Molecular and Structural Insight into proNGF Engagement of p75NTR and Sortilin

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Abstract

Nerve growth factor is initially synthesized as a precursor, proNGF, which is cleaved to release its C-terminal mature form. Recent studies suggest that proNGF is not an inactive precursor, but acts as a signaling ligand distinct from its mature counterpart. Pro- and mature NGF initiate opposing biological responses by utilizing both distinct and shared receptor components. Here, we carry out a structural and biochemical characterization of proNGF interactions with p75NTR and sortilin. We have crystallized proNGF complexed to p75NTR, and present the structure at 3.75 Å resolution. The structure reveals a 2:2 symmetric binding mode, as compared to the asymmetric structure of a previously reported crystal structure of mature NGF complexed to p75NTR, and the 2:2 symmetric complex of Neurotrophin-3 and p75NTR. Here we discuss the possible origins and implications of the different stoichiometries. In the proNGF/p75NTR complex, the pro regions of proNGF are mostly disordered, and two hairpin loops (L2) at the top of NGF dimer have undergone conformational changes in comparison to mature neurotrophin structures, suggesting possible interactions with the pro-peptide. We further explore the binding characteristics of proNGF to sortilin using surface plasmon resonance and cell-based assays, and determine that calcium ions promote the formation of a stable ternary complex of proNGF/sortilin/p75NTR. These results, together with previous structural and mechanistic studies of neurotrophin-receptor interactions, suggest the potential for distinct signaling activities through p75NTR mediated by different neurotrophin-induced conformational changes.

Keywords

Nerve Growth Factor; receptor; structure; signaling; prohormone

Introduction

Nerve growth factor (NGF) was initially identified for its survival and growth-promoting characteristics on neurons.^{1; 2} Like many other growth factors, NGF is synthesized as an

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precursor that can be proteolytically cleaved intracellularly by furin and other proconvertases, to release its C-terminal mature form,³ which was thought until recently to be the only biologically active form. Due to their apparently transient existence, the pro-peptides of NGF and other neurotrophins (NTs) have been thought to function only to promote protein folding and regulate neurotrophin secretion.^{4; 5} However, recent studies demonstrate that proNGF can be released by cells and is a specific signaling ligand to mediate cellular apoptosis.⁶ This was a surprising finding not only because the unprocessed form is biologically active ligand in its own right, but because the mature and pro-forms of NGF execute opposing actions.

Subsequent studies demonstrated that pro- and mature NGF mediate their functions via binding to distinct receptor complexes. NGF binds to two classes of transmembrane receptor: TrkA, a Tyrosine Kinase receptors superfamily member⁷ and p75NTR, a Tumor Necrosis Factor (TNF) receptor family member.⁸ All NTs bind to p75NTR while each NT preferentially interacts with a different Trk receptor subtype (TrkA, B, or C). Although p75NTR can respond to mature neurotrophins independently, Trk receptors are the key mediators of the trophic function, while p75NTR appears to enhance the binding affinity and specificity of Trk receptors for neurotrophins, as well as mediate apoptotic functions.^{9; 10} With regards to structural studies, NGF crystal structures have been available in its free form,¹¹ in complex with p75NTR,¹² and in complex with TrkA.^{13; 14}

In contrast to NGF, both in vitro and in vivo studies demonstrate that proNGF preferentially interacts with p75NTR, but not TrkA, and plays an important role in cell death induction.^{6; 15} Increased proNGF is observed in pathological conditions resulting in neuronal cell death, such as seizures, spinal cord injury (SCI) and the Alzheimer's disease.^{16; 17; 18; 19} Delivery of antibodies to proNGF, chemicals preventing proNGF binding to p75NTR, or drugs inhibiting proNGF synthesis significantly attenuates neuronal death.^{17; 19; 20} In addition, sortilin, a member of a novel Vps10p-domain receptor family, was identified as a co-receptor in a proNGF-induced apoptosis complex.¹⁵ Sortilin is an important component, as demonstrated by the reduction in proNGF-induced retinal ganglion cell death in sortilin-deficient embryos,²¹ and its co-expression with p75NTR in neurons undergoing selective elimination.^{22; 23; 24; 25} These functional studies, together with crosslinking analysis, indicate that proNGF simultaneously binds sortilin and p75NTR, creating a ternary complex that activates neuronal apoptosis.¹⁵ Therefore, the choice between life and death of neurons may be a delicate balance between cleavage or non-cleavage of proNGF, and expression of TrkA or p75NTR/sortilin receptor complexes on the cell surface.

The molecular basis underlying the signaling by the proNGF-mediated death complex largely remains elusive, but it is known that the pro- and mature domains of proNGF bind to sortilin and p75NTR, respectively.¹⁵ Two crystal structures available for mature NTs (NGF and NT-3) complexed to p75NTR have shown asymmetric (2:1) and symmetric (2:2) binding stoichiometries with p75NTR.^{12; 26} Unlike the ligand-induced dimerization mechanism for NGF activation of TrkA through 2:2 receptor complexes,^{13; 14} a ligand-induced conformational change in a preformed p75NTR dimer is the likely activation mechanism,²⁷ raising the question of the implications of the 2:1 NGF/p75NTR versus the 2:2 NT-3/p75NTR complexes. For proNGF, crystallographic analysis of the pro-peptide domain has been hindered because of its intrinsic flexibility and susceptibility to cleavage. Information to date using *E.coli*-derived proNGF indicates a flexible structure, with a possible intramolecular interaction of the NGF pro-peptide domain with the mature region,²⁸ and a crab-like overall shape predicted by a combination of antibody epitope-mapping and Small-Angle X-ray Scattering (SAXS).²⁹ Despite the significant advance of a crystal structure of sortilin with the neuropeptide neurotensin,³⁰ no information is available on how sortilin interacts with proNGF. The aim of the present study is to characterize biochemically and structurally proNGF in complex with p75NTR and in the full ternary complex with sortilin, to gain insight into the

docking geometry and stoichiometry of the complex, and to begin to reconcile recent structural data on the contrasting stoichiometries of the NT-3/p75NTR and NGF/p75NTR complexes. Our structural, biochemical and cell-based studies thus improve the current understanding of the molecular mechanisms by which proNGF and mature neurotrophins interact with their receptors.

Results

Properties of cleavage-resistant triple-mutant proNGF

ProNGF can undergo post-translational cleavage *in vivo* and *in vitro* to yield several products, making it difficult to obtain sufficient quantities of stable and biologically active proNGF for structural and cellular studies. Thus, most studies on proNGF structure have used material refolded from *E. coli*, despite being substantially less potent compared to that secreted in active form from a eukaryotic source. Insect cell-derived proNGF, in which one or more of the potential dibasic amino acid cleavage consensus sites have been mutated, does not require refolding and is biologically active³¹. To obtain sufficient quantities of recombinant proNGF for crystallographic analysis, we performed alanine mutagenesis for three processing sites of mouse proNGF and expressed this material in insect cells (Figure 1A).

Compared to a single processing site mutant proNGF (mutated amino acids -1, -2) (data not shown), triple-site mutant proNGF (mutated amino acids -1, -2, -40, -41, -70, -71; Figure 1A) was more homogenous and stable judged by chromatography and SDS-PAGE (Figure 2A, 2B), in accordance with a previous study.³¹ N-terminal sequencing showed that the processing site after the signal peptide was Ala -104, indicating the presence of an intact pro region. As the predicted size of non-glycosylated proNGF is 25 kDa, it is likely that the protein produced in insect cells, which migrates at approximately 32 kDa, is glycosylated, however the very basic nature of proNGF may be another factor in its slower migration in SDS-PAGE. The insect-derived proNGF eluted as a homodimer (~60 kDa) by size exclusion chromatography (Figure 2A), in agreement with the fact that mature NGF is also a dimer.

To determine whether the introduction of point mutations to enhance protein stability altered bioactivity, apoptosis assays were undertaken with triple-site mutant proNGF. Previous studies using a triple-site mutant proNGF, purified with a different technique, indicated that concentrations of 2–10 nM were required to induce death of cultured cells lines (Schwannoma or PC12 cells),³¹ whereas single-site mutant proNGF is effective in inducing apoptosis of sympathetic ganglion neurons (SCG) at subnanomolar concentrations.⁶ Using SCG neurons which have been well characterized as responding to proNGF in a p75NTR- and sortilin-dependent manner,^{15; 21} triple- and single-mutant proNGF were comparable in inducing cell death, with a greater than 50% enhancement following exposure to picomolar concentrations. NGF, as expected, does not kill, but in fact promotes survival (Supplementary Figure 1).

We next assessed binding of triple-mutant proNGF to cellular sortilin and p75NTR. Stable clones of human fibrosarcoma HT-1080 cells expressing p75NTR, sortilin, or both receptors were generated and the receptor expression was confirmed by Western blot analysis (Figure 1B). To examine uptake of proNGF, Alexa-594-conjugated triple-mutant proNGF was added to HT1080 cells expressing either p75NTR or sortilin, or both receptors (Figure 1C and D). Triple-mutant proNGF uptake in cells expressing sortilin alone or p75NTR alone was moderate and perinuclear or cytoplasmic in location, respectively. Uptake was significantly enhanced in cells co-expressing both p75NTR and sortilin, and little uptake was observed in wild-type cells expressing neither p75NTR nor sortilin. These results are consistent with a previous study using single mutant proNGF, and suggest that amino acid changes at positions -1, -2, -40, -41, -70, and -71 do not significantly impair binding to p75NTR, sortilin or co-receptor complexes.

ProNGF/p75NTR complex and crystallization

Several solution biochemical studies have characterized the interaction between proNGF and p75NTR. Through activity assays, Lee *et al.* demonstrated that proNGF generated from HEK-293 cells was active at subnanomolar concentrations on p75NTR expressing cells.⁶ In equilibrium binding studies with cells expressing p75NTR and sortilin, proNGF exhibited higher affinity binding (160 pM) to few sites, whereas mature NGF exhibited lower affinity binding (1 nM) to many sites, suggesting that the interactions of proNGF versus mature NGF with cell surface exposed p75NTR/sortilin are distinct. However, studies where surface plasmon resonance was employed, using surfaces on which only one receptor species was probed yielded different conclusions. Generally in these studies *E. coli* refolded proNGF showed lower affinity binding to p75NTR than mature NGF.^{15; 29} The affinity of mature NGF to p75NTR has also been reported over a broad range of K_D from ~1 nanomolar to hundreds of nanomolar depending on the assays employed.^{12; 26; 32; 33} As questions have also arisen regarding a potential role of p75NTR glycosylation in altering p75NTR:ligand interactions, here we utilized both glycosylated and non-glycosylated p75NTR. To generate the glycosylated ectodomain of wild type rat p75NTR from insect cells, tunicamycin treatment that was employed in a previous study¹² was omitted. In parallel, a mutant p75NTR was also generated where the single potential p75NTR glycosylation site at Asn32 was substituted with Asp. Wild-type and glycosylation site mutant p75NTR revealed similar migration behaviors on size exclusion chromatography, both alone and complexed with proNGF (Figure 2A). This is consistent with previous mutagenesis studies, where Asn32 to Asp mutant retained NGF binding activity comparable to wild-type p75NTR, and displayed normal transport to the cell surface.^{33; 34} Application of a mixture of proNGF saturated with p75NTR, either wild type or mutant, to size exclusion columns reproducibly resulted in three peaks comprising a minor higher order complex (11 ml), a major complex eluting at 13 ml (~100 kDa), and excess p75NTR at 16 ml (Figure 2A, B). In our prior study, we found that non-glycosylated p75NTR eluted from gel filtration column as a mixture of monomer and dimer in a concentration-dependent equilibrium. The Asn32 mutant, and the fully glycosylated p75NTR, exhibit less tendency to dimerize, although at very high protein concentrations a minor dimer peak can be detected.

The p75NTR we used for crystallization is the non-N-linked glycosylated form generated from the Asn32 to Asp p75NTR mutant. The role of N-linked glycosylation of p75NTR on the affinity of NT interactions has been discussed extensively,^{26; 35} and we will address this important issue later. Despite the previously suggested flexibility of the proNGF pro region,^{15; 28; 36} we were readily able to crystallize the major proNGF/p75NTR complex purified by gel filtration, and observed diffraction to 3.75 Å resolution (Table 1). Despite extensive optimization of both crystallization conditions and data collection, we were unable to improve resolution. Importantly, crystals of the proNGF/p75NTR complex harvested and run on SDS-Polyacrylamide gels demonstrate that the proNGF polypeptide is intact (Figure 2C). No detectable fragments are observed at 13 kDa, corresponding to mature NGF. We could not obtain crystals of proNGF alone.

Crystal structure of proNGF/p75NTR

The C-terminal mature region of proNGF and the p75NTR in the complex were located using the asymmetric structure of NGF/p75NTR (¹², PDB code: 1SG1). Following placement of one asymmetric complex model in the asymmetric unit, we independently located a second p75NTR in the complex using the coordinates of p75NTR alone extracted from the PDB file. Thus, there is one symmetric complex (i.e. 2 proNGF and 2 p75NTR) per asymmetric unit cell (discussed below) (Figure 2D–F). It is important that we independently located the second p75NTR, rather than enforcing the symmetric model through a symmetric molecular replacement model, such as NT-3/p75NTR. All p75NTR and NGF subunits had very clear

electron density, enabling us to refine the structure (Table 1) with confidence guided by the maps and the published higher resolution models for all the components (Figure 2D). While amino acid sequence assignments would not have been feasible at this resolution had these been unknown structures, the availability of the models and the use of omit maps in refinement allowed us to position side chain locations in the majority of the structure, although not to a high degree of atomic precision. Nevertheless, the resulting model allows us to make several important conclusions about the overall structure of the proNGF/p75NTR complex with respect to stoichiometry, locations of domains, and main chain position. Therefore we restrict our analysis to issues that are appropriate at this resolution.

With respect to the pro region, there are sparse fragments of electron density that were not assigned in the asymmetric unit, and no more than two p75NTR and two mature NGF can be located. Absence of significant electron density for the pro region of NGF, results in a large 'empty space' in the crystal lattice in the region that would be occupied by this domain (Figure 2D). The absence of a defined trace of the pro region is most likely due to its flexibility that has been suggested by previous solution-based studies.^{28; 29} Importantly, the structure has been refined to an R_{free} of 27.4% (Table 1) even with almost 30% of the model comprising the pro region (240 out of 800 residues) missing, which reinforces the conclusion that the pro domain contributes very little to crystal diffraction. The crystal packing in each direction is primarily mediated by the rigid p75NTR molecule, rather than by NGF, enabling proNGF to pack in such a manner that the flexible pro regions contribute minimally to crystal lattice contacts. (Figure 2E). Thus, in the crystal structure we essentially visualize a 'fluid' state of the pro regions that are connected to the stably packed p75NTR and NGF molecules, and binding to p75NTR does not induce a stable tertiary structure to the pro domain. However, crystal packing provides useful information about the location of the pro domain relative to mature NGF, as there are large channels between complexes in the lattice near the mature NGF lateral surface and topside, suggesting that the pro domains reside here (Figure 2D, 2E). The 'hole' in the lattice has a roughly oval-shape with dimensions of $61\text{\AA} \times 56\text{\AA} \times 82\text{\AA}$, which is sufficient space for the number of residues corresponding to two pro domains. The size of the holes suggests that a large portion of the pro domain flanks and shields the side of the NGF surface, reflecting the observation that mature NGF is not involved in crystal packing.

Symmetric binding mode

In the structure (Figure 2F), proNGF exists as a homodimer with the dimerization interface mediated by the mature NGF domain of proNGF, suggesting the mature domain is necessary and sufficient to induce proNGF dimerization. The crystal structure of mature NGF complexed to non-glycosylated p75NTR revealed a 2:1 binding stoichiometry between NGF and p75NTR (Figure 3A), wherein the binding of a p75NTR to one side of the NGF dimer appeared to result in crippling of the second symmetry-related p75NTR binding site, resulting in an 'asymmetric' binding mode.¹² In contrast, the proNGF/p75NTR structure has 2:2 symmetric binding stoichiometry. Identical to that seen in the NGF/p75NTR asymmetric structure, the p75NTR receptors bind along the two seams formed by the edges of the proNGF dimerization interface, aligning along the respective two-fold axes, and 'embracing' the proNGF dimer like brackets. Allowing for slight differences between mouse and human NGF sequences, the p75NTR contact interactions with NGF are well conserved between the two complex structures (Figure 3A).

Symmetric binding between neurotrophins and p75NTR has been supported by a solution-state study of an NGF/p75NTR complex,³⁵ by the crystal structure of an NT-3/p75NTR complex,²⁶ and by a recent study investigating the mechanism of p75NTR dimer signaling.²⁷ We decided to further show that the symmetric proNGF/p75NTR complex exists in solution using multi-angle light scattering, which measures molecular mass independent of the radius or shape

of a molecular species (Figure 4). We expected to observe dimeric NGF, dimeric proNGF, and monomeric p75NTR at 26.5, 60.8, and 20.6 kDa, and our measurements match very closely: 28.5, 62.1, 18.5 kDa (Figure 4A, 4B, 4C, respectively). 2:1 and 2:2 complexes of mature NGF bound to p75NTR have expected sizes of 45.0 and 63.4 kDa, respectively, and what we observe in this study is a broad peak indicative of a mixture of both species, which can be seen as the molar mass measurement spanning the range between the expected sizes, with the number-averaged molar mass calculated as 59.4 kDa (Figure 4D). This mass value indicates a higher proportion of 2:2 complexes than 2:1 under the conditions of this experiment. For proNGF/p75NTR complexes, 2:1 and 2:2 complexes would be 79.3 and 97.8 kDa, respectively, and we measure molecular species with a number-averaged molar mass of 88.3 kDa (Figure 4E), similar to the NGF/p75NTR result. The variation between the expected and observed molecular masses for the proNGF/p75NTR and NGF/p75NTR complexes suggest a dynamic equilibrium between interchanging 2:1 and 2:2 complexes. Collectively, these results are consistent with our, and others' observations of such symmetric 2:2 complexes in crystallographic and solution studies, and that an equilibrium of 2:1 and 2:2 solutions might exist for NT/p75NTR complexes.

Superposition of the NT-3/p75NTR structure onto the proNGF/p75NTR structure, in which the NT-3 dimer and the proNGF dimer were aligned, demonstrates a globally similar geometry (Figure 3B). Only minor conformational changes occur within individual domains of p75NTR, with root mean square deviations (r.m.s.d.) of 0.43 Å, 0.63 Å, 0.50 Å and 0.45 Å for CRD1 to CRD4, respectively. However, the r.m.s.d. for the whole p75NTR is 3.84 Å, indicating that interdomain movements occur. The displacements occur at the two ends of the p75NTR molecules, in the center the major binding sites I and II, formed by CRD2 and CRD3 remain consistent in all structures.^{12; 26} However, the extent of interactions between the p75NTR terminal CRD1 and CRD4 domains with neurotrophins vary in each structure; in particular, contact between p75NTR CRD4 and NT-3, not being seen in proNGF/p75NTR or NGF/p75NTR complex structures, likely reflects real differences between the NGF and NT-3 modes of interaction with p75NTR (Figure 3B). Also, interactions between p75NTR CRD1 and NGF in proNGF/p75NTR structure are not formed in the NT-3/p75NTR complex. Collectively, while the overall receptor-ligand contacts of proNGF, NGF, NT-3 and likely other NT's with p75NTR will be highly similar, there are small variations in contacts that are NT-dependent.

Given the above structural comparisons between NGF, proNGF and NT-3/p75NTR complexes, how do their respective p75NTR signaling activities compare? We evaluated the relative efficacy of NGF, proNGF and NT-3 as apoptotic ligands using primary SCG neurons, which express p75, sortilin and TrkA, but not TrkB or TrkC. NGF-deprived neurons were maintained in depolarizing media to detect the pro-apoptotic actions of mature neurotrophins, which activate p75NTR but not TrkA. This experimental paradigm has been utilized by others to investigate the downstream mechanisms of p75NTR activation following exposure to mature BDNF.^{37; 38} As shown in Figure 3C, proNGF elicits sympathetic neuron death and overcomes the survival promoting effects of membrane depolarization. In contrast to mature BDNF³⁷ and KKT (data not shown), which induces apoptosis, mature NT-3 at both low (5 ng/ml; data not shown) and high concentrations (100 ng/ml) failed to induce SCG death (Figure 3C). These cell-based results suggest that even though both NT-3 and proNGF bind symmetrically to a p75NTR dimer, differences in ligand:receptor interactions can result in distinct biological consequences and further suggests that p75NTR dimerization *per se* is not sufficient to activate pro-apoptotic signaling.

Open state of proNGF L2 loop

Superposition of the NGF dimer structure in the proNGF/p75NTR complex to the NGF dimer structure in mature NGF/p75NTR complex shows very little conformational variance, except for significant changes in the loops at the top of the NGF dimer formed by residues 41–49

(Figure 5A–E). This hairpin loop has been designated as loop 2 (L2) in previous studies.^{14;}
²⁶ In the mature NGF/p75NTR structure, the L2 loops bend toward the center of the NGF dimer interface, whereas in the proNGF/p75NTR structure, the L2 loops are pointing upward and away from the NGF dimer interface, which we refer as the ‘open’ state of L2 loops (Figure 5A, 5C). Although some side chain positions of the L2 loops cannot be built with confidence, the path of the main chain is observed without ambiguity in electron density maps (Figure 5D). Extensive interactions exist between the amino acids at positions 43–45 of the two L2 loops in the closed state. In contrast, the distance between Ile44 of the two L2 loops is up to 14 Å in the open state L2 loops in proNGF, preventing the L2 loops from interacting with each other (Figure 5D).

In both mature NT/p75NTR complex structures, despite the different stoichiometries, the L2 loops are in the closed form (Figure 3A, 3B, 4A, 4B). Comparison of L2 loops in several structures of mature NGF and other mature neurotrophins, demonstrates that, though flexible, they all adopt a closed state similar to mature NGF and NT-3 (Figure 5D).^{11; 12; 13; 14; 26; 39} We propose that open state of the L2 loops in the proNGF/p75NTR structure is a consequence of local effects of the pro domain. We designate the wider end of the NGF dimer, and CRD 1 and 2 of p75NTR as the ‘top’ of the proNGF/p75NTR complex, and the tapered end and CRDs 3 and 4 of p75NTR as the ‘bottom’ of the complex (Figure 3A).

ProNGF pro-domain and the L2 loops

The highly unstructured nature of the pro domain of proNGF prevents us from unambiguously concluding how the pro domains could effect the movement of the L2 loops from a closed to open state. However we can speculate on several possibilities based on collective structural information from this structure, and from previous biophysical and biochemical investigations of proNGF. One possibility is that the pro domains interact with the top region of the NGF dimer and L2 loops move to avoid steric clashes. In support of this, significant unaccounted-for electron density is located between of the two opened L2 loops (Figure 5C, E), suggesting that regions of two pro-peptides from both proNGF molecules may converge at the top of NGF dimer, and that the L2 loops are in an open state as a consequence of pro-peptides convergence at the top of NGF dimer, in effect serving as gates. This would be consistent with the overall shape information of proNGF obtained through a SAXS approach, predicting a crab-like model, wherein unstructured pro-peptide domains loosely flanked the side of the centrally located rigid mature NGF dimer.²⁹ Based on the lack of defined pro domain structure in the crystal structure, it seems clear that the pro domains are not tightly associated with the mature NGF domain, and that these crab-like appendages representing the pro regions could, like wings, converge over the top of the NGF molecule, displacing the L2 loops. Another explanation that we cannot exclude for the unaccounted-for density located between the two open L2 loops is conformational sampling of the L2 loop of both the open and closed forms in proNGF. However, such conformational flexibility was not observed in any of the NT structures.

The scenario proposed above has been substantiated by an earlier study in which tryptophan residues were observed to be in pro domain and mature domain contact regions.²⁸ That study pinpointed the residue Trp21 among all the tryptophan residues (Trp21, Trp76 and Trp99) in mature NGF to be at the interface between the pro and mature domains, since it was solvent accessible. However, Trp21 is involved in binding to both p75NTR and TrkA,^{11; 12; 13; 14} which casts doubt on the role of Trp21 in the pro domain/mature domain interface. In our proNGF/p75NTR structure, L2 loop movements would perturb the environment for Trp99 of both NGF molecules. In the L2 loop closed-state structure of mature NGF, Trp99 is buried underneath the two L2 loops (Figure 5A, 5B, 5E), whereas with L2 open in proNGF/p75NTR

structure, two Trp99 residues (Figure 5C, 5E) are exposed and are accessible to interact with regions of the pro domain that might be in the vicinity.

Other supportive evidence comes from mature NGF and proNGF binding studies with anti-NGF antibodies. The epitope of monoclonal anti-NGF antibody AD11 is the loop 2 region of mature NGF.⁴⁰ SPR experiments show that AD11 binds to mature NGF with very high affinity but with a thousand-fold lower affinity to proNGF.²⁹ Correspondingly, in vivo experiments also show AD11 efficiently neutralizes mature NGF activity without effects on cell death induced by proNGF. These observations can be explained by a conformational change and the binding of the pro domain at the L2 loop, as we suggest.

Interactions of proNGF with sortilin

Sortilin is a member of the family of mammalian Vps10p-domain type-1 receptors⁴¹ that regulates the intracellular trafficking of multiple ligands such as lipoprotein lipase,⁴² apolipoprotein AV,⁴³ neurotensin,⁴⁴ receptor associated protein (RAP),⁴¹ and proneurotrophins.^{15; 45} Sortilin is indispensable for proNGF-initiated cell death in several classes of neurons. The crystal structure of sortilin has been recently solved in complex with neurotensin, but less is known about how sortilin interacts with proNGF,³⁰ although prior studies indicate that the pro domain binds with higher affinity than the mature domain of NGF to immobilized sortilin.¹⁵

To further probe sortilin and proNGF interactions, the sortilin extracellular region was expressed in HEK-293 cells, and directionally immobilized at the C terminus, via an engineered C-terminal peptide that serves as a substrate for biotin ligase, on a Streptavidin biosensor chip for Surface Plasmon Resonance (SPR) experiments. Directional coupling is the preferred way to carry out SPR measurements compared to random chemical crosslinking on a CM5 chip, since it reflects how a receptor is presented on the cell. Random chemical coupling usually destroys a substantial proportion of the immobilized protein's activity since any Lysine residues in the active or binding regions will be covalently modified. Additionally, the sortilin N-terminal propeller domain is the pro-peptide binding region of the protein, so C-terminal immobilization preserves the binding activity of the molecule and presents the binding domain freely accessible. Potential interacting partners, including mature NGF, proNGF, proNGF/p75NTR complex, and p75NTR, were applied at a series of concentrations. Sortilin binds to mature NGF with weak affinity ($K_d = 8 \mu\text{M}$) characterized with fast on- and fast off-rates (Figure 6C). In comparison, the affinity of proNGF for sortilin was about 10 times higher ($K_d = 770 \text{ nM}$) resulting from a much slower dissociation rate (Figure 6A), suggesting that the pro domain is responsible for the higher affinity binding between proNGF and sortilin. Moreover, the affinity of the proNGF/p75NTR complex for sortilin is five times higher ($K_d = 140 \text{ nM}$) than that of proNGF alone, indicating a cooperative or composite binding interaction in the ternary complex (Figure 6E). To determine whether this increased affinity resulted from a summation of interactions of both proNGF and p75NTR with sortilin, we tested for an interaction between p75NTR alone and sortilin, and observed none (Supplementary Figure 3A, 3B), strongly suggesting a synergistic effect on proNGF binding to sortilin and p75NTR simultaneously.

Prior studies with sortilin indicate that neurotensin is a competitive antagonist of proNGF, albeit at micromolar concentrations.¹⁵ To probe the specificity of interaction between proNGF or proNGF/p75NTR and sortilin, neurotensin was added to the mobile phase along with the ligand in the SPR experiment. The binding of proNGF and proNGF/p75NTR to sortilin were both inhibited by neurotensin (Figure 6B, 6F). With titration of neurotensin concentrations, the response generated by proNGF and proNGF/p75NTR binding to sortilin diminished, and was almost completely inhibited at $10 \mu\text{M}$ neurotensin. These results are consistent with cell-based studies, in which $10 \mu\text{M}$ neurotensin is sufficient to abrogate neuronal apoptosis.¹⁵ In

contrast, NGF binding to sortilin was not affected by neurotensin (Figure 6D), with minor changes in response units only reflecting the increasing amounts of neurotensin bound. Similarly, no significant effect of neurotensin was observed in experiments testing for an affinity between p75NTR and sortilin (Figure 6F). These results suggest that a specific region of sortilin, likely the N-terminal propeller domain, binds to the pro domain of proNGF to mediate the high affinity binding, whereas mature domain of proNGF interacts with sortilin more in a 'bystander' fashion.

To determine whether proNGF forms a stable ternary solution complex with sortilin and p75NTR, we mixed recombinant proNGF, sortilin and p75NTR and then subjected the mixture to size-exclusion chromatography. However, this did not result in an appreciable formation of a new species indicative of a ternary complex, but rather a simple summation of the profiles of individual components. This result is in accord with the SPR result in which proNGF interacts with sortilin and p75NTR in an affinity range 0.1~1 μ M, which is at the limit of binding strength to yield stable complexes by size-exclusion chromatography. However, it was surprising to find that in the presence of a physiological concentration of calcium ions (1 mM), a high molecular weight assembly (~500 kDa) was observed (Figure 7A). Three components, sortilin (molecular weight 75 kDa), proNGF (27 kDa) and p75NTR (18 kDa), are present within the same peak as demonstrated by SDS-PAGE, suggesting a complicated binding stoichiometry. To test the stability and calcium dependence of higher molecular complexes, the ~500 kDa assembly fractions were re-applied in parallel to size-exclusion columns in the presence or absence of calcium ions. In the presence of 1 mM calcium, the ternary complex at ~500 kDa was retained (Figure 7B). In contrast, when calcium was omitted, size-exclusion chromatography resulted in a broad peak, comparable to simple summation of sortilin and proNGF/p75NTR complex eluting peaks (Figure 7C), strongly suggesting that the high order ternary complex is calcium dependent. Addition of calcium ions did not alter the chromatography patterns of individual components or the proNGF/p75NTR complex, suggesting that calcium altered the interaction between the pro domain and sortilin (Supplementary Figure 3C).

To assess the calcium sensitivity of interactions of proNGF with sortilin in cell-based assays, we co-expressed proNGF and sortilin in heterologous cells, and immunoprecipitated sortilin from cell lysates in calcium-supplemented or calcium-depleted conditions followed by Western blot analysis (Figure 7D–F). The interaction between proNGF and sortilin in the cell lysates is moderately increased when calcium is supplemented throughout the immunoprecipitation conditions, as compared to conditions that include EDTA to chelate calcium (Figure 7D, lane 2 compare with lane 1). Quantification of Western blots, corrected for the level of sortilin immunoprecipitation, indicated a 2.3-fold increase in proNGF:sortilin interaction with calcium supplementation (Figure 7D). To evaluate the effects of calcium in stabilizing the interaction of proNGF and the shed ectodomain of sortilin in these cell cultures, media was collected, and immunoprecipitation was performed with calcium supplementation of wash conditions, or with calcium chelation. Again, depletion of calcium abolishes the association of proNGF and the ectodomain of sortilin that are co-secreted by cells (Figure 7E, lane 1). To determine whether calcium stabilizes the interaction of proNGF with p75NTR and sortilin present on the cell surface, HT-1080 cells stably expressing p75NTR and sortilin were treated with recombinant proNGF at 37°C to permit binding and internalization. Cell lysates were then immunoprecipitated with a p75NTR antibody in calcium-supplemented or depleted conditions (Figure 7F). As demonstrated by immunoblotting with proNGF antibodies, the level of cell-associated proNGF is equivalent in total cell lysates (Figure 7F, input). However, the interaction of p75NTR with sortilin and proNGF are significantly reduced when calcium is depleted (Figure 7F, lane 1). Thus, these results suggest that the proNGF-sortilin interaction is regulated by calcium concentration, and calcium stabilizes proNGF-sortilin-p75NTR complexes in cells.

Discussion

The purpose of this study is to extend our understanding of the molecular basis of proNGF as a ligand for p75NTR and sortilin, and the relationship of the proNGF interaction with p75NTR compared to mature neurotrophins. The two available crystal structures for mature neurotrophins bound to p75NTR have different binding stoichiometries, with mature NGF bound to one p75NTR ectodomain (2:1), and mature NT-3 bound to two p75NTR ectodomains (2:2).^{15; 26} Here we describe a proNGF dimer bound to two p75NTR ectodomains (2:2), and examine both technical aspects and the physiological relevance of the different complex stoichiometries.

From a technical standpoint, the symmetrical mode of the NT-3/p75NTR complex has led to statements that mature NTs bind *only* in a symmetrical mode to p75NTR,^{26; 27; 35} similar to the 2:2 binding observed with Trk receptors.^{13; 14} The asymmetric binding mode in the crystal of mature NGF complexed with p75NTR has been attributed to the use of non-glycosylated p75NTR. Deglycosylation is a common, often essential strategy to enable crystallization of glycosylated proteins, but in this case, the 2:1 stoichiometry is suggested as an artifact of deglycosylation, lowering the affinity of NGF for p75NTR, and resulting in incomplete occupancy of the complex. A prior mutagenesis study of p75NTR documented similar binding affinity of a p75NTR Asn32 to Asp glycosylation mutant as the wild type p75NTR, indicating that deglycosylated p75NTR would be appropriate for structural studies.³³ Structurally, neither complex structure reveals a mechanism for how the Asn-linked glycan could influence stoichiometry, since neither neurotrophin is in contact with this glycan, and there do not appear to be major structural differences between glycosylated and non-glycosylated p75NTR. Importantly, the symmetric complex (2:2) of proNGF with non-glycosylated p75NTR reported here demonstrates that *both* glycosylated and glycan-free p75NTR can form symmetric and asymmetric complexes (Figure 2A). Thus, the role of glycans in p75NTR binding to mature neurotrophins remains unclear.

An important difference between the two complexes is the use of different neurotrophins. We show here that, despite both proNGF and NT-3 forming similar symmetric complexes, only proNGF effectively induces apoptosis, demonstrating that dimerization alone is not sufficient for p75NTR signaling. In addition, NT-3 binds to p75NTR in neurons in the picomolar range, a higher affinity than the nanomolar range observed for NGF, likely due to the slower dissociation rate of NT-3, as compared to NGF for p75NTR.^{32; 46} Previous studies demonstrate that p75NTR expression enhances the affinity of TrkA for NGF; in contrast, p75NTR reduces the affinity of TrkA for NT-3. It is also important to note that while NT-3 can activate TrkA in sympathetic neurons, NT-3 does not confer survival-promoting actions on these cells. Instead, NT-3 acts as a neurotrophic factor for nascent axons.⁴⁷ Therefore, our finding that NT-3 does not induce SCG neuron apoptosis suggests proNGF and mature NT-3 may have subtle unique receptor interactions that mediate distinct biological functions.

The most parsimonious explanation for the 2:1 NGF/p75NTR complex is that it is an intermediate in the path to, or from, a 2:2 complex, and that there exists a dynamic equilibrium of stoichiometries during receptor activation and/or ligand passing consistent with its fast on- and off-rate, as previously suggested.^{8; 48} The asymmetric structure has probably resulted from trapping of the 2:1 complex in a crystal lattice, from a solution of exchanging 2:1 and 2:2 complexes. The NT-3 complex, for reasons related to the presence of the glycan and NT-3 higher affinity, captures the more stable 2:2 complex in the crystal lattice.²⁶ A range of stoichiometries for p75NTR/NGF complexes has been proposed to exist in an equilibrium that reflects the local concentrations of NT's and relative expression levels of p75NTR and Trk receptors.⁸ While a symmetric complex was proposed in one solution study,³⁵ examination of that mass spectrometry data also indicated a heterogeneous collection of NGF/p75NTR

stoichiometries in solution. Ultimately, we do not know the true “cell surface” stoichiometry of full-length p75NTR/NT complexes. Unlike cytokine receptors, ligand induced dimerization may not be necessary for p75NTR signaling, since cell-surface p75NTR exists as a preformed dimer. Rather, conformational change of a p75NTR disulfide-linked dimer appears to be a trigger.²⁷ As such, at high NGF concentrations it is possible that each ‘arm’ (i.e. ectodomain) of the p75NTR dimer could be bound to a single NGF homodimer (2:1), and this could trigger a different ‘conformational change’ than a single NGF dimer binding p75NTR in the symmetric mode (2:2). Such a scenario could explain the different concentration-dependent effects of NGF.

Further, it is not known if a stepwise binding mechanism occurs during p75NTR engagement of NTs, mediated through sequential engagement of low- and high-affinity sites, as seen in the assembly of cytokine receptor complexes, where crystal structures of 1:1 and 2:1 complexes represent partial and complete signaling complexes, respectively. Thus, while the crystal structures of the asymmetric NGF/p75NTR complex, and the symmetric proNGF/p75NTR and NT-3/p75NTR complexes indicate important differences, the fact that p75NTR is not known to require ligand induced association for signaling suggests that it is premature to ascribe any complex form as an artifact, and that all could be relevant to different stages of p75NTR signaling and function.

Interaction of proNGF with sortilin

The receptor-ligand contacts we visualized in proNGF/p75NTR complex are well conserved and correspond to contacts in the mature NGF/p75NTR complex and NT-3/p75NTR complex, indicating the pro-peptide domain does not disturb the sites of ligand/p75NTR interaction. The mature NGF region of proNGF molecules is both necessary and sufficient to mediate binding to p75NTR. We found that presence or absence of the pro domain resulted in no significant conformational changes in p75NTR. Nevertheless, we found additional unaccounted electron densities, very likely caused by pro-peptides, in several regions close to the p75NTR surface (data not shown), likely indicating there were bystander interactions between pro-peptides and p75NTR. This result is in agreement with prior studies that suggest that the pro domain selectively interacts with sortilin.¹⁵ From these studies we expect that the pro-peptide would fold upon engagement with sortilin. Indeed, we observe a synergistic effect in proNGF binding to sortilin, when proNGF is in a preformed complex with p75NTR. It is possible that interaction between the pro domain and sortilin is facilitated when proNGF is in a 2:2 complex with p75NTR through a p75NTR-induced folding or stabilization of the pro domain. The current studies cannot distinguish whether each pro domain binds to one sortilin, or whether two pro domains bind to one sortilin. The neurotensin binding region of sortilin is inside a tunnel surrounded by a ten bladed -propeller. Thus we can envision a ‘sandwich’ scenario for the sortilin/proNGF/p75NTR ternary complex, in which two pro domains are flanking the mature domain of NGF, with the insertion of part of the pro domain into the tunnel of sortilin.

The observation that calcium ions stabilize the sortilin/proNGF/p75NTR ternary complex in vitro, and in cell-based assays (hereafter named Ca-ligation) was unexpected. The structural implications of Ca-ligation have been best resolved in Ca²⁺-dependent cytoplasmic enzymes,⁴⁹ or cadherin extracellular domains that respond to fluctuations in extracellular Ca²⁺ concentrations to elicit dynamic perturbations in protein structure, from a globular calcium-deficient moiety to a rigid, rod-like figure upon Ca-ligation.⁵⁰ Calcium binding pockets in cadherins are formed by acidic amino acid stretches, such as DAD or LDRE sequences.⁵¹ Although our studies cannot distinguish any Ca²⁺-binding pockets in the proNGF pro domain or sortilin, potential pockets are detectable in the pro domain (in box 1 and box 4 as described by Suter *et al.*),⁵² and potentially suitable acidic regions are exposed in the sortilin structure.³⁰ Future structure-function analysis will be needed to define how Ca-ligation stabilizes the

dissociation of proNGF from sortilin, and the physiological implications of Ca^{2+} -dependent stabilization of the proNGF/p75NTR/sortilin complex. Despite the well-characterized structural basis of cadherin-mediated adhesion, far less is known as to how these structures are regulated by changes in extracellular calcium in normal and pathophysiological states. However, there are dynamic fluxes of Ca^{2+} in endosomal compartments, and therefore calcium may regulate stabilization of proNGF/p75NTR/sortilin ternary complexes following receptor internalization, as our cell-based studies suggest; such events could provide a novel mechanism to potentiate, or attenuate receptor signaling.

Materials and Methods

Production of recombinant p75NTR and proNGF

cDNAs encoding mouse pre-proNGF and rat p75NTR were used as templates for PCR. proNGF constructs cover residues -121 to 118. p75NTR constructs cover residues 29 to 190, corresponding to the extracellular portion. ProNGF and p75NTR were both fused with C-terminal hexahistidine tags and cloned into the pAcGP67A baculovirus transfer vector (PharMingen, San Diego, CA). All mutations were introduced using PCR-based mutagenesis, and all furin site mutations were done to alanine. Constructs were used to produce recombinant baculovirus via recombination with the baculovirus genome (Sapphire Baculovirus DNA; Orbigen, San Diego, CA) after co-transfection into Sf9 insect cells using Cellfectin (Invitrogen, Carlsbad, CA). To produce recombinant protein (proNGF or p75NTR), High Five cells (Invitrogen, Carlsbad, CA) cultured in Insect-Xpress media (Cambrex, Charles City, IA) were incubated for 72 hours with the recombinant virus, and the secreted protein was purified from the supernatant with Ni-NTA beads (Qiagen, Valencia, CA). Proteins were eluted from the Ni-NTA beads with HBS-Imidazole (10 mM HEPES, pH 7.2, 150 mM NaCl, 0.02% NaN_3 , 200 mM Imidazole). Proteins were purified by size-exclusion chromatography with a Superdex 200 gel filtration column (GE Healthcare, Piscataway, NJ) equilibrated with HBS (10 mM HEPES, pH 7.2, 150 mM NaCl). proNGF and p75NTR were separately purified and protein concentrations were determined by absorption at 280 nm. The two proteins were then mixed in equal molar amounts and immediately loaded onto a Superdex 200 column for gel filtration purification of the complex.

Crystallography

Crystals of the proNGF/p75NTR complex were grown in sitting drops in 0.1 M sodium acetate, pH 4.6, 16% polyethylene glycol 4000 at 22°C. Diffraction data was collected at the Advanced Light Source beamline 8.2.1 (Berkeley, CA), and data was indexed and scaled with HKL2000⁵³. PHASER⁵⁴ was used to place p75NTR and NGF by molecular replacement. CNS and PHENIX were used for refinement of the model,^{55; 56} and inspection of electron density maps and model building were performed with Coot.⁵⁷ High-resolution limit to the data was determined by plotting σ_A vs. resolution using SIGMAA in the CCP4 suite.⁵⁸ A significant drop in σ_A values was observed beyond 3.75 Å, indicating data to a high resolution limit of 3.75 Å could be used for refinement.⁵⁹ Tight NCS restraints were applied throughout the model refinement process, except for residues 143–147 of p75NTR and terminal residues, where significant differences were observed between NCS-related molecules. Geometry was followed with PROCHECK and MOLPROBITY,^{60; 61} and was used to guide the refinement process. The only residues that were outliers in the Ramachandran analysis were two residues of the L2 loop, for which we had no prior structural model due to the conformational change observed. Data and refinement statistics are listed in Table 1.

Expression and purification of sortilin

We subcloned the human sortilin extracellular domain residues 1–725 into the pVLAD6 BacMam vector.⁶² The pVLAD6-sortilin construct was co-transfected into Sf9 cells with

linearized baculovirus DNA using Cellfectin according to the manufacturer's recommendations. The primary virus was harvested after 4 days and used for further amplification. To produce sortilin, the appropriate volume of virus was added to suspension HEK-293 cell culture when the cells reached a density of 2×10^6 cells/ml. Sodium butyrate was added to a final concentration of 10 mM and the cells were left shaking for 72 h at 37°C. Sortilin was extracted by Ni-NTA beads and then sequentially purified by anion exchange and size-exclusion chromatography.

Multi-Angle Light Scattering

Refolded NGF was a gift from Genentech; p75NTR and proNGF were produced as explained above. Measurements were done using a DAWN EOS multi-angle light scattering detector and Optilab DSP Refractometer (Wyatt Technology Corporation, Santa Barbara, CA) downstream of an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA) running a Superdex 200 gel filtration column at 0.35 ml/min. The running buffer was HBS. The data was analysed with ASTRA version 5.3.2 (Wyatt Technology Corporation). dn/dc values used in the mass calculations for non-glycosylated proteins (p75NTR and NGF) were 0.185, a value well-established for pure protein samples. For proNGF and proNGF/p75NTR complexes, we used 0.181 and 0.183, respectively, to reflect the sugar content by weight-averaging known sugar and protein dn/dc values.

Surface Plasmon Resonance

Affinity measurements were performed in HBS-P buffer with a Biacore T100 (Biacore AB, Uppsala, Sweden). Recombinant sortilin with genetically engineered C-terminal biotinylation peptide acceptor site (Avidity, Denver, CO) were produced in HEK-293 cells and isolated and purified as described above for sortilin. Biotinylation of sortilin was performed according to the manufacturer's protocol (Avidity), and biotinylated sortilin was subsequently purified with gel filtration. Five hundred response units of each receptor were immobilized onto the surface of a streptavidin-coated chip (SA chip, Biacore). To conduct equilibrium-binding measurements, sortilin was injected over flow cells at 25°C, the first an empty reference. The measurements were conducted over a range of ligand concentrations (Figure 6), and the amount of binding was calculated as the difference in the response at equilibrium in the sortilin and control flow cells. Equilibrium K_d values were determined assuming a 1:1 interaction model.

Primary cultures and assessment of apoptosis

Dissociated superior cervical ganglion (SCG) neurons were prepared from postnatal day 0–1 (P0–1) rats. Neurons were plated on collagen-coated Permanox slides and maintained for 9 days in NGF as described previously.^{15; 37} On the day of the experiment, replicate cultures were rinsed six times with NGF-free medium and treated with the indicated ligands. After 36 hr, SCG cultures were counterstained with anti-neuronal-specific α -tubulin (Tuj1; Covance, Berkeley, CA) and 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Neurons were scored for condensed/fragmented nuclei under each treatment condition. At least 200 cells were counted for each culture condition in a blinded manner.

Alexa594 labeling of proNGF

20 μ g of purified proNGF was added to 10 μ l of PBS and 7 μ l of reconstituted Alexa594 (according to product protocol, Molecular Probes) and incubated at room temperature, in the dark, for 15 minutes. 50 mM Tris pH 8.0 was added to the mixture to stop the labeling reaction. Labeled proNGF was then dialyzed against PBS (Phosphate-Buffered Saline) at 4°C in the dark. Labeled proNGF was diluted in serum free media, and added to HT-1080 cells that were cultured in 8-chamber slides. Cells were incubated with labeled proNGF overnight, briefly rinsed with PBS, fixed with 4% paraformaldehyde for 10 minutes, rinsed 3 times with PBS,

and then counterstained with DAPI for 3 minutes. Cells were analyzed for uptake of Alexa594-conjugated proNGF using fluorescence microscopy, and intensity per cell area was quantitated by investigators blinded to condition using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at <http://rsb.info.nih.gov/nih-image/>).

Ca²⁺-dependent proNGF/sortilin interaction

HEK-293 cells were co-transfected with C-terminal myc-tagged sortilin and N-terminal HA tagged-proNGF plasmids and maintained in Opti-MEM I media (Invitrogen) for 44 hours post-transfection. Cells were then harvested in Ca²⁺-supplemented (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40 and 2 mM CaCl₂) or Ca²⁺-depleted lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40 and 2 mM EDTA) with protease inhibitors, and cell lysates (0.3 mg) were immunoprecipitated with anti-Myc affinity matrix (Santa Cruz Biotechnology). To detect sortilin-proNGF complexes in the cellular media, culture media (Opti-MEM I; 1.5 ml) was harvested, cleared by centrifugation, and immunoprecipitated with a biotinylated sortilin antibody (BAF2934, R&D Systems) followed by streptavidin pull-down in Ca²⁺-supplemented (with 2 mM CaCl₂ and protease inhibitors) or Ca²⁺-depleted conditions (with 2 mM EDTA and protease inhibitors). Immunocomplexes from cell lysates and media were then washed 5 times with Ca²⁺-supplemented or Ca²⁺-depleted lysis buffer, eluted in 0.1 M glycine, pH 2.5 and subjected to Western blot analysis. To detect complexes of proNGF, p75NTR and sortilin upon addition of recombinant proNGF, HT-1080 cells stably expressing p75NTR and sortilin were treated with 500 ng/ml of recombinant proNGF for 1 hour at 37°C in Ca²⁺-containing serum-free DMEM media and lysed in Ca²⁺-supplemented or Ca²⁺-depleted lysis buffer as described above. Cell lysates (1 mg) were immunoprecipitated with p75NTR antibody (9993),⁶³ washed 5 times with the same lysis buffer, and immune complexes were isolated by elution with 0.1 M glycine, pH 2.5 followed by Western blot analysis using anti-sortilin (BD Bioscience), anti-proNGF (413; detects pro domain),¹⁷ or anti-p75NTR (9993). To determine the relative sortilin-proNGF interaction in calcium-supplemented, or EDTA-containing conditions, proNGF bands co-precipitated with sortilin were normalized by the band intensity of sortilin in the immunoprecipitates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Characterization of recombinant proNGF activity

(A). Schematic diagram of proNGF primary structure. Signal peptide was colored in green, pro-peptide was colored in white and mature NGF was colored in gray. Three furin cleavage sites located at positions -1, -2, -40, -41, -70, and -71 were mutated to alanine residues. There are two predicted N-glycosylation sites located in pro-peptide domain positions -8 and -53. (B). Western blot analysis of receptor expression in HT1080 cell line that was transfected with p75NTR, or sortilin or both, with wild type HT1080 cells as control. (C). Uptake of Alexa-conjugated proNGF triple mutant by HT1080 cells expressing different receptors: (1) none (WT), (2) sortilin, (3) p75NTR and (4) p75NTR/sortilin. (D). Quantitation of uptake of proNGF triple mutant. Results are representative of five independent experiments.

Figure 2. Biochemical and crystallographic analysis of proNGF/p75NTR complex

(A) Chromatographic profiles on a Superdex 200 HR 10/30 size-exclusion column. Curves were shown in different colors: Blue dashed line for p75NTR Asn32 mutant; Orange dashed line for proNGF; Magenta dashed line for proNGF + p75NTR wild type; Black solid line for proNGF + p75NTR Asn32 mutant. The column molecular weight calibration with globular protein standards is shown at the top. (B). SDS-PAGE gel corresponding to proNGF/p75NTRmut (Asn32 mutant) purification (black solid curve in panel (A)). Molecular weight standards are shown at the left side. Lanes are labeled as eluting volume from gel filtration column. ProNGF migrated at ~32 kDa and p75NTR migrated at ~20 kDa, as indicated by arrows at the right side of gel. (C). Crystal gel for proNGF/p75NTR crystal. Samples were the starting material used for crystallization, and then only from washed crystals. Crystals were washed by reservoir solution several times to eliminate carry over of starting material. ProNGF appears at ~32 kDa, indicating it is in an intact state in crystal. (D). Composite omit electron density in the asymmetric unit. Map is shown at contour level 1.2 σ . All molecules in the asymmetric unit are shown in ribbon representation. Mature NGF is shown in green and p75NTR molecules are shown in magenta. Symmetry related molecules are grey. All structural

representations in this work were generated with PyMOL (DeLano Scientific LLC). **(E)**. Molecular packing in the crystal lattice is mediated by p75NTR. Mature NGF is in green and p75NTR is in magenta. Crystal lattice in other directions is shown in Supplementary Figure 2. **(F)**. Crystal structure of proNGF/p75NTR complex. Mature regions of the two proNGF molecules are colored in green and the two p75NTR molecules are colored in magenta. N and C terminus of mature NGF in proNGF were labeled as green.

Figure 3. Symmetric and asymmetric binding in neurotrophin complexes with p75NTR
(A). Superposition of proNGF/p75NTR structure with mature NGF/p75NTR structure (PDB code: 1SG1) using mature NGF as the superposition template. Both structures are represented as ribbons. For proNGF/p75NTR complex, mature NGF is green and p75NTR is magenta. For NGF/p75NTR structure, NGF is yellow and p75NTR is cyan. L2 loops of one NGF molecule in each structure are indicated. The inset shows the asymmetric NGF/p75NTR structure. **(B).** Superposition of proNGF/p75NTR structure and mature NT-3/p75NTR structure (PDB code: 3BUK). ProNGF/p75NTR structure is colored as in panel (A). For NT-3/p75NTR structure, NT-3 is blue and p75NTR is cyan. N-Glycans at Asn32 are highlighted as cyan sticks. The inset shows the NT-3/p75NTR structure. **(C)** Cell apoptosis assay for NGF, proNGF, BDNF, and NT-3. Replica cultures of SCG neurons (DIV 9) were washed free of NGF and were treated with no additive (None), 10 ng/ml NGF, 5 ng/ml proNGF, 100 ng/ml BDNF, or 100 ng/ml recombinant NT-3 in the presence of 12.5 mM KCl. Thirty-six hours later, cultures were processed and scored for apoptotic neurons as described (Materials and Methods). The data were normalized to the number of dying neurons under 12.5 mM KCl treatment. Results were summarized from three independently conducted experiments. Vertical error bars represent S.E.M.

Figure 4. Molecular masses of NGF and proNGF alone and in complexes with p75NTR
Molar masses of NGF (A), proNGF (B), p75NTR (C), NGF/p75NTR (D), and proNGF/p75NTR (E) are measured with multi-angle light scattering, and are plotted against elution time from Superdex 200 gel filtration column. Peaks are visualized as unitless relative refractive index measurements. Grey lines represent the linear fitting of the molar mass measurements.

Figure 5. Conformational changes of L2 loops in mature neurotrophins versus proNGF

Top view of molecular surfaces of (A) NGF/p75NTR, (B) NT-3/p75NTR and (C) proNGF/p75NTR. p75NTR is magenta in all structures. Neurotrophins are colored in light grey, except for L2 loops. L2 loops are yellow for NGF/p75NTR in (A), blue for NT-3/p75NTR in (B) and green for proNGF/p75NTR in (C). Composite omit map electron densities between L2 loops of proNGF are shown at contour level 1.2 σ . Tryptophan residues at position 99 are shown as green sticks. (D). Comparison of L2 loops in proNGF/p75NTR structure with L2 loops in uncomplexed structures of neurotrophins. L2 loops in proNGF/p75NTR structure are green, with composite omit map of this region at contour level 1.2 σ . L2 in other structures include unliganded NGF (1BET) in gray, NGF complexed to p75NTR (1SG1) in yellow, NGF complexed to TrkA-D5 (1WWW) in magenta, NGF complexed to TrkA (D1-D5) (2IFG) in cyan, NT-4 (1B98) in slate, brain-derived neurotrophic factor (1B8M) in orange, and NT-3 complexed to p75NTR in blue (3BUK). Distance between the L2's of the proNGF dimer is measured as 14 Å and indicated by black dashed line. (E) Side view of L2 loop region of proNGF. Composite omit map densities were shown for the region around L2 loops. For comparison, L2 loops of NGF and NT-3 are also shown in yellow and blue respectively. Trp99 is shown in green sticks.

Figure 6. Dissecting the assembly of the proNGF/sortilin/p75NTR complex by surface plasmon resonance

Sortilin was immobilized on a streptavidin (SA) chip. Different ligands were flowed over the chip surface including (A) proNGF, (C) NGF, and (E) proNGF/p75NTR complex. proNGF used is the triple-mutant form; other forms of proNGF are not stable enough for SPR.

Experiments were performed over a series of concentrations as shown in each panel.

Competition experiments were done by adding increasing amounts of the specific antagonist neurotensin to proNGF (B), NGF (D), and proNGF/p75NTR complex (F).

Figure 7. Calcium-dependent association of proNGF and sortilin

(A) The three components (proNGF, p75NTR and sortilin) were mixed and applied to a Superose 6 gel filtration column with 1 mM Calcium included both in the sample mixture and in the running buffer. Inset shows the SDS gel for fractions from 11 ml to 18 ml as labeled at the top of gel. Column calibration with globular protein molecular weight standards is shown at top of the panel. In panel (B), fractions from 11 ml to 14 ml in panel (A) were pooled, and then divided into two equal aliquots which were re-applied to the Superose 6 column with Calcium (B) or without Calcium (C) included in running buffer. Gels for fractions 11 ml to 18 ml are shown as insets in each panel. For comparison, profiles for individual components are also shown in panel (C) with green dash for sortilin, orange dash for proNGF and blue dash for p75NTR. HEK-293 cells were transfected with proNGF and sortilin, and cell lysates (D) or culture media (E) were immunoprecipitated with sortilin antibody in calcium-supplemented or depleted conditions followed by Western blot analysis. (F) HT-1080^{P/S} cells were treated with proNGF (500 ng/ml), and cell lysates were subjected to immunoprecipitation with p75NTR antibody in calcium-supplemented or depleted conditions. Lysates, 10 µg/lane.

Table 1

X-ray crystallographic data and refinement statistics for proNGF/p75NTR

<i>Data Collection</i>	
Space Group	<i>P</i> 3 ₂ 21
Wavelength (Å)	1.0000
Cell Dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	145.41, 145.41, 114.25
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90, 90, 120
Resolution (Å) ^a	50 – 3.75 (3.88 – 3.75)
No. of unique observations	14782
No. of total observations	158749
Completeness (%) ^a	100.0 (99.7)
Multiplicity ^a	10.7 (8.1)
<i>R</i> _{merge} (%) ^a , <i>b</i>	13.2 (86.5)
<i><I / σ (I)></i> ^a	17.5 (1.8)
<i>Refinement</i>	
Resolution (Å) ^a	50 – 3.75 (4.04–3.75)
<i>R</i> _{work} (%) ^a , <i>c</i>	25.6 (30.1)
<i>R</i> _{free} (%) ^a , <i>c</i>	27.4 (31.4)
Wilson <i>B</i> factor	120.2
No. of atoms	4092
<i>B</i> factors	166.9
RMS deviations	
Bond lengths (Å)	0.013
Bond angles (°)	1.6
Ramachandran Statistics	
Favored (%)	89.7
Outliers (%)	0.75

^aNumbers in parantheses refer to the shell of highest resolution^b $R_{\text{merge}} = \sum |I_j - \bar{I}| / \sum I_j$, where *I_j* is the intensity of an individual reflection, and *I* is the average intensity for that reflection.^c $R_{\text{work}} = \sum ||F_O| - |F_C|| / \sum |F_O|$, which excludes 5% of reflections used in the calculation of *R*_{free}.