The 100-kDa Neurotensin Receptor Is gp95/Sortilin, A Non-G-Protein-coupled Receptor*

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Jean Mazella‡, Nicole Zsürger‡, Valérie Navarro‡, Joëlle Chabry‡, Mourad Kaghad‡, Daniel Caput‡, Pascal Ferrara‡, Natalio Vita‡, Danielle Gully‡, Jean-Pierre Mafrand‡, and Jean-Pierre Vincent‡

From the ‡Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, UPR 0411, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France and §Sanofi Recherche, BP 137, 31676 Labège Cedex, France

In this work, the 100-kDa neurotensin (NT) receptor previously purified from human brain by affinity chromatography (Zsürger, N., Mazella, J., and Vincent, J. P. (1994) Brain Res. 639, 245–252) was cloned from a human brain cDNA library. This cDNA encodes a 833-amino acid protein 100% identical to the recently cloned gp95/sortilin and was then designated NT3 receptor-gp95/sortilin. The N terminus of the purified protein is identical to the sequence of the purified gp95/sortilin located immediately after the furin cleavage site. The binding of iodinated NT to 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid-solubilized extracts of COS-7 cells transfected with the cloned cDNA was saturable and reversible with an affinity of 10–15 nM. The localization of the NT3 receptor-gp95/sortilin into intracellular vesicles was in agreement with previous results obtained with the purified receptor and with gp95/sortilin. Affinity labeling and binding experiments showed that the 110-kDa NT3 receptor can be partly transformed into a higher affinity (Kd = 0.3 nM) 100-kDa protein receptor by cotransfection with furin. This 100-kDa NT receptor corresponded to the mature form of the receptor. The NT3/gp95/sortilin protein is the first transmembrane neuropeptide receptor that does not belong to the superfamily of G-protein-coupled receptors.

The neuropeptide neurotensin (NT)† exerts distinct central and peripheral effects in mammals (see Ref. 1 for review). Central administration of NT modulates dopaminergic transmission and triggers hypothemeral and naloxone-insensitive angesic responses, whereas in the periphery, NT induces hypotension, decreases gastric acid secretion, and activates lipid digestion (1). Both central and peripheral actions of NT are initiated by association of the peptide to specific receptors located on the plasma membrane of target cells. Two different NT receptors have been cloned in the last years and shown to belong to the family of G-protein-coupled receptors (2–5). The use of the recently developed nonpeptide NT antagonist SR48692 (6) allowed us to demonstrate that the NT-induced modulation of midbrain dopaminergic pathways could be attributed to the high affinity NT receptor (NT1) that was cloned first (2). However, SR48692 was unable to block a series of NT effects including central analgesia and hypothermia (7), suggesting that these effects could be mediated either by the lately cloned low affinity NT2 receptor (4, 5) or by another form of NT receptor not yet cloned.

We previously described the solubilization and purification of a 100-kDa NT receptor from mouse and human brain (8–10). This 100-kDa receptor protein has been observed in primary cultures of neurons from embryonic mice together with the NT1 receptor and shown to be implicated in the internalization mechanism of NT (11). The 100-kDa protein is initially localized in an intracellular vesicular compartment and appears to the plasma membrane only after the NT-induced sequestration of the NT1 receptor, which is initially present at the cell surface (11). In addition, only the 100-kDa protein can be solubilized under an active form by the detergent CHAPS, explaining why this receptor has been selected in our purification studies based upon CHAPS solubilization and affinity chromatography (9, 10).

We describe in this work the cloning and the expression of the 100-kDa NT receptor. This receptor was found to be 100% homologous to the recently cloned gp95/sortilin (12), a protein involved in the sorting of receptors and identified by its ability to interact with a receptor-associated protein (13). Sortilin structurally differs from the two other cloned G-protein-coupled NT receptors and is similar to the mannose 6-phosphate receptor bearing a single transmembrane domain (14). Binding and photoaffinity labeling experiments carried out on COS-7 cells transfected with the cloned cDNA allowed us to demonstrate that sortilin is the 100-kDa NT receptor.

EXPERIMENTAL PROCEDURES

Isolation of the 100-kDa Human NT Receptor and cDNA Cloning—The 100-kDa human NT receptor was purified from CHAPS-solubilized brain membranes by affinity chromatography (10). After a final purification step on SDS-PAGE and protein transfer on Immobilon membrane, the N-terminal sequence of the purified protein was determined by the Edman degradation procedure. The nucleotide sequence derived from the N-terminal peptide was found to be 91.3% identical to a cDNA identified from a human retina library. Various oligonucleotides were derived from the cDNA sequence and used as probes to screen a human brain library (15). Positive clones were identified and sequenced as described elsewhere (15).

Transfection and Solubilization of NT3 and Binding Assay—Transient transfections were performed with 2–4 μg of recombinant plasmid by the DEAE-dextran precipitation method (16) onto semi-confluent COS-7 cells grown in 100-mm cell culture dishes. Homogenates were prepared from COS-7 cells after approximately 60 h of transfection as described previously (17). Cell homogenates were reconstituted in 25 mM Tris-HCl, 10% glycerol (w/v), 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 mM iodoacetamide, 5 mM EDTA, pH 7.5 (Tris glycerol buffer). Solubilization was carried out in the Tris glycerol buffer containing 0.6% CHAPS and 0.12% CHS as initially described (8). Protein concentration was determined by the Bio-Rad procedure with ovalbumin as a standard. CHAPS-solubilized extracts (25 μg of protein) were incubated with [125I]-Tyr)neurotensin (2000 or 50 Ci/mmol) for 1 h at
RESULTS

After purification by affinity chromatography and SDS-PAGE (10), the 100-kDa human brain NT receptor was identified by affinity labeling (10) then directly sequenced by Edman degragation. The first 23 amino acid residues identified were found to be identical to the protein sequence derived from a cDNA sequence of the data base. Oligonucleotides corresponding to this cDNA sequence were used as probes to screen a human brain cDNA library. Hybridization-positive clones were then purified. Sequence analysis revealed an homology of 100% with the previously cloned protein sortilin (12). The open reading frame of this cDNA encoded a protein of 833 residues (gp95/sortilin) containing a signal peptide, a putative cleavage site of furin and a single transmembrane domain (Fig. 1). The amino acid sequence obtained after purification of the human brain NT receptor is positioned just after the putative cleavage site of the protein convertase furin (Fig. 1), indicating that furin cleavage does occur in brain and that our sequence corresponds to the N terminus of the naturally expressed protein (10).

To demonstrate that sortilin is indeed able to selectively bind NT, COS-7 cells were transfected with the recombinant plasmid encoding the cloned cDNA. Whole cells as well as membrane homogenates were found to be devoid of specific $[^{125}\text{I}-\text{Tyr}^3]\text{neurotensin}$ binding, indicating that no NT receptor was expressed at the surface of COS cells. However, when transfected cells were solubilized by CHAPS under conditions identical to those used for the purification of mouse or human NT receptor (9, 10), $[^{125}\text{I}-\text{Tyr}^3]\text{neurotensin}$ bound specifically to solubilized cell extracts according to a rapid association kinetic that reached a plateau value within 3–5 min at 0 °C (Fig. 2). The inset of Fig. 2A shows that a semilogarithmic plot of the data is linear, as expected for a pseudo-first order reaction. The rate constant of the association is: $k_a = k_n[^{125}\text{I}-\text{Tyr}^3]\text{neurotensin} + k_d$, where $k_n$ and $k_d$ represent the second order rate constant of association and the first order rate constant of dissociation of the $[^{125}\text{I}-\text{Tyr}^3]\text{neurotensin}$-receptor complex, respectively (6). The value of $k_a$ calculated from the slope of the semi-logarithmic plot was found to be $2.2 \pm 0.3 \times 10^{-5} \text{M}^{-1} \text{s}^{-1}$. Fig. 2B demonstrates that the binding of $[^{125}\text{I}-\text{Tyr}^3]\text{neurotensin}$ to CHAPS-soluble extracts is a reversible process. Dissociation of bound $[^{125}\text{I}-\text{Tyr}^3]\text{neurotensin}$ was initiated by addition of 1 µM unlabeled NT after the plateau value of the association kinetics was reached. As shown by the linearity of the semilogarithmic representation (inset, Fig. 2B), the dissociation is a first order process with $k_d$ as a rate constant. The value of $k_d$ calculated from the slope was $1.8 \pm 0.2 \times 10^{-2} \text{ s}^{-1}$, not significantly different from $k_a$. When association and dissociation kinetics were performed with 5.5 nM $[^{125}\text{I}-\text{Tyr}^3]\text{neurotensin}$, identical $k$ and $k_d$ values were obtained (not...
NT3 alone (solubilized extracts from COS-7 cells transfected either with extracts from COS-7 cells transfected with NT3 alone). The data from saturation experiments carried out on CHAPS-solubilized cross-linking experiment on the corresponding extract using 50 nM [125I-Tyr3]neurotensin in the absence (lane a) or in the presence (lane b) of 1 μM unlabeled NT. B, Scatchard plot of the data from saturation experiments carried out on CHAPS-solubilized extracts from COS-7 cells transfected with NT3 plus furin. inset, affinity cross-linking experiment on the corresponding extract (lanes a and b) or nontransfected cell extracts (lanes c and d) using 50 μM DSS and 0.5 nM [125I-Tyr3]neurotensin in the absence (lanes a and c) or in the presence (lanes b and d) of 1 μM unlabeled NT.

shown), indicating that the term $k_d [125I-Tyr^3]neurotensin was always negligible as compared with $k_a$ and that $k$ was representative only of the $k_a$ value. In these conditions, it was not possible to calculate the $k_a$ value and then to estimate the dissociation constant $K_d = k_d/k_a$.

Saturation experiments were carried out to determine directly the $K_d$ value for the binding of [125I-Tyr3]neurotensin to CHAPS-solubilized extracts of cells transfected with the cloned cDNA. CHAPS-solubilized extracts (15–30 μg of protein) were incubated for 10–20 min at 0 °C with increasing concentrations of [125I-Tyr3]neurotensin (50 Ci/mmol, from 0.2 to 15 nM), and the bound radioactivity was determined by filtration. The binding of [125I-Tyr3]neurotensin was specific and saturable (Fig. 3), and the corresponding binding parameters were $K_d = 10 \pm 3$ nM and $B_{max} = 4 \pm 1$ pmol/mg.

The difference observed between the $K_d$ value (10 nM) of the cloned NT3 receptor expressed in COS cells and the $K_d$ value (0.3 nM) determined for the purified human brain NT receptor (10) could result from the absence of cleavage at the furin site for the expressed NT3 receptor. To check this hypothesis, saturation experiments were performed on solubilized extracts prepared from COS-7 cells transfected either with the NT3 recombinant plasmid alone or with both the NT3 plasmid and a plasmid expressing the protein convertase furin (Fig. 4). Scatchard representation of the binding experiments carried out on soluble extracts of COS cells transfected only with the NT3 plasmid was linear and corresponded to a single class of NT-binding sites with a $K_d$ of 10 nM (Fig. 4A). By contrast, CHAPS-soluble extracts of cells cotransfected with both the NT3 and furin plasmids gave a curvilinear Scatchard representation with an upward concavity (Fig. 4B). These data could be perfectly fitted to the equation describing the binding of a ligand to two different classes of independent binding sites (19). Each site is represented by a straight line in Fig. 4B. Binding parameters determined in three different experiments were $K_d^H = 0.7 \pm 0.1$ nM, $B_{max}^H = 320 \pm 45$ fmol/mg and $K_d^L = 15 \pm 4$ nM, $B_{max}^L = 3.2 \pm 0.8$ pmol/mg for the high (H) and low (L) affinity NT-binding sites, respectively. Thus cotransfection of NT3 with furin into COS cells partly convert the expressed receptor ($K_d^f = 10$ nM) into an higher affinity form ($K_d^f = 0.4$ nM).

Soluble extracts prepared from cells transfected with NT3 alone or with NT3 plus furin were covalently labeled at 0 °C with [125I-Tyr3]neurotensin using the chemical reagent disuccinimidyl suberate (DSS). Fig. 4A shows autoradiograms of the corresponding SDS-PAGE experiments. In the absence of furin, a single protein band with a $M_r$ of about 110 kDa was selectively labeled because it can be protected by the addition of 1 μM unlabeled NT in the incubation medium (Fig. 4A, inset). In the presence of furin, although the same protein band was observed, a second band with a $M_r$ of 100 kDa was also specifically labeled (Fig. 4B, inset), indicating a partial maturation of the receptor by the protein convertase. Note that autoradiogram of the covalent labeling experiment carried out on soluble extracts prepared from nontransfected COS-7 cells was totally devoid of specifically labeled protein (Fig. 4B, inset).

To determine the binding specificity of the cloned human
brain NT receptor, we tested the ability of NT agonists or antagonists or of unrelated peptides to inhibit the binding of [125I-Tyr]neurotensin to the CHAPS-solubilized NT3 receptor (Fig. 5). NT and Trp11NT inhibited the specific binding of [125I-Tyr]neurotensin with identical potencies ($K_d = 17 \text{ nM}$). Neuromedin N and DTrp11NT were 3.5- and 4-fold less potent with $K_d$ of 63 and 72 nM, respectively. The nonpeptide antagonist SR48692 was poorly effective with an $K_d$ of 3 $\mu M$, and levoocabastine was unable to inhibit [125I-Tyr]neurotensin binding up to 10 $\mu M$. We also verified that neuropeptides unrelated to NT such as vasoactive intestinal peptide, somatostatin, and deltorphin were totally devoid of activity (not shown).

**DISCUSSION**

The 100-kDa NT receptor (NT3) cloned in this work corresponds to the 95-kDa protein designated gp95/sortilin previously purified by receptor-associated protein affinity chromatography and cloned (12). The structure of this protein bearing a single transmembrane domain is related to known sorting receptors as the yeast Vps10p and the CI-Mepr receptor (14, 20) but is totally different from the two G-protein-coupled NT receptors previously cloned. As far as we are aware, the NT3/gp95/sortilin protein described here is the first neuropeptide receptor that does not belong to the family of the G-protein-coupled receptors.

The 100-kDa NT-binding protein purified from human brain occurred in neurons as a truncated form cleaved at the furin site present in the N-terminal part (amino acids 41–44) of the receptor. When solubilized by the detergent CHAPS, this mature protein bound [125I-Tyr]neurotensin with a high affinity ($K_d = 0.3 \text{ nM}$) (10). In the present study, the affinity of iodinated NT for CHAPS-solubilized homogenates prepared from cells transfected with the cDNA encoding the total protein was calculated to be between 10 and 15 nM. To determine whether this relatively low affinity was the consequence of the absence of furin cleavage in the cell line used for expression, we co-transfected the cDNA of NT3/gp95/sortilin and the cDNA encoding the protein convertase furin. In these conditions, Scatchard plot from saturation experiments revealed the appearance of a high affinity NT-binding component ($K_d = 0.7 \text{ nM}$), which represented 8–10% of the total NT-binding sites (Fig. 4B). These results strongly suggested that although total maturation was not effective, a portion of the protein was truncated to give a receptor able to bind NT with a higher affinity. Interestingly, this hypothesis was corroborated by affinity labeling experiments performed on CHAPS-solubilized extracts from co-transfected cells. In that case, a lower molecular weight band ($M_r = 100,000–105,000$) was detected together with the protein of 110 kDa already observed in extracts from cells transfected with NT3 alone (Fig. 4B, inset). The 100–105-kDa labeled protein most probably corresponded to the furin-cleaved form of the 110-kDa protein. To definitely demonstrate this proposal, we are trying to find cotransfection conditions leading to a more complete maturation of the low affinity 110-kDa protein into its high affinity, naturally occurring form.

Taking into account the difference of affinity between the cloned and the purified NT3 receptor, structure-function relationships toward NT analogs or antagonist are similar except for Trp11-NT, which displays a potency identical to that of NT on the cloned CHAPS-solubilized receptor, whereas Trp11NT was 10-fold less potent than the native peptide on the purified CHAPS-solubilized receptor (10).

As expected from our previous study carried out on primary cultures of neurons (11), no detectable binding of [125I-Tyr]neurotensin was observed on whole cells or on crude homogenates from cells transfected with NT3, indicating that the receptor is not expressed at the cell surface. This result is in agreement with the localization by confocal microscopy of gp95/sortilin in the endoplasmic reticulum and the Golgi compartment and with the absence of detectable protein on the cell surface after transfection in COS-1 cells (12). The NT3/gp95/sortilin protein could recognize NT by its extracellular binding site only after translocation to the plasma membrane. By contrast, the binding site of NT3 localized in vesicular structures is directed toward the inner part of vesicles and remains inaccessible to NT in the absence of CHAPS solubilization. Both the subcellular localization and properties after CHAPS treatment of the NT3/gp95/sortilin protein are in agreement with our previous purification data (10).

The NT3/gp95/sortilin protein is colocalized with the glucose transporter Glut4, the insulin-like growth factor/mannose 6-phosphate (IGF-II/Man-6-P) receptor, and the insulin-regulated aminopeptidase in 3T3-L1 adipocyte vesicles (21). These proteins are cotranslocated to the plasma membrane in response to insulin (21, 22). Moreover, the cytoplasmic tail of NT3/gp95/sortilin contains a nine-residue sequence identical with the phosphorylation site and sorting signal in the C terminus of the IGF-II/Man-6-P receptor. Taken together, these data strongly suggest that the NT-regulated function of the NT3/gp95/sortilin receptor could be very similar to the IGF-II-regulated function of the IGF-II/Man-6-P receptor in adipocytes. Both receptor proteins may serve to clear their specific ligand from the circulation, to regulate the translocation of Glut-4-containing vesicles via their phosphorylation, or to be involved in the biogenesis of these vesicles (21).

In conclusion, cloning and expression of the NT receptor previously purified in this laboratory allowed us to demonstrate that the purified 100-kDa receptor is identical to gp95/sortilin. These data open the way to future studies devoted to the elucidation of the physiological function of the NT3/gp95/sortilin protein.

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