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The expression system affects the binding affinity between p75NTR and proNGF

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A R T I C L E I N F O

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A B S T R A C T

ProNGF (nerve growth factor) is a precursor of NGF and a signaling peptide exerting opposite effects on neuronal cells, i.e., apoptotic or neuritogenic. The conflicting biological activity of proNGF depends on the relative levels of two membrane receptors, TrkA and p75NTR. The effect of proNGF depends on the expression levels of these receptor proteins and their affinity to proNGF. Since the affinity of proteins has been studied with various recombinant proteins, it is worth comparing the affinity of these proteins within one experiment with the same method. This study examined the affinity between a recombinant proNGF and p75NTR expressed in common systems: bacterial, insect, and mammalian cells. The extracellular domain of p75NTR expressed in the insect or mammalian systems bound to native mature NGF, with a higher affinity for the insect receptor. The uncleavable proNGF was expressed in the three systems and they showed neuritogenic activity in PC12 cells. These recombinant proteins were used to compare their binding affinity to p75NTR. The insect p75NTR showed a higher binding affinity to proNGF than the mammalian p75NTR. The insect p75NTR bound proNGF from the insect system with the highest affinity, then from the mammalian system, and the lowest from the bacterial system. Conversely, the mammalian p75NTR showed no such preference for proNGF. Because the recombinant proNGF and p75NTR from different expression systems are supposed to have the same amino acid sequences, these differences in the affinity depend likely on their post-translational modifications, most probably on their glycans. Each recombinant proNGF and p75NTR in various expression systems exhibited different mobilities on SDS-PAGE and reactivities with glycosidases and lectins.

1. Introduction

The nerve growth factor (NGF), a member of the neurotrophin family, plays an essential role in the survival, differentiation, and neurite outgrowth of neuronal cells by binding to the TrkA receptor on the cell surface [1,2]. The affinity of NGF for TrkA is increased by the coexisting pan neurotrophin receptor, p75NTR [3]. NGF arises from its precursor, proNGF, via the release of the N-terminal "pro" region of proNGF under the action of proteases, e.g., furin intracellularly [4] and plasmin or matrix metalloproteinases extracellularly [5,6]. However, the intact proNGF is reported to be the predominant form of NGF in mouse, rat, and human brain tissue, and is upregulated in patients with neurodegenerative diseases such as Alzheimer's disease [7–9], Parkinson's disease [10], and spinal injuries [11]. The proNGF has a high affinity for p75 and their binding was reported to induce apoptosis of neuronal cells [6]. This effect is shown to be strengthened by the coexistence of another membrane receptor, sortilin [12]. On the other hand, other researchers described NGF-like effects for proNGF, such as neuronal differentiation and neurite outgrowth [13]. These conflicting findings have been explained by the relative expression levels of the two membrane receptors, TrkA and p75NTR on target cells, with the former mediating the neurotrophic signal for survival and the latter initiating the process of apoptosis [14,15]. Thus, the effect of proNGF is based on the balance of the intensity of the two conflicting signals. In addition to the amount of these receptors, the affinity of proNGF for the receptors is a crucial factor for balancing the signal intensity.

Since p75NTR concerns apoptotic process, it could be a target to prevent cell death. The affinity of p75NTR to NGF or proNGF has been studied using isothermal titration calorimetry (ITC) [12] or surface plasmon resonance (SPR) assay [16–21] using rat or human sequence p75NTR and human or mouse sequence NGF. Most of the p75NTR used were extracellular domain expressed in insect cells, and most of NGF and proNGF were expressed in bacteria. The affinity (K_d) of proNGF to p75NTR has been reported to be 15 and 23.5 nM for human combination

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[12,21] and 18.3 nM for human p75NTR and mouse proNGF [19]. The difference between the expression systems could affect the binding affinity of proNGF and p75NTR. The preceding studies have used proNGF derived from bacteria, and the bacterial proteins are supposed not to be glycosylated. Since there are two possible N-glycosylation sites in the propeptide of mouse NGF, the glycans of proNGF could affect its affinity to the receptors. Glycans could influence the structural conformation and spatial arrangement of the peptide chains which could affect the binding character of proteins. To compare the affinity of proteins from various sources, we obtained recombinant proteins from common expression systems such as bacterial, insect, and mammalian cells, and applied them to SPR assay.

2. Materials and methods

2.1. Plasmids encoding proNGF and p75NTR

A DNA encoding a maturation-resistant mouse proNGF (R49A, R50G, K79D, R80A, R81D, R118Q, K120S, and R121Q) with a C-terminal hexahistidine sequence (UCproNGF) was obtained by PCR amplification using the following oligonucleotides as the primers: 5′-GAGCTCGAGAATTCATGTCCATGTTGTTCT-3′ and 5′-TTCTGCAGT-CAGTGATGGTGATGGTGATGGCCTCTTCTTGTA-3′; and the previously constructed plasmid QSSQ-NGF-C1x-C2x [22] as the template. The DNA fragment was cloned into the EcoRI and PstI sites of pGW1 [23] and pFastBac1 (Invitrogen, Waltham, MA, USA), to construct pGW/UC-proNGF for mammalian expression and pFB/UCproNGF for baculovirus vector, respectively. For bacterial expression, the same DNA fragment lacking the N-terminal signal peptide (M1-A18) was amplified by PCR using the 5′-GGCCATATGGAACCGTACACAGATAGC-3′ and 5′-AGGACCTCGAGGCCTCTTCTTGTAGCC-3′ as the primers and pGW/UCproNGF as the template, then cloned into the NdeI and XhoI site of pET-21a(+) (Novagen, Waltham, MA, USA) (pET/UCproNGF).

A DNA fragment encoding the extracellular region including signal sequence (M1-N251) of rat p75NTR was obtained from rat PC12 cell line using the BcaBEST RNA PCR Kit (Takara, Shiga, Japan) and primers, 5′-TACCAAGCTTATGAGGAGGGCAGG-3′ and 5′-TACCAAGCTTGT TGTCGGTGGTGCCG-3′. The DNA fragment, together with a hexahistidine-coding sequence, was cloned into pGW1 to form pGW/p75NTRex. This plasmid encoded the C-terminal hexahistidine-tagged extracellular region of rat p75NTR (p75NTRex). Similarly, pFB/p75NTRex was also constructed in pFastBac1.

2.2. Expression using a mammalian system

COS7 monkey kidney cells were used as the host. The cells were cultured at 37 °C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were cultured to 60%–80% confluency, then transfected with pGW/UCproNGF or pGW/p75NTRex using Lipofectamine 2000 (Life Technologies, Waltham, MA, USA), according to the manufacturer's protocol. The culture medium was collected and replaced with fresh medium at 2, 4, 6, and 8 days after transfection. The collected media were centrifuged at 15,000×*g* for 15 min at 4 °C to remove cells and insoluble matter. To the supernatant (160 mL), Ni Sepharose 6FF gel (2 mL) was added and incubated at 4 °C for 30 min with gentle stirring, followed by packing into an empty column. The column was washed with buffer A (50 mM Tris-HCl, 500 mM NaCl, pH 8.0) containing 30 mM imidazole. Next, the recombinant protein was eluted from the column over a linear gradient of imidazole, from 60 to 500 mM. Finally, the fractions containing the recombinant proteins were collected and then concentrated using an Amicon Ultracel 10-kDa filter unit, and the solvent was replaced with phosphate-buffered saline.

2.3. Expression using an insect system

Baculoviruses were prepared according to the manufacturer's protocol for the Bac-to-Bac system (Invitrogen). pFB/UCproNGF or pFB/p75NTRex was introduced into bacterial DH10 cells, and the extracted bacmid was then introduced into Sf9 cells using the Cellfectin II Reagent (Life Technologies), to obtain passage 1 baculoviruses (P1 baculoviruses). The P1 baculoviruses were harvested after incubation of the transfected Sf9 cells at 28 °C for 3 days. The suspension culture of Sf9 cells was infected with P1 baculoviruses at 28 °C in serum-free Sf900 III medium (Life Technologies) and harvested at 3 days to obtain passage 2 baculoviruses (P2 baculoviruses). The suspension culture of Sf9 cells was infected with P2 baculoviruses at 28 °C in serum-free Sf900 III medium and incubated for 3 days before harvesting. The collected media were centrifuged at 500×*g* for 5 min, and the supernatant was further centrifuged at 15,000×*g* for 10 min. The supernatant was loaded onto a DEAE Sephadex A-25 column pre-equilibrated with 50 mM Tris-HCl, pH 8.0 (bed = 10 mL) for p75NTRex or Q-Sepharose FF column pre-equilibrated with the same buffer (bed = 10 mL) for UCproNGF. The absorbed proteins were eluted using a linear gradient of NaCl, from 0 to 500 mM. The fractions containing the recombinant protein were combined and then incubated with Ni Sepharose 6FF (1.5 mL) at 4 °C for 1 h with constant stirring, followed by packing into an empty column. Subsequently, the column was washed with buffer A containing 5 mM imidazole. Then, the recombinant protein was eluted from the column over a linear imidazole gradient from 10 to 500 mM. Finally, the fractions containing the recombinant proteins were collected and concentrated, and the solvent was replaced with phosphate-buffered saline.

2.4. Expression using a bacterial system

The SHuffle T7 *Escherichia coli* cell line (New England Biolabs, Ipswich, MA, USA) was used as the host for bacterial expression. *E. coli* cells harboring pET/UCproNGF were grown in LB medium at 37 °C to an OD_{600} of 0.4. Protein expression was then induced using 1 mM IPTG and vigorous shaking for 16 h at 22 °C. The nonsecretory form of proNGF, UCproNGF, was produced inside the bacterial cells. The bacterial pellet obtained from a 1-L culture was resuspended in 60 mL of 20 mM Tris-HCl (pH 8.0) supplemented with 1 mM phenylmethylsulphonyl fluoride and homogenized via sonication at 0 °C. The insoluble matter was removed by centrifugation at 15,000×*g* for 10 min at 4 °C. The cleared lysate was adjusted to 35 mM Tris-HCl (pH 8.0), 250 mM NaCl, and 30 mM imidazole, followed by loading onto a Ni Sepharose 6 FF column (bed = 5 mL) pre-equilibrated with buffer A containing 30 mM imidazole. After washing the column with a 4-bed volume of buffer A containing 30 mM imidazole, UCproNGF was eluted over a linear imidazole gradient from 30 to 500 mM. Finally, the solvent was replaced with phosphate-buffered saline, and the protein was concentrated to approximately 10 mg/ml.

2.5. Neurite outgrowth

PC12 cells (2×10^5 cells/well) were grown on collagen-coated 6-well plates in DMEM supplemented with 5% FBS and 10% horse serum at 37 °C in a humidified 5% CO_2 incubator, followed by the addition of UCproNGF or mature NGF (50 ng/mL). After 6-day incubation, the morphology of the cells was examined using phase-contrast microscopy, and the cells expressing neurites that were longer than their cell bodies were counted as being positive. Three fields in each well per treatment group were examined. Mature NGF was purified from mouse submaxillary glands [24].

2.6. SPR assay

An affinity analysis of UCproNGF with p75NTRex was performed using the Biacore 3000 system (Biacore AB, Uppsala, Sweden). Purified

p75NTRex was immobilized on a CM5 sensor chip (Biacore) until the resonance units reached approximate saturation. The binding of UCproNGF or NGF to p75NTRex was measured in buffer BC (10 mM HEPES–NaOH, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% Tween-20) at 25 °C with a flow rate of 20 μL/min. An increasing amount of UCproNGF from was applied to CM5 sensor chips covered with p75NTRex. The regeneration of the sensor chip was carried out using 10 mM glycine–HCl (pH 2.0 or 1.5) with a flow rate of 20 μL/min for 30 s. The BIA evaluation software ver. 3.2 was used for data analysis.

2.7. Glycan structure analyses

Purified recombinant proteins were denatured at 100 °C for 10 min in 0.5% SDS and 40 mM dithiothreitol, then digested with peptide-*N*-glycanase F (PNGaseF, New England Biolabs) or endoglycosidase H (EndoH, New England Biolabs) according to the manufacturer's manual. The samples were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (PALL, Port Washington, NY, USA) before incubation with the PVDF Blocking Reagent (TOYOBO, Osaka, Japan). Immunoreactive or lectin-reactive bands were visualized using a LAS-3000 apparatus (Fuji Film, Tokyo, Japan) and an ECL Western blotting substrate (Promega, Madison, WI, USA). The antibodies and lectins used in this study were as follows: rabbit anti-mouse NGF antibody [25], rabbit anti-rat p75 antibody (Millipore AB1554), biotinylated wheat-germ agglutinin (WGA), *Ricinus communis* agglutinin I (RCA), concanavalin A (ConA), *Dolichos biflorus* agglutinin (DBA), and *Ulex europaeus* agglutinin I (UEA-I). The antibodies and biotinylated lectins were reacted with goat anti-rabbit HRP-conjugated IgG (Promega) and the Vectastain ABC HRP kit (Vector Laboratories, Newark, CA, USA), respectively. Western blotting and lectin blotting experiments were performed several times, and representative results are shown in the figures.

2.8. Statistical analyses

All data are expressed as the mean ± S.D. Statistical significance was determined by one-way ANOVA, followed by the Tukey–Kramer multiple comparison test, or by Student's t-test for paired results using EZR version 1.6.1. (Saitama Medical Center, Jichi Medical University) [26], which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 4.2.2); more specifically, it is a modified version of R commander (version 2.8-0) that was designed to add statistical functions that are frequently used in biostatistics. Significance was set at $P < 0.05$.

3. Results

3.1. Expression of recombinant p75NTRex and affinity to NGF

In our experiments, the recombinant proteins were obtained from rat p75NTR and mouse proNGF. NGF was first purified from mouse submaxillary gland [27] and its biological effect was extensively studied with rat PC12 cells. Such combinations of different species including humans have been widely used till now.

The extracellular region of p75NTR with a C-terminal hexahistidine tag (p75NTRex) was expressed in insect and mammalian cells. The usage of the extracellular region of p75NTR is quite common to analyze its affinity to neurotrophins or its crystal structures [16–18,28]. The secreted p75NTRex was purified from the culture medium and SDS-PAGE was performed (Fig. 1a). In both expression systems, two main bands were observed and p75NTRex from the insect cell system (p75NTRex-i) moved faster than the mammalian system (p75NTRex-m). Since the amino acid sequences were defined by the transfected DNA, the two protein bands for each expression system and the difference between the expression systems depend likely on the post-translational modification. To address the functional relevance of p75NTRex, the interaction between p75NTRex and mature NGF purified from mouse submaxillary glands was investigated. The purified p75NTRex-i or p75NTRex-m was covalently immobilized on the surface of the sensor chip, and NGF was injected across the surface as the analyte. A reversible binding response was observed in an analyte-concentration-dependent manner for both p75NTRex (Fig. 1b and c), indicating that the purified p75NTRex molecules were functional in the ligand binding ability, although their dissociation constants (K_d) were remarkably different, i.e., 0.011 ± 0.00058 μM (n = 3) for p75NTRex-i and 4.2 ± 1.7 μM (n = 4) for p75NTRex-m.

3.2. Expression of recombinant UCproNGF

Since proNGF of native structure is susceptible to proteases which creates mature NGF intracellularly or extracellularly, we expressed a maturation-resistant version of mouse proNGF, UCproNGF with eight amino-acid substitutions. Such substitution has been widely used for proNGF by many researchers [6,13,18,29]. Still, the number of our mutations is more than those reported because we previously studied the cleavage of proNGF with various mutations and found 8 mutations were preferable to get intact proNGF [22]. When expressed in insect and mammalian cells, the signal sequence leads the UCproNGF out of the cells via the host's protein secretory pathway. In these cases, UCproNGF was purified from the supernatant of the culture media, as described in the Materials and Methods. In the bacterial system, as protein targeting

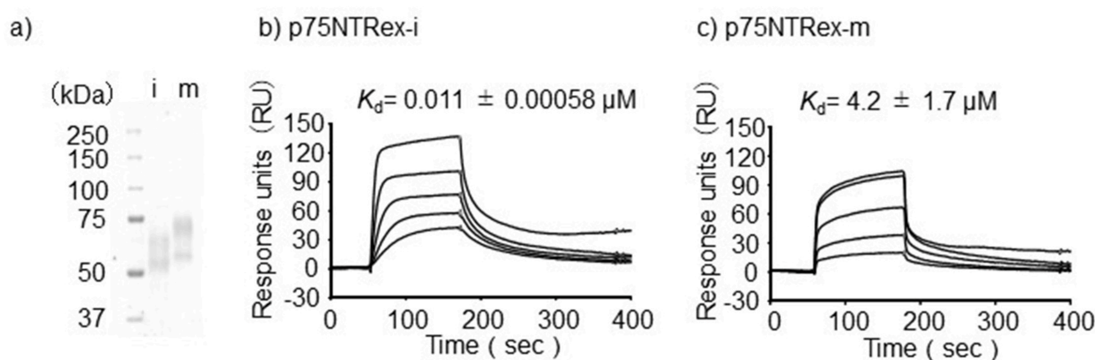

Fig. 1. Purification of p75NTRex proteins and kinetic analysis of their binding to mature NGF.
a, Purified p75NTRex expressed in insect (i) or mammalian (m) cells were applied to SDS-PAGE and the gel was stained with CBB. The left-most lane shows molecular weight markers. b, c, Kinetic analysis using SRP. p75NTRex-i (b) or p75NTRex-m (c) was immobilized on a CM5 sensor chip (20.2 fmol/mm^2 for p75NTRex-i and 74.4 fmol/mm^2 for p75NTRex-m), and an increasing amount of mature NGF (b, 2.5–40 nM; and c, 0.25–2.0 μM) was applied. The experiment was performed three or four times and the obtained K_d values are shown in the figure.

across the cytoplasmic membrane to periplasmic is limited [30,31], UCproNGF was produced without the signal sequence and was obtained from the bacterial lysate. To distinguish the UCproNGFs produced by the bacterial, insect, and mammalian expression systems, they were designated as UCproNGF-b, UCproNGF-i, and UCproNGF-m, respectively. Although the theoretical molecular weight of the polypeptide part of UCproNGF is 25.6 kDa, the purified UCproNGFs exhibited different mobilities on SDS-PAGE depending on the expression system used (Fig. 2a). UCproNGF-b yielded a sharp band at 28 kDa, whereas UCproNGF-i and UCproNGF-m produced smeared bands around 31 and 36 kDa, respectively. These mobility shifts may have been caused by different glycoforms, as described in the section "Glycoform analysis of UCproNGFs and p75NTRexs".

The purified UCproNGFs were examined for their neuritogenic activities toward PC12 cells. ProNGF was shown to have neurotrophic activity depending on the receptors [14,32]. All three UCproNGFs caused neurite outgrowth from PC12 cells under the conditions used in our experiment (Fig. 2b and c). These results demonstrated that the recombinant UCproNGFs were properly folded to exert biological activities. Neurites were observed in 52%–73% of cells after stimulation with 50 ng/mL UCproNGFs for 6 days. There was no significant difference among the activities of the three UCproNGFs. The neuritogenic activity of proNGF is supposed to be dependent on TrkA, and the interaction between proNGF and TrkA should be studied next.

3.3. Affinities among UCproNGFs and p75NTRexs

To examine the affinity between UCproNGF and p75NTRex expressed in different systems, the UCproNGFs were injected into sensor chips covered with p75NTRex-i or p75NTRex-m (Fig. 3a and b). The K_d values among these proteins are summarized in Fig. 3c and Table 1 p75NTRex-i showed a higher binding affinity to UCproNGFs than p75NTRex-m. Among UCproNGFs, the affinity of UCproNGF-b was lower than those of UCproNGF-i and UCproNGF-m, and UCproNGF-i had a smaller K_d value than UCproNGF-m, although no significant difference was observed. Regarding p75NTRex-m, because of the large

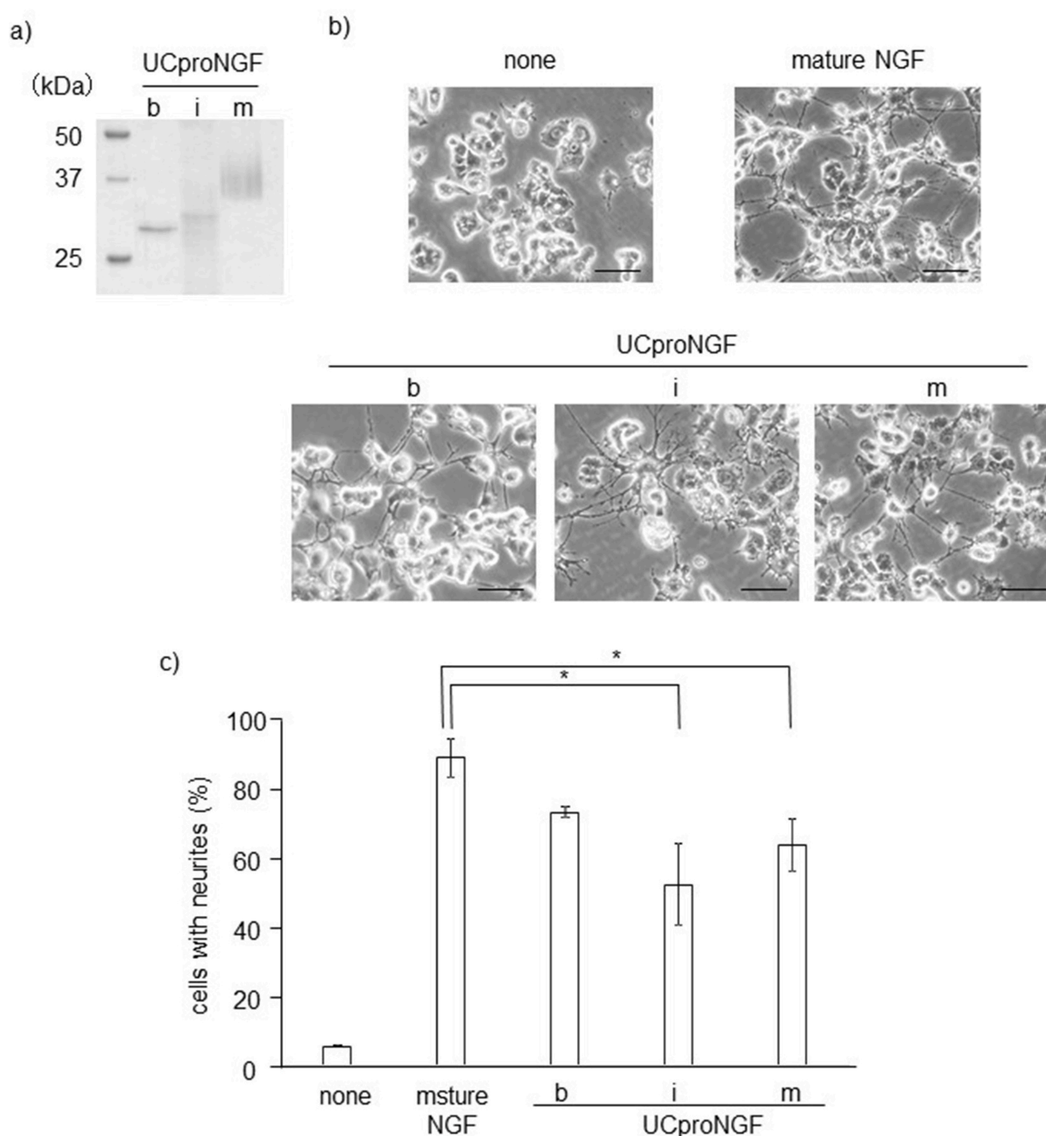

Fig. 2. Purification of UCproNGF proteins and their neurite-extending activity on PC12 cells.
a, Purified UCproNGF expressed in bacteria (b), insect (i), or mammalian (m) cells were applied to SDS-PAGE and the gel was stained with CBB. The left-most lane shows molecular weight markers. b, Purified UCproNGF or mature NGF (50 ng/mL) was added to PC12 cells and incubated for 6 days. Control cells without UCproNGF or mature NGF are shown as "none." c, Neurite-extending cells were counted in three fields for each well. (*$P < 0.05$, Tukey–Kramer multiple comparisons, n = 3).

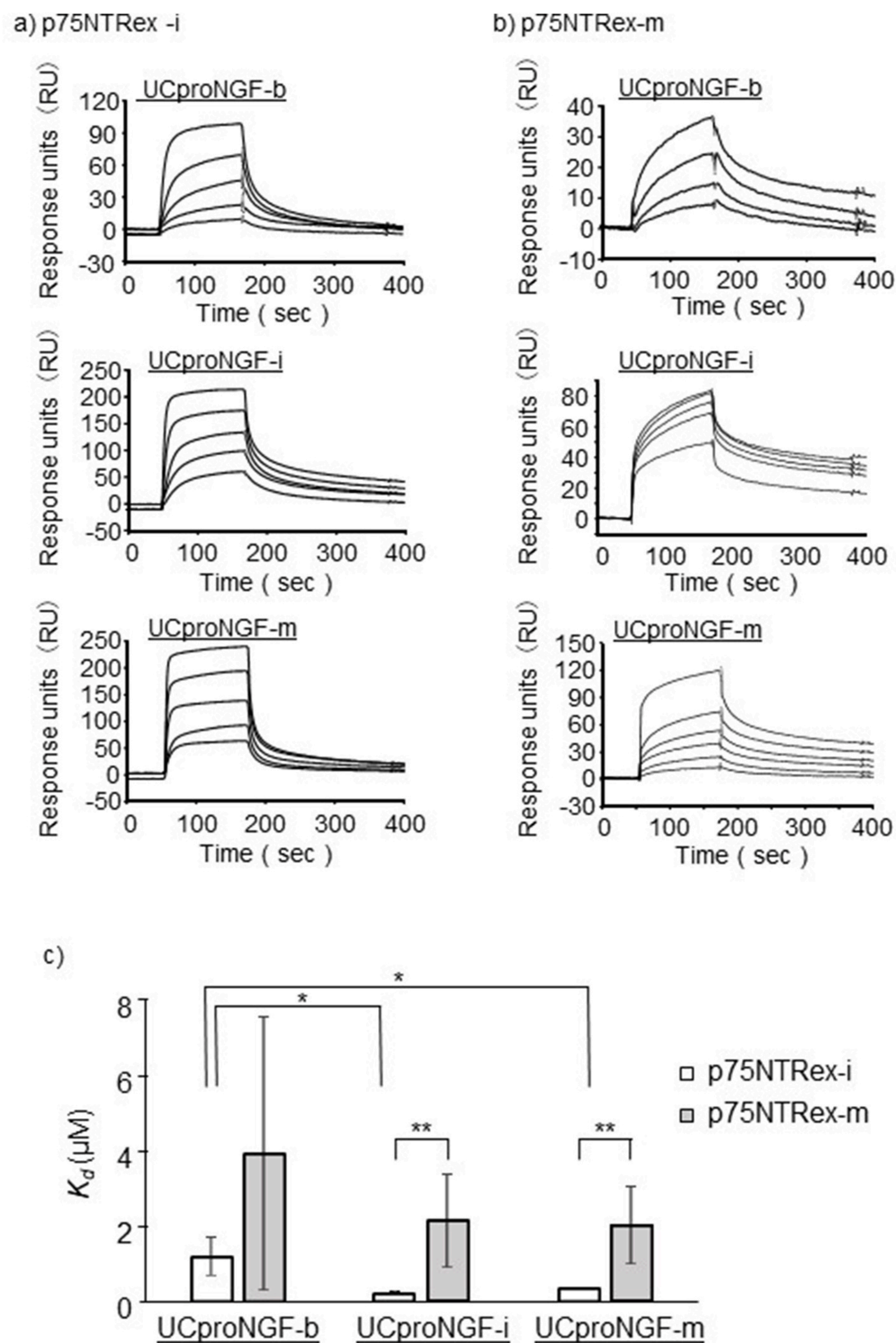

Fig. 3. Analysis of the binding kinetics between proNGF and p75NTR in various expression systems.
a, b, An increasing amount of UCproNGF from bacteria, insect, and mammalian cells was applied to CM5 sensor chips covered with p75NTRex-i (a) or p75NTRex-m (b). For p75NTRex-i, UCproNGF proteins were applied at 0.063–1.0 μM; for p75NTRex-m, UCproNGF-b was applied at 0.25–1.5 μM, UCproNGF-I, at 0.5–1.6 μM, and UCproNGF-m, at 0.25–2 μM. c, The experiment was performed three or four times and the obtained K_d values are shown in the bar graph. *$P < 0.05$ by Tukey–Kramer multiple comparison, **$P < 0.05$ by Student's t-test.

Table 1
Affinity between UCproNGF and p75NTRex from the various expression systems.
K_d values (μM) obtained from Fig. 3 are shown.

	p75NTRex-i	p75NTRex-m
UCproNGF-b	1.2 ± 0.51 (n = 4)	3.9 ± 3.6 (n = 3)
UCproNGF-i	0.21 ± 0.017 (n = 3)	2.1 ± 1.2 (n = 4)
UCproNGF-m	0.33 ± 0.005 (n = 3)	2.0 ± 1.0 (n = 3)

variance in the data, no significant difference was detected for UCproNGFs, but UCproNGF-b tended to exhibit a low affinity compared with UCproNGF-i and UCproNGF-m. Our K_d values were rather large compared to the reported values for proNGF and p75NTR [12,19,21], which might be caused by the differences in the recombinant protein mutations, expression systems, or assay systems.

3.4. Glycoform analysis of UCproNGFs and p75NTRexs

The difference in the affinity between p75NTRexs and UCproNGFs or

NGF in the various expression systems may reflect their post-translational modifications. The difference in their mobility observed in SDS-PAGE (Figs. 1 and 2) suggested that their glycoforms were different. Both p75NTRex and the "pro" region of proNGF contain N-linked glycosylation motifs (-Asn-Xaa-Ser/Thr-). Each protein has two such sites; N61 and N71 for p75NTR and N69 and N114 for proNGF (the amino acid numbers include signal sequences). Therefore, we analyzed the glycoforms of UCproNGFs and p75NTRexs via a combination of glycosidase digestion and lectin blotting.

On SDS-PAGE, UCproNGF-b appeared at 28 kDa, even after the PNGase F digestion, indicating it had no glycan, whereas the size of UCproNGF-i and UCproNGF-m was decreased after the digestion (Fig. 4a). The size of the digested UCproNGF-i was close to that of UCproNGF-b, whereas that of the digested UCproNGF-m was slightly larger than that of UCproNGF-b, indicating some other modification might have occurred to the protein. This result revealed that UCproNGF-i and UCproNGF-m retained *N*-glycoside-type glycans. Because they were resistant to EndoH digestion (Fig. 4a), their glycoforms are not high-mannose type or EndoH-susceptible hybrid type.

Both p75NTRex-i and p75NTRex-m were also shown to have glycans since they were susceptible to PNGaseF digestion (Fig. 4b). The two protein bands of each expression system were both susceptible to PNGaseF. On digestion of p75NTRex-m by EndoH, the lower band increased its mobility which indicated it retained EndoH susceptible glycan, and the upper band became smear, indicating partial existence of EndoH susceptible glycan in this band. Since the size of the EndoH-digested lower band was a little bigger than that of PNGaseF-digested lower band, the lower band likely contained at least two kinds of glycans, EndoH susceptible and resistant. On the other hand, the EndoH digestion of p75NTRex-i had little effect on their mobility on SDS-PAGE. The intensity of the lower band of p75NTRex-i increased slightly which may be caused by the existence of a small amount of EndoH susceptible glycan in p75NTRex-i. Anyway, the glycan of p75NTRex-i is mainly resistant to EndoH.

The glycoform of proteins was further analyzed using lectin blotting in combination with glycosidase digestion. The proteins from the insect system, UCproNGF-i and p75NTRex-i, bound ConA, but not RCA or WGA, regardless of neuraminidase digestion (Fig. 5). It is known that glycans of insect cell glycoproteins are predominantly of the paucimannose-type [33] and it is also the case for the glycan of the extracellular domain of p75NTR expressed in insect cells [28]. The resistance of the paucimannose-type glycan to EndoH digestion is reported [34,35]. Thus, the glycoform of UCproNGF-i and p75NTRex-i was considered mainly paucimannose-type. Since a small portion of p75NTRex-i seemed EndoH-susceptible (Fig. 4), there might exist glycans having more mannose residues than paucimannose-type glycan.

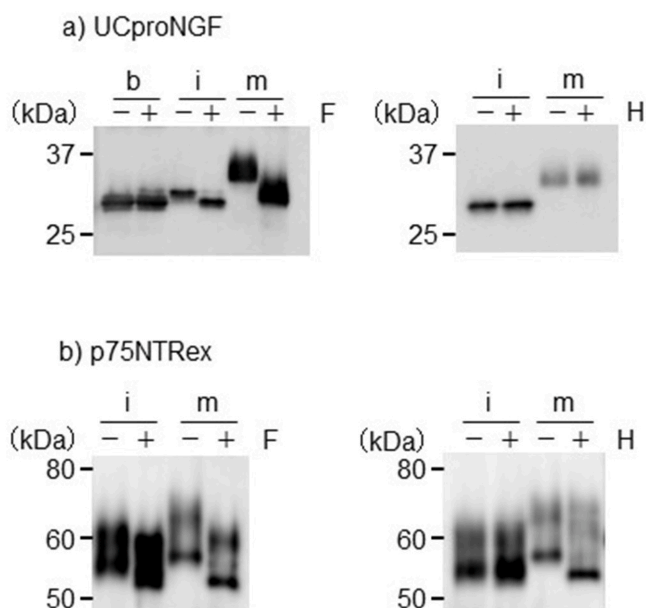

Fig. 4. Susceptibility of UCproNGF (a) and p75NTRex (b) to PNGaseF and EndoH.
UCproNGF and p75NTRex from the various expression systems (bacteria (b), insect (i), or mammalian (m) cells) were digested with PNGaseF (F) or EndoH (H) and detected by Western blotting.

Regarding the mammalian proteins, UCproNGF-m and the upper band of p75NTRex-m bound WGA and RCA, and the binding to RCA was more evident after neuraminidase digestion with weaker binding to WGA after the digestion (Fig. 5). WGA is known to have a high affinity not only to *N*-acetyl glucosamine (GlcNAc) but also to sialic acid [36]. These results suggest that they have sialic acid (SA)–galactose (Gal) sequences in their glycans, which is a typical structure of complex-type glycans. On the other hand, they exhibited different behaviors in binding to ConA; i.e., the both upper and lower band of p75NTRex-m bound to ConA, whereas UCproNGF-m did not. These results suggest that UCproNGF-m retained only the complex-type glycan, and that p75NTRex-m retained at least two different types of glycans. The upper band of p75NTRex-m might have EndoH-resistant complex-type and hybrid or high-mannose-type glycans, which are susceptible to EndoH. The lower band of p75NTRex-m, without binding to RCA or WGA, did not retain complex-type glycan but contained high-mannose or hybrid-type glycans. The binding of ConA to p75NTRex-m diminished after EndoH digestion, indicating ConA bound to EndoH susceptible glycans. The existence of complex-type glycans might cause the upper band of p75NTRex-m. The difference between the two bands of p75NTRex-i. is not clear.

4. Discussion

The interaction between NGF and its receptors has been studied extensively, and in those experiments, recombinant proteins from various sources have been frequently used. However, even if the amino acid sequences were the same, the proteins could show different properties depending on the expression systems. This study aimed to compare the affinities between p75NTR and proNGF derived from different expression systems, and as a result, their affinities were shown to be different depending on their expression systems. In our experimental condition using mutated proNGF and extracellular domain of p75NTR, we found that p75NTRex from insect cells showed higher affinity to NGF or UCproNGF than p75NTRex from mammalian cells. Among UCproNGF, UCproNGF-i exhibited the highest affinity toward p75NTRex-i, followed by UCproNGF-m, and proNGF-b exhibited the lowest affinity toward p75NTRex-i. Conversely, there was no significant difference in the affinity of these UCproNGFs to p75NTRex-m. These recombinant proteins were based on the same DNA sequences and should have the same amino acid sequences. Hence, these differences in affinity are likely reflecting their post-translational modifications, especially their glycans, which may influence the structural conformation and spatial arrangement of the proteins and affect the binding character between proteins. The glycans from the different expression systems were shown significantly different. The insect proteins carried paucimannose-type glycans, whereas the mammalian UCproNGF had complex-type glycans and mammalian p75NTRex had complex and high-mannose or hybrid-type glycans.

The inhibition of glycosylation of the NGF receptor by tunicamycin was reported to decrease the cellular responses of PC12 cells [37]. p75NTR expressed in Sf9 cells treated with tunicamycin exhibited a lower affinity to NGF [16] and NT-3 [17]. These indicate the significance of glycans of the receptors for their binding character. In contrast, the substitution of Asn61 of p75NTR with Asp was reported to have an insignificant effect on its binding to proNGF [18]. Thus, the role of glycan at Asn61 is not clear. Rat p75NTR has another N-glycosylation

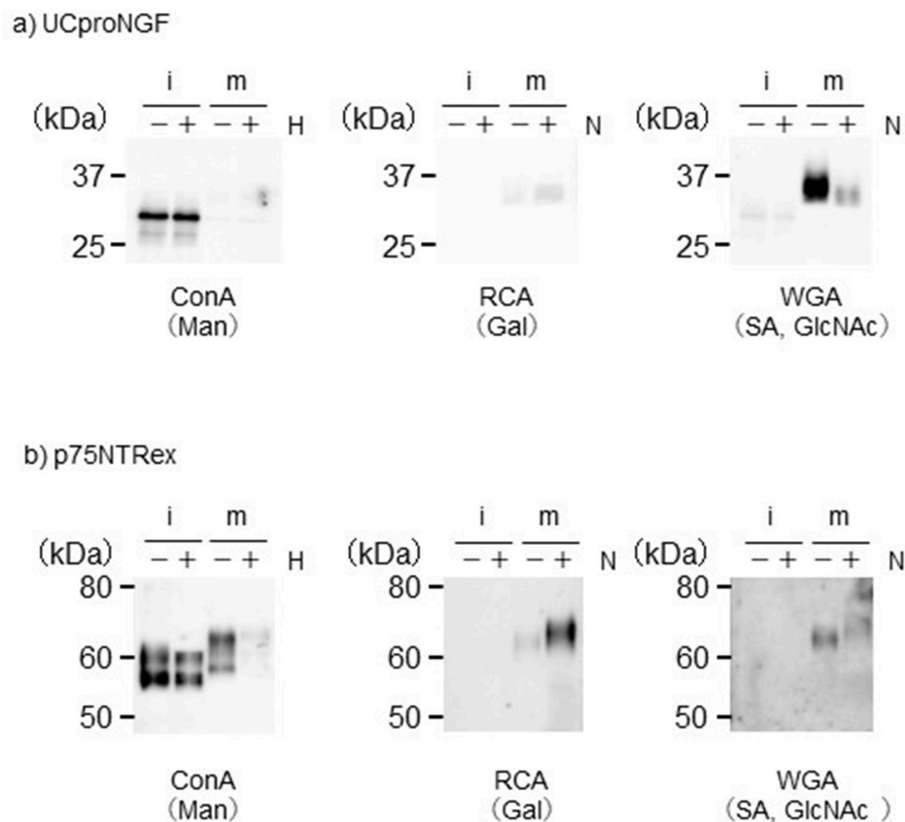

Fig. 5. Lectin binding to UCproNGF (a) and p75NTRex (b).
UCproNGF and p75NTRex expressed in insect (i) or mammalian (m) cells were treated with EndoH (H) or neuraminidase (N), and their binding to lectins was analyzed after SDS-PAGE and blotting to a PVDF membrane. The specificity of lectins against sugars is indicated in parentheses. Man: mannose, Gal: galactose, SA: sialic acid, GlcNAc: *N*-acetyl glucosamine.

site, Asn71, although this glycosylation site is not conserved in mouse or human p75NTR. According to a crystal structural study, Asn71 of p75NTR was in the vicinity of the binding site to NGF [16] or NT3 [17]. Therefore, the difference in the glycoform on Asn71 might influence the binding affinity of p75NTR. Our result that the insect proteins had high affinity suggests that small paucimannose-type glycans might be beneficial for obtaining a preferable conformation for binding. Such impact of the glycan moiety on the binding affinity has been reported in other systems, for example, the binding of IgG-Fc and Fc-gamma-R III [38].

The glycans on proNGF also affected its binding affinity to p75NTR. According to a crystallographic study, the prodomain of proNGF was unstructured and was not tightly associated with the mature NGF domain [18]. However, proNGF was shown to have a higher affinity toward p75NTR than mature NGF [6,18], which suggests the glycan in the prodomain of proNGF might have some effect on its affinity toward p75NTR.

This study showed that the affinity between p75NTR and proNGF depended on their expression systems which could cause some variation in the expressed proteins. We showed differences in the glycoform of these proteins from various expression systems and such differences in the glycoform likely affected their binding affinity. Other than glycans, there might be differences in the three-dimensional structure of the recombinant proteins depending on the expression systems, and it might affect the affinity. The structural changes of glycans on proteins could happen during neuronal development or depending on the physiological states of cells. If such changes could happen in p75NTR or proNGF, it would affect their interaction and modulate their signaling, which would influence the physiological states or the fate of neurons.

5. Conclusion

Recombinant proNGFs and p75NTRs were expressed in bacterial, insect, and mammalian cells, and the affinity between them in various combination was examined. The affinity was quite different depending on the expression systems. These differences in the affinity depend likely on the glycoforms of the recombinant proteins.

CRediT authorship contribution statement

Mami Hino: Methodology, Investigation, Formal analysis. **Masayuki Nakanishi:** Data curation, Methodology, Validation. **Hiroshi Nomoto:** Writing – original draft, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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