Immunological recognition of different forms of the neurotensin receptor in transfected cells and rat brain

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In this work, the molecular forms of the rat neurotensin receptor (NTR) expressed in transfected Chinese hamster ovary (CHO) cells, in infected Sf9 insect cells and in rat cerebral cortex were immunologically detected by means of an anti-peptide antibody raised against a fragment of the third intracellular loop of the receptor. Immunoblot experiments against a fusion protein indicated that the anti-peptide antibody recognized, under denaturing conditions, the corresponding amino acid sequence within the NTR. In immunoblot analysis of membranes from NTR-transfected CHO cells, high levels of immunoreactivity were observed between 60 and 72 kDa, while only a faint labelling was observed at 47 kDa, the molecular mass deduced for the rat NTR cDNA. The bands of high molecular mass were no longer observed after deglycosylation of membrane proteins by peptide N-glycosidase F, indicating that they represented glycosylated forms of the receptor. Extracts of membranes derived from baculovirus-infected Sf9 insect-cells expressing the NTR provided

a quite different immunoblot pattern, since the major band detected in that case was at 47 kDa, the molecular size of the non-glycosylated receptor. Taken together, these data show that, while most of the NTR protein was glycosylated in CHO cells, it was unglycosylated in Sf9 insect-cells. In addition, molecular sizes of the receptor proteins observed in these two cell lines differed from those obtained for the NTR endogenously expressed in the rat cerebral cortex of 7 day-old rats, where bands at 56 and 54 kDa were detected. Binding experiments carried out on membrane preparations obtained from baculovirus-infected Sf9 cells demonstrated that the immunogenic sequence was still accessible to the antibody when the receptor was embedded in the cell membrane. Immunohistochemical studies carried out on both transfected CHO cells and infected Sf9 cells confirmed this interpretation and further indicated that the antibody could be applied in the visualization of the receptor.

INTRODUCTION

Neurotensin (NT), a tridecapeptide first discovered in the bovine hypothalamus (Carraway and Leeman, 1973), was subsequently shown to be widely distributed in both the central nervous system and peripheral tissues in a variety of mammals (Carraway and Leeman, 1976). In the brain, NT acts as a neuromodulator, in particular as a modulator of dopaminergic transmission, and has been implicated in the physiopathology of several brain diseases such as schizophrenia, Huntington's and Parkinson's diseases (Garver et al., 1991; Rostene et al., 1992). In addition, NT was shown to modulate hormonal secretion by acting at the level of the hypothalamo-pituitary axis (McCann and Vijayan, 1992), and to exert potent hypothermic and analgesic effects (Bissette et al., 1976; Behbehani, 1992). In the periphery, NT was shown to be involved in the regulation of gastro-intestinal and cardiovascular systems (Al-Saffar and Rosell, 1981; Rioux et al., 1982).

Studies using radiolabelled NT have characterized the binding properties and localization of NT-binding sites in a number of central and peripheral tissues (for reviews, see Kitabgi et al., 1985; Quirion et al., 1992). These studies revealed the presence of both a high-affinity site ($K_d = 0.3$ nM), present in all tissues, and a low-affinity site ($K_d = 5$ nM), sensitive to the antihistamine levo-

cabastine in tissues such as the adult murine brain (Schotte et al., 1986). However, up to now, only the high-affinity, levocabastine-insensitive site has been shown to correspond to a functional NT receptor.

Different molecular forms of neurotensin receptor (NTR) have been purified from bovine and mouse brain by affinity chromatography (Mills et al., 1988; Mazella et al., 1989). Molecular masses of 100 and 72 kDa were found for purified mouse and bovine NTR respectively. For the rat NTR, a 55 kDa protein was recently purified from newborn rat cortex (Miyamoto-Lee et al., 1991), while previous covalent labelling experiments performed on adult rat brain membranes indicated the presence of both a 49 kDa and a 51 kDa species (Mazella et al., 1985).

Further understanding of the molecular structure of NTR has stemmed from the recent cloning, sequencing and expression of the rat NTR (Tanaka et al., 1990). These studies showed that the high-affinity NTR corresponds to a 424 amino acid protein belonging to the family of G-protein-coupled receptors with seven membrane-spanning domains. More recently, the human NTR was also sequenced and shown to possess 84% homology with the rat NTR (Vita et al., 1993).

Investigations of the various molecular forms of the NTR and their occurrence in the different functional states of this receptor would be greatly facilitated by the development of antibodies

Abbreviations used: CHO, Chinese hamster ovary; EMCS, ϵ -maleimido-caproic acid-*N*-hydroxysuccinimide ester; GST, glutathione-S-transferase; NGS, normal goat serum; NT, neurotensin; NTR, neurotensin receptor; PNGase F, peptide N-glycosidase F; MEM, minimum essential medium; PMSF, phenylmethanesulphonyl fluoride; Abi-3, anti-i3 antibody.

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toward different domains of the NTR. Moreover, the development of such antibodies is a necessary step to measure and visualize NTR proteins and to study their possible regulation.

In the present study, the cloned rat NTR was expressed in Chinese hamster ovary (CHO) cells and Sf9 insect-cells, and an antibody raised against a synthetic fragment of the third intracytoplasmic loop of this receptor was used: (1) to determine the molecular forms of the NTR produced in these two cell lines and compare it with that found in rat cerebral cortex, and (2) to detect the NTR by immunocytochemistry in cells expressing the NTR.

MATERIALS AND METHODS

Materials

The peptide i3 was synthesized by Neosystem Laboratories (Strasbourg, France). ϵ -Maleimido-caproic acid-*N*-hydroxysuccinimide ester (EMCS) was purchased from Sigma. Alpha minimum essential medium (MEM) without nucleoside and deoxyribonucleoside, and Grace's medium were purchased from Gibco-BRL. Luria-Bertani medium was purchased from Difco. The NTR cDNA plasmids CDM8 and prNTR2 were provided by Dr. S. Nakanishi, Kyoto University Faculty of Medicine, Kyoto, Japan. The baculovirus transfer vector pJVELTZ/NheI was provided by Dr. C. Richardson, Biotechnology Research Institute, Montréal, Canada. Goat anti-(rabbit IgG) conjugated with alkaline phosphatase was purchased from Dakko. Peptide *N*-glycosidase F (PNGase F) was purchased from Boehringer Mannheim.

Antibody preparation and purification

The sequence of the peptide i3 is TVGTHNGLEHST-FNMTIEPGRVQAYC and corresponds to amino acids 279–302 of the NTR plus two additional amino acids, Cys and Tyr, at the C-terminus.

The synthetic peptide was coupled to BSA (Sigma) by way of its terminal cysteine residue, using the bifunctional cross-linking reagent EMCS.

Briefly, 14 mg of BSA (12 microequivalents of amine function) dissolved in 200 μ l of 50 mM phosphate buffer, pH 7.4, were added to 7.7 mg (25 μ mol) of EMCS dissolved in 50 μ l of dimethyl sulphoxide. The mixture was incubated for 2 h at room temperature. The maleoyl-BSA was separated from free EMCS on a Sephadex G-25 M column (Pharmacia) equilibrated with 50 mM NaHCO₃ pH 6.5, and the fractions containing the maleoyl-BSA were pooled. The maleoyl-BSA (1.6 mg) was added to 2.2 mg of peptide in solution in NaHCO₃ buffer. The mixture was incubated for 2 h at room temperature. The conjugated BSA-peptide i3 was then separated from the free peptide on a Sephadex G-25 column by elution with 50 mM phosphate buffer, pH 7.4. The fractions containing the conjugate were pooled, aliquoted and stored at -20°C . The efficiency of peptide-BSA coupling was evaluated by measuring the number of thiol functions of the unreacted peptide with Ellman's reagent, and was found to reach 94%. The amino acid composition of the conjugate indicated that 24 molecules of peptide were bound per molecule of BSA.

Three pre-bled New Zealand white rabbits were first immunized with the peptide-BSA conjugate (100 μ g of peptide equivalent) in Freund's complete adjuvant. Rabbits were boosted every 3 weeks with 100 μ g of peptide emulsified in incomplete adjuvant and bled 2 weeks after each boost. Antibody production was monitored by solid-phase e.l.i.s.a. using *o*-phenylenediamine

as the chromogen substrate. Maximal titres were obtained from the second to the fourth boosts.

Purification of anti-i3 antibody from whole rabbit-serum was achieved by affinity chromatography, using peptide i3 coupled to agarose gel beads (Harlow and Lane, 1988). Antibodies were eluted by the addition of 0.2 M glycine/HCl, pH 2.8. Neutralized fractions were dialysed against PBS and concentrated using a Centricon 30 (Amicon). The purified anti-i3 antibody (purified Abi3) was stored at -20°C in 50 % glycerol.

Production of the fusion protein GST-NTR

The *Bam*HI-*Stu*I fragment of prNTR2 (Tanaka et al., 1990) was subcloned into the *Bam*HI site of the plasmid pTZ/18R. A *Stu*I site was added by oligonucleotide-directed mutagenesis immediately upstream of the initiation codon for NTR using the BioRad mutagenesis system, and the *Stu*I-*Bam*HI fragment from the resulting plasmid was subcloned into the *Sma*I site of the prokaryotic expression vector pGEX-2T encoding a 27.5 kDa fragment of glutathione-S-transferase (GST). The recombinant plasmid was verified to be in frame by double-stranded dideoxy sequencing (Sanger et al., 1977) using the Sequenase 2.0 sequencing kit (United States Biochemical). The resulting fusion protein contained GST fused to the N-terminal of the NTR.

Escherichia coli were transformed with either the recombinant or parent vector and grown at 37°C in 500 ml of the Luria-Bertani medium containing 100 μ g/ml ampicillin to an absorbance of 0.6. The synthesis of the fusion protein was then induced with 1 mM isopropylthiogalactoside for 3 h at 37°C . Bacterial cultures were harvested by centrifugation, washed with 10 ml of PBS, resuspended in 5 ml of PBS and lysed by sonication. During the whole preparation, the samples were kept on ice. A 500 μ l volume of 10 % Triton X-100 was added and the lysed cells were centrifuged for 5 min at 15000 *g* at 4°C . The supernatant containing the solubilized fusion protein was affinity purified by a batch procedure using 1 ml of reduced glutathione-agarose beads (Pharmacia), mixed for 20 min at 4°C and washed three times with 10 ml of cold PBS. The fusion protein was eluted with 5 mM reduced glutathione in 50 mM Tris/HCl, pH 8. The fractions were analysed by SDS/PAGE and the protein concentration was determined by the Bradford method (Bradford, 1976) (Bio-Rad).

Expression of NTR in CHO and Sf9 cells, and membrane preparation

CHO cells, grown in alpha MEM without nucleoside and deoxyribonucleoside, supplemented with 10 % decomplexed fetal calf serum, 2 mM glutamine and 80 μ g/ml of gentamicin, were transfected with NTR cDNA plasmid CDM8 and neomycin-resistant plasmid pRSVneo by the standard calcium phosphate precipitation technique (Wigler, 1977). The transfectants were isolated in medium containing 500 μ g/ml of G418-sulphate (Gibco).

For membrane preparation, all buffers contained 20 mM EDTA, 1 mM phenylmethanesulphonyl fluoride (PMSF) and a cocktail of protease inhibitors (0.1 mg/ml leupeptin, 0.1 mg/ml aprotinin, 0.1 mg/ml bacitracin, 0.1 mg/ml pepstatin and 1 mg/ml soybean trypsin inhibitor). The cells were scraped in PBS, collected by centrifugation at 500 *g* and lysed in 5 mM Tris/HCl, pH 7.4, by successive passages through a syringe with a 26 $\times$ G1/2 needle. The suspension was centrifuged for 10 min at 15000 *g*, the pellet resuspended in 5 mM Tris/HCl, pH 7.4, passed again

through the syringe and centrifuged as above. Finally, the membranes were resuspended in 50 mM Tris/HCl, pH 7.4, the protein concentration was determined and the suspension stored at -80°C .

The expression of the NTR in Sf9 insect cells was achieved using a recombinant baculovirus, as previously described for the β_2 -adrenergic receptor (Mouillac et al., 1992). The recombinant baculovirus was obtained by subcloning the *StuI*-*Bam*HI restriction fragment containing the entire coding sequence of the NTR (see above) into the baculovirus transfer vector pJVTETLZ/NheI (Vialard et al., 1990). The NTR-plasmid construct was then co-transfected into Sf9 cells with wild-type AcNPV viral DNA using the calcium phosphate method (Summers and Smith, 1987). Recombinant baculoviruses were purified by successive plaque-purifications using β -galactosidase activity as described (Vialard et al., 1990), amplified and the viral titre measured (Summers and Smith, 1987). Production of the recombinant NTR was achieved by infecting Sf9 cells $[(1-2) \times 10^6 \text{ cells/ml}]$, grown at 27°C in Grace's supplemented medium containing 10% fetal bovine serum, 50 $\mu\text{g/ml}$ of gentamycin sulphate and 2.5 $\mu\text{g/ml}$ of fungizone] with virus stock at a multiplicity of infection of 2. For membrane preparation, the cells were harvested 48 h post-infection, washed in cold PBS and lysed in ice-cold 10 mM Tris/HCl (pH 7.4)/2 mM EDTA. The lysates were centrifuged at 500 g for 5 min and the supernatants centrifuged at 45000 g for 30 min at 4°C . The pelleted membranes were resuspended in 50 mM Tris/HCl/2 mM EDTA, the protein concentrations measured and the suspensions stored at -80°C .

For rat-tissue membrane preparation, the cortex was removed from 7-day old rats and the liver from adult rats. The tissues were homogenized (Polytron) in 10 ml of ice-cold 50 mM Tris, pH 7.4, containing protease inhibitors as described above, and centrifuged at 60000 g for 35 min at 4°C . The pellet was resuspended in 10 ml of 50 mM Tris/HCl, pH 7.4, homogenized again and centrifuged as above. The pellet was then resuspended in Tris buffer, the protein concentrations measured and the suspension stored at -80°C until use.

Radioligand-binding assay

The binding assay for the membranes from Sf9 and CHO cells was performed at 22°C for 30 min using 3 and 10 μg of membranes from Sf9 and CHO cells respectively. The membranes were incubated with 0.1 nM ^{125}I -Tyr³NT (^{125}I -NT, 2000 Ci/mmol) prepared as previously described (Sadoul et al., 1984) in a final volume of 250 μl of binding buffer (50 mM Tris/HCl (pH 7.4)/0.2% BSA/0.8 mM 1,10-*o*-phenanthroline) in the presence of increasing concentrations of unlabelled NT (from 0.3 nM to 1 μM). The assay was terminated by centrifugation at 12000 g for 4 min at 4°C . The supernatant containing the free ligand was removed and the pelleted membranes were rinsed quickly with 500 μl of cold 50 mM Tris/HCl, pH 7.4, and centrifuged again. Radioactivity remaining on the membranes was measured in a gamma counter (Packard). The competition data were analysed using the EBDA-LIGAND program to estimate the dissociation constant (K_d) and the maximal number of binding sites (B_{max}) values.

PNGase F treatment

CHO cell membranes (80 μg) were solubilized for 2 h at 4°C in 16 μl of 0.1% SDS/0.5% Nonidet P40/100 mM Na_2HPO_4 (pH 7.2) containing 20 mM EDTA, 1 mM PMSF and a cocktail of protease inhibitors as described above. The soluble fraction was collected by centrifugation for 15 min at 15000 g , 1.2 μl of

stock PNGase F (20 units/100 μl) was added to the lysate and the mixture was incubated for 1 h at 37°C before analysis by immunoblotting.

Electrophoresis and immunoblotting analysis

Solubilized samples containing 0.1–1 μg of the fusion protein, 5 μg of Sf9, 30 μg of CHO cell membranes or 30 μg of rat-tissue membranes were subjected to SDS/10% or 8% PAGE (Laemmli, 1970), and proteins were transferred to nitrocellulose membrane (0.45 μm , Bio-Rad). Non-specific sites were blocked by 3% BSA in TBS [10 mM Tris/HCl/0.15 M NaCl (pH 7.4)] for 1 h at 37°C . Crude anti-i3 antiserum (1:200), preimmune serum (1:200) or affinity-purified Abi3 (1:300–1:750) was incubated overnight at 4°C in TBS containing 3% BSA (w/v) and 1% (v/v) normal goat serum (NGS). The blots were then washed in TBS containing 0.05% Tween 20, incubated for 2 h at 22°C with alkaline phosphatase-conjugated goat anti-(rabbit IgG) (1:300) in TBS containing 3% BSA and 1% NGS, washed with TBS and then treated for 10 min with Nitroblue tetrazolium (0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.16 mg/ml) in 100 mM Tris/HCl (pH 9.5)/100 mM NaCl/5 mM MgCl_2 . The reaction was terminated by washing the blots in water containing 20 mM EDTA.

Binding of antibody to Sf9 cell membranes

The affinity-purified Abi3 (1:100 to 1:100000 in 1% NGS and 0.05% Triton X-100 in TBS, pH 7.4) was incubated overnight at 4°C with membranes (6 μg) from Sf9 cells expressing the NTR or from wild-type cells, the mixture was centrifuged at 15000 g for 10 min and the pellet washed with 0.05% Tween 20 in TBS, pH 7.4. Antibodies bound to the membranes were then revealed using the biotin-avidin immuno-peroxidase method (Vectastain kit; Vector Laboratories), with *o*-phenylenediamine as a chromogenic substrate, and the absorbance was determined at 450 nm.

Immunocytochemistry

Sf9 cells infected 12 h earlier with NT receptor-encoding viruses were collected by centrifugation, washed with 100 mM Tris/HCl/2 mM EDTA (pH 7.5) and fixed in 4% paraformaldehyde for 20 min at 22°C . The cells were then washed again in Tris buffer, incubated overnight with crude anti-i3 antiserum or preimmune serum (1:200–1:500) in Tris buffer containing 0.15 M NaCl, 1% NGS and 0.05% Triton X-100, washed in Tris-saline, incubated for 45 min at room temperature with goat anti-(rabbit IgG) tagged with Texas Red (1:100), washed again in Tris-saline and mounted on gelatinized glass slides in a drop of Aquamount.

Histochemical staining of CHO cells transfected with NTR cDNA as well as wild-type, non-transfected cells was performed with purified Abi3 (1:200–1:500) according to the same procedure.

All immunolabelled cells were then examined under a Leitz Diaplan fluorescent microscope equipped with dichroic filters for detection of Texas Red.

RESULTS

Recognition of the fusion protein

To determine whether the antibody raised against the peptide i3 recognized the immunogenic sequence when the latter was present in the NTR, its ability to react with the fusion protein GST-NTR was assessed by immunoblotting (Figure 1). In Coomassie Blue

Figure 1 Immunoblot analysis of the fusion-protein GST-NTR

(a) Coomassie Blue staining of the 10% SDS/PAGE analysis of NTR fusion protein affinity-purified with glutathione-Sepharose. Approximately 1 μ g of GST encoded by the pGEX-2T parental plasmid (lane 1) and 1 μ g of GST-NTR fusion protein (lane 2) were loaded. Migration of molecular mass markers (in kDa) is indicated on the left. (b) Immunoblot of the GST-NTR fusion protein with the anti-peptide antibody. GST-NTR (100 ng) was transferred onto nitrocellulose and incubated with the crude anti-i3 antiserum (1:200, lane 1), the preimmune serum (1:200, lane 2), the affinity-purified Abi3 (1:300, lane 3), or the affinity-purified Abi3 (1:300) preincubated with 10^{-5} M of peptide i3 (lane 4).

stains of the fusion-protein preparation (Figure 1(a), lane 2), a faint band was apparent at approximately 74 kDa (arrow), corresponding to the expected molecular mass of the full-length fusion product. Several bands of lower molecular mass were also visible in this preparation: one of 27.5 kDa corresponding to GST (see also Figure 1(a), lane 1) and bands of molecular masses ranging from 29 to 70 kDa, which probably represent breakdown products of the whole fusion protein. Such breakdown products have indeed been observed previously with the expression of other recombinant pGEX-2T plasmids, even in the presence of protease inhibitors (Levey et al., 1991).

As shown in Figure 1(b), the crude anti-i3 antiserum recognized preferentially the complete fusion protein GST-NTR (band at 74 kDa; Figure 1(b), lane 1) and two other proteins (65 and 62 kDa). Interestingly, the 74 kDa protein was heavily labelled by the antiserum, while it was only slightly visible in Coomassie Blue staining (Figure 1(a), lane 2). The anti-i3 antiserum also labelled, to a lesser extent, some other components of the fusion-protein preparation. No signal was obtained with the preimmune serum (lane 2). When the purified Abi3 was used, only two bands corresponding to 74 and 62 kDa remained highly labelled, and a lower staining was observed at 65 kDa (lane 3). In competition experiments in which the purified Abi3 was preadsorbed with an excess of peptide i3, all these three bands disappeared (lane 4).

Molecular forms of the NTR expressed in CHO cells, Sf9 cells and rat cerebral cortex

In membranes derived from NTR-transfected CHO cells [Figure 2(a)], immunoreactive proteins were detected from 42 to 90 kDa by the purified Abi3 in Western-blot analysis (lane 1). The highest levels of immunoreactivity were observed between 60 and 72 kDa. Only a faint labelling was observed at 47 kDa, the molecular mass deduced from the amino acid sequence of the transfected NTR. No signal was obtained in membranes derived from the parent untransfected cells (Figure 2(a), lane 2). Similarly, when the experiments were performed with the purified Abi3 preadsorbed with the peptide i3 (10^{-5} M), the labelling was no longer observed in membranes from NTR-transfected cells (lane 3).

Immunoblot analysis was then performed after deglycosylation of the membrane proteins of the NTR-transfected CHO cells by PNGase F (Figure 2(a), lane 4). Application of the deglyco-

Figure 2 Immunoblot analysis of membranes from recombinant CHO and Sf9 cells

(a) Membranes (30 μ g) derived from transfected CHO cells (lanes 1, 3 and 4) or from untransfected cells (lane 2) were transferred onto nitrocellulose. Lanes 1 and 2: the purified Abi3 was used at a dilution of 1:750. Lane 3: the purified Abi3 was preincubated with 10^{-5} M of peptide i3. Lane 4: the membranes were treated with PNGase F before immunoblot analysis. Migration of molecular mass markers (in kDa) is indicated on the left. (b) Membranes (5 μ g) derived from Sf9 cells infected with a baculovirus encoding the NTR (lane 1) or infected with the wild-type baculovirus (lane 2) were transferred onto nitrocellulose. The purified Abi3 was used at a dilution of 1:750.

Figure 3 Immunoblot analysis of membranes from rat tissues

Lane 1: 7-day-old rat cortex (Cx). Lane 2: adult rat liver (L). Each sample (30 μ g of protein) was applied to 10% SDS/PAGE, transferred onto nitrocellulose and then probed with Abi3 (1:750).

sylation procedure in the absence of PNGase F did not modify the pattern of immunoreactivity (results not shown). After treatment with PNGase F, the bands of high molecular mass were no longer observed and high levels of immunoreactivity were present at 47 and 36 kDa. The 47 kDa molecular mass corresponds to that expected for the deglycosylated NTR. Although experiments were carried out in the presence of the protease inhibitors, the band at 36 kDa might represent a degraded form of deglycosylated NTR. It should be noted that such a proteolysis was not observed in the control experiments performed in the absence of the enzyme, since, as was mentioned above, the deglycosylation procedure without the enzyme did not modify the pattern of immunoreactivity.

Figure 2(b) illustrates the results of Western-blot analysis obtained when the NTR was expressed in Sf9 insect cells. In this case, a major immunoreactive band was observed at 47 kDa (lane 1). Additional bands were seen at higher (i.e. 65 kDa) and lower (i.e. 35 kDa) molecular masses, and lower levels of immunoreactivity were detected in-between. No signal was obtained under the same conditions using membranes from cells infected with wild-type AcNPV baculovirus (lane 2). Similarly, when the experiments were performed on NTR-expressing Sf9 cells with Abi3 preadsorbed with an excess of peptide i3 (10^{-5} M), the labelling was completely abolished (results not shown).

In membranes derived from the cerebral cortex of 7-day-old rats, two immunoreactive bands of very close molecular masses were detected, at 54 and 56 kDa (Figure 3). Preabsorption of the

Table 1 Binding parameters of ^{125}I -NT-binding to membranes from Sf9 and CHO cells expressing the NTR

The dissociation constant (K_d) and the maximal binding capacity ($B_{\max}$) values were determined in competitive binding assays using increasing concentrations of unlabelled NT. Results are the mean $\pm$ S.E.M. of *seven or †three independent experiments.

Cell	K_d (nM)	$B_{\max}$ (pmol/mg protein)
Sf9-NTR	$1.28 \pm 0.13^*$	$9.6 \pm 1.1^*$
CHO-NTR	$0.44 \pm 0.01^\dagger$	$1.96 \pm 0.52^\dagger$

Figure 4 Binding of Abi3 antibody to Sf9 cell membranes

The membranes (6 μg of protein/tube) from Sf9 cells infected with a baculovirus encoding the NTR ($\blacktriangle$) or from wild-type Sf9 cells ($\square$) were incubated overnight at 4 $^\circ\text{C}$ in the presence of increasing dilutions of purified antibody. The bound antibody was revealed by the biotin-avidin immuno-peroxidase method with α -phenylenediamine as chromogenic substrate, and the absorbance was determined at 450 nm. Each value represents the mean of duplicates.

antibody with an excess of peptide i3 (10^{-5} M) led to the complete disappearance of the labelling (results not shown). In the rat liver, a tissue expressing very low amounts of NTR (Muraki et al., 1987), only a very faint signal was observed at 56 kDa. In contrast to what was observed in CHO cells, no decrease in molecular masses could be obtained in membranes from cerebral cortex following the same treatment with PNGase F and endoglycosidase F.

^{125}I -NT binding to membranes from Sf9 and CHO cells

As seen in Table 1, despite the differences in the molecular forms of the NTR expressed in Sf9 and CHO cells, affinities of ^{125}I -NT for NTR in these two systems were quite similar. In the Sf9 cells, where most of the NTR was unglycosylated, the affinity for ^{125}I -NT remained in the nM range.

Immunological detection of the NTR in unfixed cell membranes and fixed cells

In order to test whether the sequence of the NTR corresponding to peptide i3 (amino acids 279–302) was still accessible to antibodies when the receptor was inserted in the cell membranes, binding experiments were performed on Sf9 membranes. The bound antibody was revealed by colorimetry using the biotin-avidin immuno-peroxidase method.

As shown in Figure 4, high absorbance values were obtained when Abi3 was used against Sf9 membranes expressing the NTR whereas low values were observed on membranes derived from

Figure 5 Immunocytochemistry of the NTR

Immunofluorescent staining of the Sf9 insect cells infected with a baculovirus encoding the NTR (a,b) and of CHO cells transfected with the NTR cDNA (c,d). (a) Sf9 cells incubated with crude i3 antiserum. (b) Control cells incubated with preimmune serum. (c) NTR-transfected CHO cells incubated with Abi3. (d) Wild-type CHO-cells incubated with the same antibody. Scale bar: 25 μm .

wild-type cells, indicating that the antibody still recognized the corresponding sequence in the NTR. The amount of specific Abi3-binding was dependent on the antibody dilution, with an EC_{50} value of 1:5000.

As illustrated in Figure 5, the binding ability of the antibody to the NTR was also retained after cell fixation. Thus, NTR-expressing Sf9 cells, immunoreacted with the crude anti-i3 antiserum, exhibited intense immunostaining [Figure 5(a)], whereas much lower levels of fluorescence were observed when the cells were incubated with the preimmune serum [Figure 5(b)]. Similarly, NTR-transfected CHO cells incubated with purified Abi3 exhibited a clear-cut immunolabelling [Figure 5(c)], which was not obtained when the antibody was tested on wild-type CHO cells [Figure 5(d)].

DISCUSSION

In this work, we present the first immunological detection of the molecular forms of rat NTR expressed in different cell lines and in rat cerebral cortex.

The antibody used in the present study was directed against a peptide corresponding to a fragment of the third intracellular loop of the rat NTR, the sequence of which was deduced from the nucleotide sequence of the cloned rat NTR (Tanaka et al., 1990).

The immunization of rabbits with synthetic peptides has proven an effective method for the production of antibodies against many G-protein-coupled receptors, including β -adren-ergic (Wang et al., 1989), D2-dopaminergic (McVittie et al., 1991; Boundy et al., 1993a) or muscarinic (Wall et al., 1991) receptors. An alternative approach to the generation of anti-receptor antibodies consists in the use of fusion proteins as immunogens (Levey et al., 1991; Boundy et al., 1993c). Indeed, this was our original goal in producing a fusion protein consisting of the whole sequence of the NTR and a fragment of GST. However, antisera obtained by immunizing rabbits with this fusion protein presented a low specificity toward the NTR (H. Boudin, P. Forgez, W. Rostene and D. Pelaprat, unpublished

work). Nevertheless, this fusion protein proved useful here for the preliminary screening of the different antisera. Furthermore, its use in immunoblot experiments indicated that the anti-peptide antibody cross-reacted, under denaturing conditions, with the 279–302 aminoacid sequence of the NTR.

A positive signal was also obtained when the antibody was tested by Western immunoblot analysis on extracts of membranes derived from CHO and Sf9 cells expressing the NTR, but not on those originating from the corresponding untransfected control cells, indicating that the antibody recognized the denatured forms of the NTR expressed in these cell lines. We also showed that the antibody could bind to unsolubilized membranes of these cells, indicating that the sequence of the NTR corresponding to peptide i3 was still accessible when the receptor was embedded in the cell membranes. Moreover, we showed that the interaction of the antibody with the NTR was not destroyed by cell fixation, and enabled the receptor to be visualized by immunocytochemistry in Sf9 and CHO cells.

When the NTR was expressed in CHO cells, only a faint labelling was observed in Western-blot analysis at 47 kDa, the expected molecular mass deduced from the rat NTR cDNA (Tanaka et al., 1990), whereas multiple bands were detected at higher molecular masses, up to 90 kDa. Similar diversity of high molecular mass forms had been previously observed with the D2-dopaminergic (McVittie et al., 1991) or the substance P (Shigemoto et al., 1993) receptors expressed in CHO cells and had been attributed to variations in the glycosylation state of the receptors, suggesting that the high-molecular-mass components observed in our study might represent glycosylated forms of the NTR. Indeed, deglycosylation of the membrane proteins by PNGase F led to the disappearance of the high-molecular-mass forms associated with the reinforcement of the labelling at 47 kDa.

Under those conditions, however, a heavily labelled band was also observed at 36 kDa, which probably represents a truncated form of the unglycosylated receptor. Its recognition by the antibody shows that it still contains the 279–302 amino acid sequence. This band was observed in spite of the presence of protease inhibitors during the whole experimental procedure, and might reflect insufficient protection of the receptor during the membrane preparation, or enhanced sensitivity of the NTR to proteases after deglycosylation. However, this truncated receptor could also have already been present in glycosylated form within the cell membrane, as a result of intrinsic cellular degradation processes.

The pattern observed in extracts of membranes from recombinant Sf9 cells was quite different from that obtained in CHO cells. In the case of recombinant Sf9 cells, the major band revealed by the antibody was at 47 kDa, the molecular size of the non-glycosylated receptor. Only faint bands were observed at higher molecular masses, suggesting that only a small fraction of receptor proteins were glycosylated. Secondary bands were also seen under 47 kDa, and in particular at 36 kDa, the size of the degradation product observed in CHO cells under deglycosylation conditions.

Taken together, these data show that the NTR protein was mainly glycosylated when expressed in CHO cells but almost entirely unglycosylated when expressed in Sf9 cells. The lower degree of glycosylation observed here in Sf9 cells has been previously demonstrated for other receptors, such as the D3 dopaminergic (Boundy et al., 1993b) or the β_2 -adrenergic (George et al., 1989) receptors, when expressed in this cell type. Moreover, it has been previously demonstrated that Sf9 cells only incompletely process the N-linked oligosaccharides (Luckow and Summers, 1988), which could explain the lower molecular

masses attained by the glycosylated forms of the NTR in this cell line (up to 65 kDa) when compared with CHO cells (up to 90 kDa).

When immunoblot analysis was carried out on membrane extracts from the cerebral cortex of 7-day-old rats, two immunoreactive bands were detected with the anti-i3 antibody: one at 54 kDa and the other at 56 kDa. This two-band pattern was reminiscent of the one previously obtained in photoaffinity labelling experiments on adult rat-brain membranes, where two proteins with molecular masses of 49 and 51 kDa were identified (Mazella et al., 1985). Moreover, a protein of 55 kDa having the characteristics of the high-affinity NTR was recently purified from the cerebral cortex of newborn rat (Miyamoto-Lee et al., 1991), which probably corresponds to one of the two bands observed in our study. Our second band may conceivably correspond to a form of the NTR which would have lost its binding ability during the purification procedure. Such a loss of activity, restricted to some of the molecular forms of the receptor, has already been reported in the case of the mouse NTR, where components of 50, 60 and 100 kDa were identified by photoaffinity labelling while only the 100 kDa protein could be purified in the active form (Chabry et al., 1993; Mazella et al., 1989). The lack of labelling at 47 kDa in rat cerebral-cortex membranes suggested that most of the endogenously expressed NTR was glycosylated, similarly to that expressed in transfected CHO cells. However, the molecular sizes of the main receptor-forms were different in the two systems, probably reflecting differences in the processing of the oligosaccharide chains. Moreover, in contrast to CHO cells, attempts to deglycosylate the NTR of rat cerebral-cortex membranes with PNGase F were unsuccessful. Another N-glycanase, endoglycosidase F, was also inefficient (H. Boudin, D. Pelaprat and W. Rostene, unpublished work). A possible explanation is that, in the brain, the NTR is not N-glycosylated but O-glycosylated.

We have demonstrated that there are large differences in the molecular forms of the NTR in membranes from rat cerebral cortex, CHO cells and Sf9 cells. Nevertheless, we have found that ^{125}I -NT binding-affinity in these transfected cell models is very close to the binding-affinity to rat brain homogenates (Kitabgi et al., 1985). These data suggest that, for the NTR, glycosylation does not seem to play an essential role in the binding of ^{125}I -NT to NTR.

The antibody directed against the NTR obtained in this study allowed the heterogeneity of the molecular forms of the rat NTR expressed in different systems to be demonstrated. In addition, the NTR has been visualized by immunocytochemistry in transfected CHO and Sf9 cells. This antibody should therefore prove a useful tool for investigating the characteristics and regulation of the NTR protein at both the molecular and cellular levels.

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