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Phosphatidylinositol (3,4,5)-trisphosphate binds to sortilin and competes with neurotensin: Implications for very low density lipoprotein binding

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Abstract

Sortilin is a multi-ligand sorting receptor that interacts with B100-containing VLDL and LDL as well as other ligands including neurotensin (NT). The current study investigates the hypothesis that phosphatidylinositol (3,4,5)-trisphosphate (PIP3) generated downstream of insulin action can directly bind to sortilin. NT binds to sortilin at a well characterized site via its carboxy terminus (C-term). Using a crystal structure of human sortilin (hsortilin), PIP3 is predicted to bind at this C-term site. Binding of PIP3 to hsortilin is demonstrated using surface plasmon resonance (SPR) flowing PIP3 nanodiscs over immobilized hsortilin. Studies were performed using SPR where dibutanoyl PIP3 is shown to compete with NT for sortilin binding. Rat VLDL and LDL were evaluated for PIP3 content immunologically using monoclonal antibodies directed against PIP3. Rat plasma VLDL contained three times more immunoreactive PIP3 than LDL per μg of protein. Because VLDL contains additional ligands that bind sortilin, to distinguish specific PIP3 binding, we used PIP3 liposomes. Liposome floatation assays were used to demonstrate PIP3 liposome binding to sortilin. Using SPR and immobilized hsortilin, the C-term NT tetrapeptide (P-Y-I-L) is shown to bind to hsortilin. A compound (cpd984) was identified with strong theoretical binding to the site on sortilin involved in NT N-terminal binding. When cpd984 is co-incubated with the tetrapeptide, the affinity of binding to sortilin is increased. Similarly, the affinity of PIP3 liposome binding increased in the presence of cpd984. Overall, results demonstrate that sortilin is a PIP3 binding protein with binding likely to occur at the C-term NT binding site. The presence of

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multiple ligands on B100-containing lipoproteins, VLDL and LDL, raises the interesting possibility for increased interaction with sortilin based on the presence of PIP3.

Keywords

sortilin; VLDL-B100; neurotensin; surface plasmon resonance

1. Introduction

Sortilin is a multi-ligand binding protein with numerous binding partners [1]. Sortilin binds neurotensin (NT) at two sites: the carboxy terminus (C-term) site 1, and the amino terminus (N-term) site 2 [2]. Sortilin also binds B100-containing very low density lipoprotein (VLDL) and LDL [3, 4]. We previously demonstrated that sortilin facilitates the secretion of VLDL-B100 by insulin-sensitive McArdle RH7777 (McA) cells [5]. We have further shown that insulin enhances B100 binding to sortilin in McA cells [6] preceding autophagic destruction [7]. Considering the association of sortilin with VLDL binding and with VLDL-B100 degradation in response to insulin, we explored the possibility for insulin-generated phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to interact directly with sortilin. We specifically tested the potential of PIP3 binding to NT defined binding sites on sortilin. Our results suggest a possible role for PIP3 present on VLDL to directly modulate lipoprotein-sortilin interactions.

2. Materials and methods

2.1. Materials

Plasma from fasted Sprague Dawley rats was obtained from BioreclamationIVT, (Westbury, NY) and used to prepare rat lipoproteins. Bovine serum albumin (BSA) (A7285) was from Sigma-Aldrich Corp. (St. Louis, MO). The following materials and reagents were obtained from Echelon Biosciences, Inc. (St. Lake City, UT) including PIP3 PolyPIPosomes (Y-P039); dibutanoyl PIP3 (diC4-PIP3) (P-3904); dipalmitoyl PIP3 (diC16-PIP3) (P-3916); purified anti-PIP3 IgG (Z-P345b), and purified anti-PIP3 IgM (ZP345) monoclonal antibodies. Biotinylated goat anti-mouse IgM and goat serum (GS) were from Jackson ImmunoResearch Labs, Inc. (West Grove, PA). Streptavidin-horseradish peroxidase (HRP), HRP-conjugated sheep antimouse IgG (NXA931), HRP-conjugated donkey anti-rabbit IgG (NA9340V), ECLTM Prime western blotting detection reagent, CM7 (S series) and NiNTA (S series) sensor chips were from GE Healthcare (Buckinghamshire, UK). Human sortilin (hsortilin) recombinant protein (Ser78-Asp755) with C-terminal 6-His tag was from R&D Systems, Inc. (Minneapolis, MN). Neurotensin C-terminal fragment (P-Y-I-L) (C-term NT) was synthesized by Biomatik Corp. (Wilmington, DE). SuperBlock[®], mouse anti-6X HIS monoclonal antibody and 1-StepTM Ultra TMB-ELISA (TMB) were from Thermo Scientific (Rockford, IL). Costar[®] Assay Plates were from Corning, Inc. (Kennebunk, ME). Rabbit anti-sortilin polyclonal antibody (GTX54854) was from GeneTex, Inc. (Irvine, CA). Compound 98477898 (2S)-1-methyl-N-{3-[(3-phenylpropanoyl)-amino]phenyl}pyrrolidine-2-carboxamide (cpd984) was obtained from ChemBridge Corp. (San Diego, CA). All other materials and reagents were essentially as described previously [5].

2.2. Computational modeling

Schrödinger's Maestro program (version 9.3.5) was used as the primary graphical user interface and Release 2015-2: Maestro, version 10.2 (Schrödinger, LLC, New York, NY) was used for ligand interaction diagramming [8]. Docking of diC4-PIP3 on a grid centered at the C5 term NT (site1) of hsortilin crystal structure at excess concentration of NT PDB ID: 4PO7 was performed using Schrödinger's Glide software [9] as described [5].

2.3. Surface plasmon resonance

Surface plasmon resonance (SPR) measurements were performed on a Biacore T200 instrument equipped with two separate CM7 sensor chips, one with ~20,000 response units (RU) and another with ~27,000 RU of hsortilin covalently immobilized. HBS running buffer with and without 1% (v/v) DMSO (10 mM HEPES pH 7.4, 150 mM NaCl) was used at a flow rate of 30 µl/min and injections had an association time of 90 sec, dissociation time of 300 sec, and binding was measured in relative RU or difference in response between the immobilized protein flow cell and the corresponding control flow cell subtracting relevant blank injections of buffer alone [5]. Regeneration buffer of pH 3.0 glycine was injected after each injection for PIP3 nanodiscs and C-term NT flowed in the presence of cpd984. For all other SPR injections, regeneration consisted of injections of running buffer alone. GE BIAcore T200 evaluation software version 3.0 (BIAevaluate) was used to analyze and export results. Full length NT was injected using 2:1 NT dilutions on ~27,000 RU CM7 surface and kinetics fitted using BIAevaluate Langmuir 1:1 binding model using local Rmax exported from BIAevaluate to Microsoft Excel 2016 with fit indicated by the black line. Full length NT was flowed in the presence of diC4-PIP3 diluted directly in running buffer and RU (5 sec before injection stop) plotted in GraphPad Prism version 6.05 according to log [inhibitor] versus response [three parameter] model to obtain IC₅₀ of diC4-PIP3 with respect to full length NT. PIP3 containing nanodiscs were flowed at 2:1 dilutions to obtain kinetic data fitted using ~27,000 RU CM7 surface. Kinetic fit generated with BIAevaluate and local Rmax using Langmuir 1:1 binding model exported from BIAevaluate to Microsoft Excel 2016 with fit indicated by the black line. Cterm NT was flowed using 2:1 dilutions alone and co-injected with 100 µM cpd984 and steady state K_d obtained for full length NT using RU (45 and 10 sec after injection start, respectively) plotted in GraphPad according to One site-Total binding model.

2.4. Preparation of PIP3 nanodiscs

Lipid composition of nanodiscs consisted of 3.023 µmol dipalmitoyl phosphatidylcholine (PC), 0.098 µmol diC16-PIP3, and 0.78 µmol 1-palmitoyl, 2-oleoyl phosphatidylethanolamine (PE), which were combined, dried, and desiccated overnight. Lipids were then dissolved in 20 mM sodium deoxycholate in TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.02% (w/v) sodium azide) and sonicated. Membrane scaffold protein 1D1 (MSP1D1) was then added in a ratio of 70:1 lipid to protein and detergent removed with Bio-Beads® SM-2 (Bio-Rad). Nanodiscs were isolated using size exclusion chromatography [10]. Nanodiscs were quantified using a NanoDrop and extinction coefficient of 21,000 L mol⁻¹ cm⁻¹ for MSP1D1, and the resultant mg/ml divided by two because there are two MSP proteins per nanodisc.

2.5. Liposome floatation assay

Unilamellar liposomes containing specific lipid compositions were produced using lipids dissolved in chloroform that were mixed according to mole percentages of 2.6 μ moles total phospholipid [11, 12]. Lipids were dried and desiccated overnight followed by incubation with PBS (2.6 ml) for 1 h. Lipids were resuspended by vortexing and disrupted by sonication for 30 min. To measure binding, a liposome floatation assay was used by adding concentrations 50 nM or 100 nM hsortilin to liposomes (150 μ l) containing 5% PIP3, 20% PE and 75% PC; 10% PIP3, 20% PE, 70% PC; or 0% PIP3, 80% PC, and 20% PE as described [12, 13]. Samples containing 50 nM or 100 nM protein and liposomes in the presence and absence of cpd984 were incubated for 10 min at 30 °C and 630 μ l of 1.65 M sucrose/PBS was added. Samples were overlaid with 630 μ l of 0.75 M sucrose/PBS, and PBS to the top of the tube and centrifuged ($200,000 \times g$, 90 min, 4 °C), and floated liposomes recovered from the 0.75 M sucrose layer. Liposomes at the interface of the top and second fraction were recovered and diluted in 2 ml of PBS and re-isolated by centrifugation ($16,000 \times g$, 20 min, 4 °C). 50 μ l PBS and 20 μ L 5X SDS sample buffer was added to the final liposome pellet. Bound proteins were resolved by SDS-PAGE and detected by western immunoblotting following transfer to PVDF membranes [5].

2.6. Assay of immunoreactive PIP3 on rat plasma lipoproteins

Plasma VLDL, LDL and high density lipoprotein (HDL) from fasted rats were isolated by sequential density ultracentrifugation as previously described [14]. Immunoreactive PIP3 on plasma lipoproteins was evaluated by enzyme linked immunoassay and by immunoslot blotting. Enzyme linked immunoassay involved coating 96-well multi-well assay plates with 0.1 ml/well diluted lipoprotein or BSA in 0.05 M carbonate/bicarbonate buffer (25 μ g protein/ml) overnight at 4°C. Wells were washed three times and post-coated with SuperBlock® for 1 h at room temperature to block non-specific binding. Anti-PIP3 (IgM) diluted in 1% (v/v) GS/TBS was added (1:1000 dilution) and incubated at 4°C overnight. Wells were washed three times in 1% (v/v) GS/TBS and 0.1 ml biotinylated goat anti-mouse IgM antibody was then added and incubated for 30 min at 37 °C. Wells were washed three times in 1% (v/v) GS/TBS and 0.1 ml of streptavidin-HRP in 1% (v/v) GS/TBS was added (1:500) and incubated for 30 min at 37 °C. Wells were washed three times in 1% (v/v) GS/TBS and bound HRP was detected by addition of 0.1 ml TMB reagent with incubation at room temperature. Reaction was terminated by addition of 0.1 ml of 2 M sulfuric acid and absorbance was measured at 450 nm using the iMark™ Microplate Reader (Bio-Rad, Hercules, CA). The second method employed immunoslot blotting where rat plasma VLDL and LDL from three separate plasma pools were diluted in TBS (2.5 to 10 μ g protein/ml) and slot blotted (200 μ l/slot) onto PVDF membranes as previously described [5]. PolyPIPosomes containing PIP3 diluted 1:2000 in PBS were used as positive control. Following filtration, membranes were air dried, quickly rehydrated in methanol, reincubated in PBS and then incubated in SuperBlock® at 4°C overnight. Afterwards, membranes were incubated in 3% (w/v) BSA/PBS containing anti-PIP3 (IgG) (1:1000) overnight at 4°C. After washing three times in PBS/0.1% Tween 20 (PBST), membranes were incubated in HRP-conjugated sheep anti-mouse IgG in 3% (w/v) BSA/PBS for 90 min. Membranes were washed three times in PBST and then developed using ECL™ Prime western blotting

detection reagent. Chemiluminescence was measured using the ChemiDocXRS+ system (Bio-Rad) and band intensities quantified using Image Lab 3.0.1 software (Bio-Rad).

2.7. Statistics

Unless otherwise noted, results are expressed as mean $\pm$ S.E.M. SPR experiments were analyzed using GE BIAevaluation Software, Microsoft Excel and GraphPad Prism.

3. Results and discussion

3.1. Sortilin is a PIP3 binding protein

Sortilin binding to NT has been characterized and involves N-term (site 2) and C-term (site 1) binding sites for binding to a single molecule of sortilin [2, 15]. A compound was identified with strong theoretical binding to site 2 (cpd984), and in biologic experiments cpd984 incubation increased VLDL-B100 secretion by McA cells [5]. In Fig. 1A, N-term NT is depicted in site 2 located across the central space of sortilin from PIP3 computationally docked into site 1 of hsortilin. The theoretical binding of diC4-PIP3 was modeled for binding to site 1 with a docking score (gscore) of -10.3 kcal/mol indicating a high predicted affinity for hsortilin [8]. DiC4-PIP3 is shown binding to site 1 in Fig. 1A with phosphate groups interacting with arginine 292 (R292). The interaction diagram provided in Fig. 1B indicates a number of additional interacting amino acids. To directly test PIP3 binding to sortilin, we prepared PIP3 containing nanodiscs and performed SPR using immobilized hsortilin (Fig. 1C). Nanodiscs were used to simulate PIP3 in membranes or membrane-like surfaces as might be present on VLDL. As shown in Fig. 1C, the K_d for PIP3 nanodiscs was 17 nM. The theoretical prediction of sortilin as a PIP3 binding protein is supported by these results.

3.2. PIP3 competes with NT for binding to sortilin

To determine whether PIP3 binds to sortilin at similar sites as NT, we flowed diC4-PIP3 in the presence of full length NT on immobilized hsortilin and analyzed the response using SPR. In the absence of PIP3, the K_d for NT binding on a highly saturated surface of hsortilin was approximately 290 nM (Fig. 2A). This value is higher than the nM value previously reported using isothermal titration calorimetry [2], potentially because of unproductive crosslinking of hsortilin to the CM7 chip. As seen in Fig. 2B at 100 nM NT, there was progressive competition for NT-sortilin interaction by diC4-PIP3. An IC_{50} of approximately 750 nM was obtained for diC4-PIP3 inhibition of full length NT binding to sortilin (Fig. 2C). Structural analysis of a sortilin-related receptor, sorLA, suggests that a site exists similar to site 2 on sortilin that binds mainly hydrophobic ligands [16]. While there was some predicted binding affinity of PIP3 to site 2 of sortilin, it is unlikely to be relevant to sortilin-lipoprotein interaction as hydrophobic components of PIP3 would be anticipated to be embedded within the lipoprotein. Based on the overall data, we hypothesize that sortilin binds PIP3 at a NT related site, with binding most likely occurring at the C-term site 1.

3.3. Immunoreactive PIP3 is present on circulating lipoproteins

Previous studies have demonstrated the presence of phosphatidylinositols on circulating lipoproteins [17]. PIP3 is present in serum bound to albumin [18]. We tested whether PIP3

might also be present on plasma lipoproteins. Plasma lipoproteins have a surface monolayer of phospholipids that would orient the phosphatidylinositol head group toward the aqueous environment. We performed qualitative studies where we adsorbed to solid phase plastic wells freshly prepared rat plasma VLDL, LDL and HDL. After blocking non-specific binding, we detected PIP3 using an IgM monoclonal anti-PIP3 antibody. As shown in Fig. 3A, there was three times the immunoreactivity present in VLDL as compared with LDL, HDL or BSA. To confirm these findings, VLDL and LDL were slot blotted and PIP3 immunoreactivity determined using an IgG monoclonal anti-PIP3 antibody (Fig. 3B). Results were compared with slotted PolyPIPIsomes containing PIP3 as a positive control. Calculating the relative reactivity per microgram of three separate preparations of rat plasma VLDL and LDL, there was 4.3, 2.5 and 4.0 times more immunoreactivity present in VLDL compared with each matched LDL. (LDL 3 is not shown). Our results indicate there is immunoreactive PIP3 on circulating lipoproteins. The presence of lipoprotein PIP3 could influence the interaction of lipoproteins with corresponding cellular receptors including sortilin.

3.4. Sortilin binds PIP3 liposomes and binding is enhanced by a sortilin binding compound

Using liposome floatation assay, sortilin is shown to bind PIP3 liposomes, but not PC/PE liposomes (Fig. 4A, upper and middle panel, first lanes). A sortilin binding compound (cpd984) was developed to bind site 2 [5], and was used to probe PIP3 and C-term NT binding to sortilin. In the presence of increasing concentrations of cpd984, there was increased sortilin binding to PIP3 liposomes without change in binding to PC/PE liposomes (Fig. 4A, upper and middle panel, respectively). The enhanced binding of PIP3 liposomes to sortilin is confirmed using an anti-sortilin antibody (Fig. A, lower panel). A C-terminal NT tetrapeptide (P-Y-I-L) (C-term NT) was used in SPR experiments to examine the effect of cpd984 on site 1 binding to immobilized sortilin. The C-term NT alone had a K_d of 138 nM (Fig. 4B, upper panel). To test the effect of cpd984, we determined the K_d for the C-term NT in the presence of 100 μ M cpd984. The K_d was lowered from 138 nM to 662 pM (Fig. 4B, lower panel). The sensorgrams used to derive the K_d 's are provided in Supplemental Fig. 1. These studies indicate cpd984 enhanced the affinity of binding of PIP3 liposomes and C-term NT to sortilin. The use of liposomes as lipoprotein surrogates containing PIP3 to show binding to sortilin is important, as liposomes do not contain other potential sortilin ligands that would be present on VLDL such as B100 and apoE. We speculate that VLDL containing multiple PIP3 molecules on its surface and additional ligands could bind both sites 1 and 2 on sortilin enhancing binding as the size of spherical VLDL is large enough to span the central region of sortilin.

Considering the downstream role PIP3 plays in insulin signaling and the enigmatic role sortilin has in VLDL metabolism [19], the finding that sortilin is a PIP3 binding protein could have important implications for VLDL. We suggest that the presence of PIP3 on VLDL could influence the relative binding of B100 and apoE to various receptors including sortilin. Furthermore, the presence of PIP3 within the secretory pathway on VLDL might affect cell transit if it were demonstrated to modulate sortilin-B100 binding. It is apparent from our results that there is an interaction between at least two sites on sortilin in response to binding [5]. Because there is such a strong increase with cpd984 in site 1 ligand binding,

our data suggest that there could be a conformational change in sortilin similar to that reported for sorLA [16]. In summary, our data support that sortilin is a PIP3 binding protein that could influence VLDL interaction with sortilin based on PIP3 content. Considering PIP3 is a downstream insulin signaling molecule, the demonstration of sortilin binding and presence of PIP3 on VLDL may represent a novel signaling pathway for insulin action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PIP3	phosphatidylinositol (3,4,5)-trisphosphate
diC4-PIP3	dibutanoyl PIP3
hsortilin	human recombinant sortilin
NT	neurotensin
C-term	carboxy terminus
N-term	amino terminus
SPR	surface plasmon resonance
RU	response units
VLDL	very low density lipoprotein
B100	apolipoprotein B derived from unedited <i>APOB</i> mRNA

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Research highlights

- PIP3 theoretically binds to the C-terminal neurotensin binding site on sortilin
- PIP3 nanodiscs bind to sortilin
- diC4 PIP3 competes for neurotensin binding to sortilin
- Immunoreactive PIP3 is present on rat plasma VLDL
- A sortilin binding compound enhances neurotensin and PIP3 binding to sortilin

Fig. 1. Computational docking of PIP3 on hsortilin

(A) Computational docking was performed using Schrödinger molecular docking software Glide [8] and diC4-PIP3 on PDB ID 4PO7. The top ranking pose of PIP3 using Glide XP is shown for the C-term NT site 1 of hsortilin (right) forming salt bridges with R292 opposite the N-term NT site 2 (left) present in the reported crystal structure. The docking score (gscore) for this pose is -10.3 kcal/mol. (B) Ligand interaction diagram depicting binding of diC4-PIP3 docked in C-term site 1 indicating key amino acid residues likely to be involved in binding (blue, basic; red, acidic; green, hydrophobic amino acids). Hydrogen bonds are indicated with purple arrows. (C) SPR analysis using PIP3 nanodiscs was performed using the CM7 chip containing $\sim 27,000$ RU of hsortilin. Sensorgrams and mathematical fit (black line) of hsortilin for 2.5% PIP3 nanodisc injections at indicated concentrations in duplicate on immobilized hsortilin followed by regeneration buffer. Kinetic fit using local R_{max} and Langmuir 1:1 binding model yielded a K_d of 17 nM with k_a $1.66 \times 10^5 \pm 88$ ($M s^{-1}$), k_d of $0.0027 \pm .000006$ (s^{-1}) and chi-sq of 3.98.

Fig. 2. Surface plasmon resonance analysis of NT binding to hsortilin and competitive inhibition of binding with diC4-PIP3

(A) SPR was performed using the CM7 chip containing ~27,000 RU hsortilin. Sensorgrams with mathematical fit (black) of NT injections are displayed at concentrations in decreasing order of 1000 nM (red), 500 nM (blue), 250 nM (purple), 31 nM (blue), 16 nM (green), and 8 nM (yellow). Kinetic fit using local Rmax and Langmuir 1:1 binding model yielded a K_d of 288 nM with k_a $1.74 \times 10^5 \pm 14 \text{ (Ms}^{-1}\text{)}$, k_d of $0.05 \pm 00034 \text{ (s}^{-1}\text{)}$ and chi-sq of 4.49. (B) SPR analysis as described in 2A are shown using injections of 100 nM NT in the presence of diC4-PIP3 at concentrations of 125 μM, 31.3 μM, 3.91 μM, 976 nM 488 nM, 244 nM, 122 nM, and 31 nM. (C) Calculation of diC4-PIP3 inhibition measured at 5 sec before injection stop using sensorgrams shown in (B). GraphPad log[inhibitor] versus response [three parameter] was used to calculate IC_{50} normalizing the two series with the highest concentration of diC4-PIP3 injected of 500 μM and excluding it from the series yielding an IC_{50} of $754 \text{ nM} \pm 285 \text{ nM}$ for diC4-PIP3 inhibition of 100 nM NT.

Fig. 3. Immunoreactive PIP3 is present on circulating VLDL and LDL

(A) Rat plasma VLDL, LDL, HDL and BSA were adsorbed on plastic 96-well dishes and immunoreactive PIP3 was evaluated following incubation with mouse anti-PIP3 monoclonal antibody (IgM) and detected by enzyme linked immunoassay. Results are the average of 12 wells for each protein $\pm$ S.D. (B) For immunoslot blotting, duplicate 200 μ l samples of rat plasma VLDL ($d < 1.006$ g/ml) and LDL (1.006 g/ml $< d < 1.063$ g/ml) were slot blotted at a protein concentration of 2.5 μ g/ml or at 10 μ g/ml in TBS from 3 separate batches of rat plasma (1–3). Immunoreactive PIP3 was evaluated using mouse anti-PIP3 monoclonal antibody (IgG) and binding was detected after incubation with HRP-anti-mouse IgG followed by chemiluminescence detection.

Fig. 4. Cpd984 enhances PIP3 and C-term NT binding to hsortilin

(A) Liposome flotation assays where 5% PIP3 liposomes (upper panel) and 0% PIP3 PC/PE liposomes (middle panel) were incubated with 50 nM hsortilin in the absence and presence of increasing concentrations of cpd984 as indicated. After incubation, liposomes were separated on sucrose gradients and bound proteins analyzed by western blotting after protein separation by SDS-PAGE using anti-6X HIS tag monoclonal antibody. Liposomes containing 10% PIP3 were incubated with 100 nM hsortilin and increasing concentrations of cpd984 (lower panel). Following incubation and liposome isolation, sortilin binding was detected by western immunoblotting following protein separation by SDS-PAGE using rabbit anti-sortilin polyclonal antibody. (B) Steady state affinities were calculated using GraphPad One site - Total binding model. For C-term NT alone affinity was calculated at concentrations of 31 nM, 63 nM, 125 nM, 250 nM, 500 nM, 1 μM , 2 μM , 4 μM , and 8 μM with K_d 138 nM $\pm$ 68 nM (upper panel). For Cterm NT, affinity was calculated with 100 μM cpd984 using concentrations of 0 (cpd984 alone), 2 nM, 8 nM, 16 nM, 31 nM, and 63 nM with K_d of 662 $\pm$ 476 pM (lower panel) using sensorgrams developed at 45 sec or 10 sec as shown in Supplemental Fig. 1.