal compartment (11). Confocal imaging experiments were again carried out in the present work to locate the NTR1 after NT-induced internalization under normal conditions (i.e. when NTR1 is expressed alone) and under the influence of NEEP21 (i.e. when NTR1 is cotransfected with NEEP21). As observed in Fig. 4, in the absence of an excess of NEEP21, part of the NTR1 immunoreactivity is detected in Lysotracker-positive lysosomes (Fig. 4, A–F). When NEEP21 is overexpressed, although the NTR1 is detected in punctate intracellular compartments, no colocalization is observed with the Lysotracker (Fig. 4, G–L) indicating that in this case NTR1 is not routed to lysosomes. This qualitative inspection was also verified by image analysis. The degree of localization of NTR1 into lysosomes was quantified. In control cells 69 ± 5% (n = 7) of pixels in NTR1-positive vesicles were labeled with Lysotracker, this colocalization dropped to 8.4 ± 1.5% (n = 7) following overexpression of NEEP21.

We also examined the effect of NEEP21 overexpression or NEEP21 suppression on NTR1 and NTR2 down-regulation during prolonged incubation of cells with 100 nM NT. In control cells, persistent NT stimulation reduced the level of NTR1 on
the cell surface by 60–70% between 1 and 3 h, followed by a reappearance after 24 h to ~60% of initial values (Fig. 5, closed circles). Overexpressing NEEP21 cells showed the same behavior as control cells (Fig. 5, closed squares). By contrast, the partial recovery of surface NTR1 was completely inhibited upon suppression of NEEP21 (Fig. 5, open squares). When identical experiments were carried out for NTR2, an increase in the amount of cell surface receptors was observed in control cells (not shown) suggesting an up-regulation of the receptor under persistent exposure to NT. For this reason, we were unable to examine the effect of NEEP21 on down-regulation.

Cellular Pathways of NTR2 and NEEP21-induced NTR1 Recycling—To determine the pathways of NTR2 and NEEP21-induced NTR1 recycling, we used either wortmannin to block receptor recycling from early endosomes or brefeldin A to inhibit recycling through tubular vesicular REs. Fig. 6A clearly shows that the NEEP21-induced NTR1 recycling is altered by brefeldin A but not by wortmannin. By contrast, both drugs inhibit the recycling of NTR2 (Fig. 6B). These results indicate that at least part of NTR2 does not take the same recycling pathway as that of the NEEP21-induced NTR1 recycling.

**DISCUSSION**

Recently, Steiner et al. (6) reported that NEEP21, also called 1A75/p21 (5), is necessary for correct recycling of the Tf and AMPA receptors (6). However, nothing is known about its implication in the recycling processes of GPCRs internalized after activation by their ligands. In the present work, by using the two identified NT receptors coupled to G-proteins as a model of recycling and non-recycling receptors, we established a crucial role for NEEP21 in the modulation of the intracellular behavior of both receptors.

NEEP21, which interacts with syntaxin 13, has been proposed to localize mainly to Rab-4-positive domains on EE involved in transport to RE or to the plasma membrane (6). The endosomal NEEP21 is strongly expressed in neurons, and its highest level of expression correlates with that of AMPA receptors during postnatal development (6, 18), suggesting a role of NEEP21 during synaptogenesis. However, as shown in this study, NEEP21 is also expressed in COS-7 cells, and although its expression level is relatively low (Fig. 1), the amount of protein present in COS-7 cells appears sufficient to fulfill a functional role, because variation of NEEP21 expression strongly influences GPCRs trafficking. Indeed, although neither overexpression nor down-regulation of NEEP21 affects the internalization process of NTR1 and NTR2, its down-regulation strongly delays NTR2 recycling, whereas its overexpression allows the non-recycling NTR1 to return to the plasma membrane. The implication of NEEP21 on GPCR trafficking is in line with its role previously demonstrated on the modulation of the Tf receptor and AMPA receptor recycling. The difference is that overexpression of NEEP21 accelerates Tf internalization (6) but not the sequestration of both NTR1 and NTR2 (shown in this work).

Interestingly, the intracellular pathways taken by the natural NTR2 recycling or the NEEP21-induced NTR1 are clearly distinct. Indeed, the cellular process that mediates the recycling of the NTR2 uses both the rapid phosphatidylinositol 3-kinase (wortmannin-sensitive) and the juxtanuclear (brefeldin A-sensitive) recycling compartments. As previously described for the Tf receptor (19), the first recycling route bypasses RE and involves a direct transfer from EE to the plasma membrane. The second pathway involves the passage through RE by an unknown mechanism. These two pathways could contribute to the rapid recycling observed for the NTR2 in several studies (9, 10). The recycling process of the NTR2 resembles that observed for the angiotensin receptor-1 in that it takes both phosphatidylinositol 3-kinase-sensitive and -insensitive pathways (20). Because the NTR2 is poorly expressed to the plasma membrane (9), this process could be of importance to maintain its functionality.

By contrast, with the NTR1 being strongly expressed at the cell surface, its recycling would not be crucial to preserve its activity. Indeed, the absence of recycling for the NT-induced internalized NTR1 has been clearly established (9, 11). However, in the presence of an excess of NEEP21, the NTR1 be-
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comes able to recycle as rapidly as the NTR2. Two mechanisms could explain this behavior. The first possible mechanism is that an excess of NEEP21 could lead to an inhibition of transfer of the receptor from EE to late endosome or from late endosome to lysosomes, because NTR1 is no longer localized in lysosomes in these conditions (Fig. 4). Further characterization of the cellular localization of internalized NTR1 when NEEP21 is overexpressed remains to be performed. The second hypothesis could be that NEEP21 is capable of driving GPCRs to the recycling pathway and that its overexpression can force these receptors to take this route. It will be of interest to analyze whether NEEP21 can associate with GPCR. However, the fact that the NEEP21-induced recycling of the NTR1 is sensitive only to brefeldin A suggests that NEEP21 can favor this recycling pathway, thus preventing the sorting of the receptor by lysosomes. According to this scheme, the function of NEEP21 would be well correlated with its localization in Rab-4-positive vesicles (6) and must be upstream to the molecular events responsible for the targeting of receptors to the degradation process. From these results, we could predict an effect of over- or underexpression of NEEP21 on the down-regulation of NT receptors. Unfortunately, only NTR1 levels are decreased on the cell surface after persistent stimulation with NT. In fact, the NTR2 is slightly up-regulated upon prolonged stimulation. The recovery of part of the NTR1 binding sites after 24 h of NT treatment has been observed already (21). However, overexpression of NEEP21 alters neither the initial down-regulation nor the second phase recovery of NTR1, only the blocking of NEEP21 expression is able to inhibit the receptor recovery. Overexpression of NEEP21 can modify NTR1 trafficking from a non-recycling to a recycling behavior but does not change the overall amount of NTR1 upon prolonged exposure to NT. This might be explained by an incomplete rerouting of NTR1 from the degradation pathway to the recycling pathway leading to an overall decrease in the receptor level. An alternative explanation could be that the internalization rate in continuous presence of a saturating NT concentration is so high that efficient sorting of NTR1 to the recycling pathway by NEEP21 overexpression cannot increase receptor surface levels above the 60% level observed in control cells. In any case, NEEP21 apparently plays a role in the long term recovery of the receptor, because this process is inhibited by blocking its expression.

The fact that underexpression of NEEP21 strongly reduced NTR1 levels during prolonged stimulation might suggest that de novo synthesized receptors might also be routed by a NEEP21-dependent pathway.

In conclusion, NEEP21 is an endosomal protein that can act on the recycling mechanism of a wide range of membrane proteins including type I and channel receptors as well as GPCRs. The action of this protein occurs essentially at the level of the early endosomal pathway both in neuronal and non-neuronal cells.

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