Sortilin Facilitates Signaling of Ciliary Neurotrophic Factor and Related Helical Type 1 Cytokines Targeting the gp130/Leukemia Inhibitory Factor Receptor β Heterodimer

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Sortilin is a member of the Vps10p domain family of neuropeptide and neurotrophin binding neuronal receptors. The family members interact with and partly share a variety of ligands and partake in intracellular sorting and protein transport as well as in transmembrane signal transduction. Thus, sortilin mediates the transport of both neurotensin and nerve growth factor and interacts with their respective receptors to facilitate ligand-induced signaling. Here we report that ciliary neurotrophic factor (CNTF), and related ligands targeting the established CNTF receptor α, binds to sortilin with high affinity. We find that sortilin may have at least two functions: one is to provide rapid endocytosis and the removal of CNTF, something which is not provided by CNTF receptor α, and the other is to facilitate CNTF signaling through the gp130/leukemia inhibitory factor (LIF) receptor β heterodimeric complex. Interestingly, the latter function is independent of both the CNTF receptor α and ligand binding to sortilin but appears to implicate a direct interaction with LIF receptor β. Thus, sortilin facilitates the signaling of all helical type 1 cytokines, which engage the gp130/LIF receptor β complex.

Sortilin (40) is a member of the mammalian Vps10p domain (Vps10p-D) family of neuronal type 1 receptors that also includes SorLA (23) and SorCS1-3 (19, 27, 32). The hallmark of the family is the N-terminal Vps10p-D consisting of two minor domains and a unique, ligand-binding, 10-bladed β-propeller domain (41). The Vps10p-D constitutes the entire luminal part of sortilin and is followed by a single transmembrane segment and a short cytoplasmic domain containing functional motifs for endocytosis as well as intracellular trafficking, e.g., sorting between the Golgi apparatus and endosomes (34). Sortilin is synthesized as an inert proreceptor with a short N-terminal propeptide (amino acids 1 to 44) (38). The propeptide is removed by proteolytic cleavage in late Golgi compartments, and sortilin is thereby converted to the ligand binding mature receptor (38), which binds a growing number of competing ligands, including the receptor-associated protein (RAP), its own propeptide, and, notably, neuropeptides (e.g., neurotensin [NT]) and neurotrophins (e.g., the proforms of nerve growth factor β [NGF-β] and brain-derived neurotrophic factor [BDNF]) (30, 35, 38, 40, 45). In addition to soluble ligands, sortilin also interacts with other transmembrane proteins. Thus, sortilin has been shown to associate with the common neurotrophin receptor p75NTR and to engage both p75NTR and proneurotrophins to form a death signaling trimeric complex (24, 35, 45). Moreover, sortilin may bind neurotensin receptor 1 (NTS1) and was reported previously to modulate NT signaling and the endocytosis of NTS1 (29).

Sortilin binds apolipoprotein E (apoE) (22), which is structurally related to ciliary neurotrophic factor (CNTF) (31, 47). They both form homodimers, but the two may also combine to form functional heterodimeric complexes (17); however, the possibility that CNTF, similarly to apoE, targets sortilin has not been explored. CNTF belongs to the family of helical type 1 cytokines, which also includes interleukin-6 (IL-6), IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotoxin-1 (CT-1), neuropoietin, and the heterodimer cardiotoxin-like cytokine/receptor cytokine-like factor 1 (CLC/CLF-1). These cytokines play key roles in several physiological processes, including immune regulation, host defense, reproduction, food intake, and the regulation of neuronal growth (20). CNTF was initially identified, and named, for its ability to maintain the survival of parasympathetic neurons of chicken ciliary ganglia (2). Since then, it has been shown to support the survival of many different neuronal cell types, including sensory (44) and motor (36) neurons and neurons of cerebral and hippocampal origin (21, 28). CNTF lacks a classical signal peptide sequence and is subject to alternative secretion. It is generally believed to act as a lesion factor that is released mainly from tissues subjected to trauma or stress. In support of this notion, several studies have reported a marked change in the localization and expression of CNTF on nerve lesions (15, 42, 43).

CNTF signaling is elicited by the formation of a trimeric
receptor complex (7). At first, CNTF binds to nonsignaling glycosylphosphatidylinositol (GPI)-anchored CNTF receptor α (CNTFRα) (6). The binding of CNTF to the membrane-bound or soluble CNTFRα (sCNTFRα) is followed by the recruitment of the signaling subunits 130-kDa glycoprotein (gp130) and LIF receptor β (LIFRβ) (7). The CNTF-induced heterodimerization of gp130 and LIFRβ leads to the activation of the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway by the phosphorylation of STAT3, in addition to the Ras/mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase pathways (18, 20). CNTFRα is the specificity-conferring subunit of the trimeric receptor complex. It is well conserved across species and is highly expressed in neuronal tissue and skeletal muscle (8). However, the receptor is not an absolute requirement for signaling because CNTF at high concentrations (40 nM and above) may directly prompt and activate the gp130/LIFRβ heterodimer (16).

CNTFRα is also a receptor subunit for CLC/CLF-1 and neuropoietin, which, similar to CNTF, induce a cellular response via gp130/LIFRβ. In contrast to CNTF, CLC/CLF-1 completely depends on the initial binding of CNTFRα for signal transduction (12).

The importance of CNTFRα and the associated ligands is reflected by studies of gene deficiency. Thus, the lack of either CNTFRα or CLC/CLF-1 results in a severe loss of motor neurons and perinatal death due to a sucking defect (3, 9, 49).

The fact that no particular phenotype has been ascribed to CNTF deficiency may, on the other hand, result from a significant degree of redundancy (13).

In the present study we demonstrate that CNTF, and related ligands targeting CNTFRα, binds to sortilin with high affinity. We show that a high-affinity site in the C terminus of CNTF interacts with the sortilin β-propeller domain and demonstrate that sortilin provides rapid endocytosis and the removal of CNTF. Importantly, we find that sortilin facilitates CNTF signaling and mediates CNTF-dependent proliferation through the gp130/LIFRβ heterodimeric complex. Interestingly, this function is independent of both CNTFRα and ligand binding to sortilin but appears to implicate a direct interaction with LIFRβ. Thus, we find that sortilin facilitates the signaling of all helical type 1 cytokines that engage the gp130/LIFRβ complex but not of IL-6, which signals through the gp130 homodimeric complex.

MATERIALS AND METHODS

Reagents. Recombinant human CNTF, IL-6 (26), LIF (1), and hyper-IL-6 (hIL-6) (14) were expressed and purified as described previously. Recombinant human CLC/CLF-1, CT-1, OSM, sCNTFRα, soluble LIFRβ (sLIFRβ), recombinant mouse neuropoietin, and goat anti-CNTFRα were purchased from R&D Systems (Minneapolis, MN). Neurotensin (N6383) and murine monoclonal anti-sortilin but appears to implicate a direct interaction with LIFRβ. Thus, we find that sortilin facilitates the signaling of all helical type 1 cytokines that engage the gp130/LIFRβ complex but not of IL-6, which signals through the gp130 homodimeric complex.

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5.0 Ci/mmol; Amersham, United Kingdom) for 24 h. Cells were harvested, and incorporated [3H]thymidine was determined by scintillation counting.

**Fluorescence-activated cell sorter (FACS) analysis and antibodies.** Cells were stained with appropriate concentrations of phycocerythrin (PE)-conjugated human anti-gp130 and PE-conjugated human anti-LIFRβ antibodies (R&D Systems, MN) and Alexa 488-conjugated sortilin antibody. The sortilin antibody was labeled with the commercially available Alexa Fluor 488 monoclonal antibody labeling kit (Invitrogen, OR) according to the manufacturer’s instructions. After staining, cells were analyzed immediately with a BD FACSCalibur flow cytometer (Becton Dickinson and Company, NJ), with logarithmic recording of fluorescence and linear recording of scatter parameters. The data were analyzed by using FlowJo (version 4.6) software (TreeStar Inc., OR).

**SPR.** Surface plasmon resonance (SPR) analysis was performed with a Biacore 3000 instrument (Biacore, Sweden) equipped with CM5 sensor chips activated as described previously (5). Soluble sortilin-prosortilin densities of 66 to 86 fmol/mm², and samples for binding (40-3000 instrument (Biacore, Sweden) equipped with CM5 sensor chips activated as described previously (5)). The difference between the response obtained from the flow cell with the immobilized receptor minus the response obtained when using an activated but uncoupled chip. The overall Kd (dissociation constant) values were determined with BIAevaluation 4.1 software using a Langmuir 1:1 binding model and simultaneously fitting to all curves in the concentration range considered (global fitting).

**Immunocytochemistry.** Untransfected and transfected HEK293 cells were cultured on cover slides and incubated with 50 nM CNTF for 10 to 45 min in the absence and presence of 20 μM NT or 9 μM RAP. The cells were then washed and fixed in 4% formaldehyde (pH 7.4), permeabilized with 0.5% saponin (Sigma, MO), incubated with mouse anti sortilin and rabbit anti-CNTF, and finally stained with Alexa 488-conjugated goat anti-mouse antibody and DAPI.

**Duolink.** Cells were washed and fixed in 4% formaldehyde (pH 7.4), permeabilized with 0.5% saponin (Sigma, MO), incubated with mouse anti sortilin and rabbit anti-CNTF, and finally stained with Alexa 488-conjugated goat anti-mouse antibody and DAPI.

**C�NTF binds sortilin via a C-terminal site.** We next examined the binding of CNTF to full-length constructs of sortilin in transfected HEK293 cells. The cells were incubated with 50 nM CNTF in warm (37°C) medium, and following fixation, their uptake of CNTF was determined by immunofluorescence. No staining was observed for untransfected cells (Fig. 1F). In contrast, wild-type (wt) sortilin transfectants displayed a significant, predominantly intracellular, staining signifying a considerable uptake of ligand (Fig. 1G). This uptake was almost abolished when cells were incubated in the presence of excess NT (Fig. 1H) or RAP (not shown), and a similar lack of uptake was seen for transfectants expressing prosortilin (Fig. 1I). Finally, cells expressing a mutant sortilin incapable of endocytosis due to disrupted endocytosis motifs (Y758A/L761A and L795L796) (34) displayed staining limited to the surface of its binding site for CNTF-R

**RESULTS**

Because CNTF exhibits structural similarity to apoE (31, 47) and forms heterodimeric complexes with apoE (17), we speculated whether CNTF, similar to apoE, targets sortilin for binding (22). To clarify this, we examined the binding of CNTF to the immobilized ectodomain of sortilin (s-sortilin) using SPR analysis. As demonstrated in Fig. 1A, CNTF bound s-sortilin in a concentration-dependent manner and with an estimated Kd of about 25 nM. The binding was completely inhibited in the presence of excess NT (Fig. 1B) or RAP (Fig. 1C), and as apparent from Fig. 1D, CNTF did not interact with the immobilized sortilin precursor construct s-prosortilin, which carries an uncleavable propeptide. This demonstrates the specificity of the binding and that CNTF targets the β-pro-
Sortilin facilitates CNTF-induced phosphorylation of STAT3 and MAP kinase. To determine if sortilin might influence CNTF signaling, we initially tested the human TF-1 erythroleukemia cell line, which endogenously expresses gp130 and LIFR but not CNTFR. The cells were stably transfected with sortilin, and the surface expression of gp130 and LIFR, the absence of CNTFR, and the expression of sortilin were confirmed by FACS analysis and Western blotting (Fig. 4A).

Wild-type and transfected TF-1 cells were then stimulated with CNTF at a concentration (40 nM for 15 min) that is known to induce a cellular response even in the absence of CNTFR (16). In agreement, both cell lines responded with an increase in levels of phospho-STAT3 (relative to unstimulated cells). The level of phosphorylation, however, was significantly higher (2.5-fold) in the sortilin transfectants than in wt TF-1 cells (Fig. 4B and C), suggesting that the expression of sortilin served to facilitate CNTF signaling. A similar increase in phospho-STAT3 levels was obtained for cells transfected with a sortilin mutant lacking the cytoplasmic domain, signifying that the enhanced signaling did not depend on the sortilin tail (Fig. 4D and E).

To confirm and elaborate on this finding, we next performed a series of experiments with the murine BA/F3 cell line, which expresses neither sortilin, gp130, LIFR but not CNTFR. The cells were stably transfected with different combinations of these receptors (Fig. 5A and B), and their response in terms of the content of phospho-STAT3 was subsequently determined before and after stimulation with CNTF (40 nM for 15 min). As apparent from Fig. 5C, wt BA/F3 cells and cells expressing sortilin and/or gp130 showed no response to CNTF, and only a
minor increase in levels of phospho-STAT3 could be detected in BA/F3-[gp130/LIFRβ] transfectants, which did not express sortilin. In contrast, BA/F3-[gp130/LIFRβ/sortilin] cells and cells expressing the established CNTF signaling combination of gp130/LIFRβ and CNTFRα presented a marked increase in levels of STAT3 phosphorylation (Fig. 5C), whereas the response in BA/F3-[gp130/LIFRβ/mock] cells was comparable to that of BA/F3-[gp130/LIFRβ] cells (not shown). As determined by quantification of Western blots from 22 separate experiments, sortilin increased levels of CNTF-induced STAT3 phosphorylation in BA/F3-[gp130/LIFRβ/sortilin] cells by 2.8-fold (Fig. 5D). In agreement with these results, sortilin was also found to increase MAP kinase activation, which is an established downstream event in gp130/LIFRβ signaling (Fig. 5E).

A time course of CNTF-mediated phospho-STAT3 induction in BA/F3-[gp130/LIFRβ/sortilin] cells is shown in Fig. 5F. It is important that the high-level response in BA/F3-[gp130/LIFRβ/sortilin] cells compared with that in BA/F3-[gp130/LIFRβ] cells did not appear to result from a relative increase in gp130/LIFRβ expression levels. Thus, the simultaneous detection of STAT3 phosphorylation and the expression of sur-
face membrane receptors (by FACS) demonstrated that the sortilin transfectants displayed similar or lower levels of gp130 and LIFR than did the corresponding BA/F3-[gp130/LIFR] control cells (Fig. 6A and B).

Finally, CNTF induction of phospho-STAT3 was assessed in the presence of soluble CNTFR, which is known to promote CNTF signaling in gp130/LIFR-expressing cells. BA/F3-[gp130/LIFR] and BA/F3-[gp130/LIFR/sortilin] cells were therefore incubated with either CNTF (40 nM), sCNTFR (4 nM), or both prior to the detection of phospho-STAT3. As expected, sCNTFR had no effect on its own (not shown), whereas it strongly upregulated the response to CNTF in both cell types. However, even upon combined stimulation, the level of phospho-STAT3 remained higher in the sortilin transfec-
tants (Fig. 6C).

Evidently, these results suggest that sortilin and CNTFRα have mutually independent but additive and facilitating effects on CNTF signaling.

**Sortilin induces CNTF-dependent proliferation.** To further substantiate that sortilin promotes the biological activity of CNTF, we examined the proliferation of various BA/F3 transfectants in response to increasing concentrations of CNTF. As apparent from Fig. 7, stimulation with as much as 4 nM CNTF resulted in little or no proliferation of wt BA/F3 cells, of transfectants expressing either sortilin or gp130, and of cells coexpressing gp130 and LIFR. In contrast, enhanced proliferation was already detectable in BA/F3-[gp130/LIFR/sortilin] cells at 0.4 nM CNTF, and at 4 nM, this response was increased by as much as 5- to 6-fold. As expected, the stimulation of BA/F3-[gp130/LIFR] cells in the presence of sCNTFRα proved even more efficient (Fig. 7, inset), but the results confirm that sortilin significantly facilitates CNTF bio-
activity by a CNTFRα-independent mechanism.

**Sortilin binds CLC/CLF-1 and neuropoietin and facilitates their signaling.** Apart from CNTF, CNTFRα is also the primary receptor for neuropoietin and the heterodimeric CLC/CLF-1 (10, 12), and they both interact with sortilin (Fig. 8A). The affinity of CLC/CLF-1 for immobilized s-sortilin (Kd of ~10 nM) appeared to be even more pronounced than that of CNTF, whereas neuropoietin bound with a considerably lower affinity (Kd of ~100 nM). In each case, binding was abolished in the presence of excess NT (Fig. 8A), and as exemplified in Fig. 8B, all three ligands exhibited some degree of competition for binding. In agreement with this, HEK293 cells transfected...
with sortilin presented a specific uptake of CLC/CLF-1 (not shown).

We therefore next examined if CLC/CLF-1 and neuropoietin signaling in BA/F3 cells, similar to that of CNTF, was supported by sortilin. To that end, BA/F3-[gp130/LIFRβ] and BA/F3-[gp130/LIFRβ/sortilin] transfectants were initially stimulated with CLC/CLF-1 (40 nM), but interestingly, none of them showed any response in terms of STAT3 phosphorylation (not shown). When stimulation was conducted in the presence of sCNTFRα, however, a clear increase in phospho-STAT3 was detected in BA/F3-[gp130/LIFRβ] cells, and this response was significantly more pronounced in corresponding cells expressing sortilin (1.9-fold [standard deviation (SD), ±0.5] \(n = 2\)) (Fig. 8C). In contrast to CLC/CLF-1, even low concentrations of neuropoietin (1 nM) showed CNTFRα-independent activity, but also, in this case, the resulting increase in phospho-STAT3 levels was much more distinct in BA/F3-[gp130/LIFRβ/sortilin] cells than in BA/F3-[gp130/LIFRβ] cells (2.2-fold [SD, ±0.2] \(n = 7\)) (Fig. 8C, right). CLC/CLF-1 and neuropoietin, on the other hand, had no effect on BA/F3-[gp130], BA/F3-[gp130/sortilin], or BA/F3-[sortilin] cells (not shown).

The results confirm that the facilitating effect of sortilin is independent of CNTFRα and further suggest that this function is conditioned by an interaction between the respective ligands and the gp130/LIFRβ heterodimeric complex. Also, the appa-
ent lack of a connection between sortilin’s affinity for a particular ligand and its ability to promote signaling by the same ligand seems to reflect that in the present context, ligand binding and the facilitation of signaling are two separate functions in sortilin.

Ligand binding does not appear to be a requirement for the signal-facilitating function of sortilin. To clarify this, we first examined if the signal-facilitating effect of sortilin was abrogated in the presence of alternative ligands that target sortilin but not the gp130/LIFRβ heterodimer. To that end, BA/F3-[gp130/LIFRβ] (gray) and BA/F3-[gp130/LIFRβ/sortilin] (black) cells. A comparatively low level of gp130 was seen in the sortilin transfectants. (B) Phospho-STAT3 levels upon stimulation of the same cells with 40 nM CNTF (15 min at 37°C). Stimulation was performed in parallel with FACS analysis. (C) BA/F3 transfectants expressing the indicated receptor combinations were incubated with or without 40 nM CNTF and 4 nM sCNTFRα for 15 min at 37°C. The cells were then lysed, and the amounts of phospho-STAT3 and β-actin contained in the lysates were determined by Western blotting.

by the CNTF-tr construct, which binds to CNTFRα but not to sortilin. Findings with TF-1 (Fig. 10A) and BA/F3 (Fig. 10B) transfectants clearly demonstrated that the expression of either CNTFRα or sortilin in combination with gp130/LIFRβ strongly upregulated the response to the truncated cytokine. Moreover, the sortilin binding C-terminal peptide of CNTF was unable to alter CNTF signaling in BA/F3-[gp130/LIFRβ/sortilin] cells (Fig. 10C).

It can be concluded that sortilin promotes signaling without having to engage in ligand binding.

Sortilin promotes gp130/LIFRβ-mediated signal transduction. Given the findings described above, it seemed plausible that sortilin could promote the cellular response to other CNTF-related helical type 1 cytokines that target the gp130/LIFRβ dimer for signaling. We therefore tested STAT3 phosphorylation in TF-1 and BA/F3 cells stimulated with CT-1, LIF, and OSM. These ligands are considered not to bind the CNTFRα (20) and exhibit weak binding to s-sortilin (Table 1). However, in agreement with our assumption, cells expressing gp130/LIFRβ responded to all three ligands, and in each case, the level of phospho-STAT3 was further enhanced upon coex-
expression with sortilin (in TF-1 cells, 3.9-fold with CT-1 [SD, ±0.6] [n = 3], 2.9-fold with LIF [SD, ±0.1] [n = 3], and 1.5-fold with OSM [SD, ±0.1] [n = 3], and in BA/F3 cells, 1.8-fold with CT-1 [SD, ±0.3] [n = 4], 1.8-fold with LIF [SD, ±0.3] [n = 4], and 1.7-fold with OSM [SD, ±0.3] [n = 4]) (Fig. 11A and B).

Considering that signal induction by hIL-6 in TF-1 cells (Fig. 11C) and in BA/F3-gp130 cells (Fig. 11D) was unaltered upon transfection with sortilin (sortilin transfected-to-untransfected ratio of 0.9 [SD, ±0.1] [n = 4] for both cell lines), it appears that the facilitating effect of sortilin is restricted to the gp130/LIFRβ heterodimer, with particular reference to the function of the LIFRβ chain.

**Sortilin and LIFRβ interact in cells.** To elaborate on this idea, experiments were set up to provide evidence of a possible direct interaction between gp130/LIFRβ and sortilin. SPR analysis demonstrated that while the extracellular (C-terminally tagged) domain of gp130 did not bind to immobilized s-sortilin, the corresponding domain of LIFRβ (sLIFRβ) did (Fig. 12A). The interaction was not inhibited by a peptide (V819GPEKSMYVVTKENS) covering the C-terminal sequence of sLIFRβ and therefore did not originate from the “artificial” C terminus generated by receptor truncation (not shown). Notably, even a large surplus of NT or CNTF could inhibit binding only by about 30% (not shown), which seems in good agreement with the inability of NT to abrogate sortilin’s facilitating effect on signaling (Fig. 9A and B).

As determined by the subcellular fractionation of untransfected HEK293 cells that express both LIFRβ and sortilin, the localization of the two receptors overlapped (Fig. 12B). Notably, both were found (in CNTF-stimulated and unstimulated cells) in fractions containing flotillin-1, a marker for lipid rafts, which was suggested previously to be a functional site in gp130/LIFRβ signaling (4).

Further evidence of a possible interaction between sortilin and LIFRβ at the cellular level was next obtained by using fluorescence microscopy and Duolink, a method that visualizes interactions and/or close colocalization of single molecules. As apparent from Fig. 12C, staining with a combination of anti-sortilin and anti-gp130 did not provide (elicit) a detectable (fluorescence) signal in either untransfected HEK293 cells or in sortilin transfectants. In contrast, staining with antisortilin and anti-LIFRβ generated a strong signal in the transfectants as well as a significant (although comparatively weaker) signal in untransfected cells carrying endogenous levels of both sortilin and LIFRβ. Similar results confirming the close colocalization of the two receptors were obtained with BA/F3 cells, yet attempts to cross-link and coimmunoprecipitate sortilin and LIFRβ proved unproductive (not shown).

Taken together, the above-described results support a model in which sortilin facilitates gp130/LIFRβ-mediated signaling by interacting with LIFRβ and, e.g., increasing its affinity for...
ligands and promoting the assembly of the gp130/LIFRβ heterodimer.

**DISCUSSION**

It is well known that CNTF binds CNTFRα and subsequently recruits the gp130 and LIFRβ receptors to form a heterotrimeric signaling complex (6, 7). Here we report that a high-affinity site in the C terminus of CNTF interacts with the sortilin β-propeller domain and that sortilin may serve at least two functions in relation to CNTF. In the first place, it provides rapid cellular uptake and endocytosis of the ligand, and second, it facilitates the CNTF-mediated induction of gp130/LIFRβ signaling. Our findings further demonstrate that other ligands of CNTFRα, notably CLC/CLF-1, also interact with sortilin and that sortilin, independent of ligand binding, facilitates the signaling (STAT3 phosphorylation) of all helical type 1 cytokines targeting the gp130/LIFRβ heterodimer. Based on analysis by SPR and immunofluorescence, we propose that the latter is brought about by a direct interaction between sortilin and LIFRβ.

CNTF is internalized by cellular sortilin and targets sortilin and CNTFRα via separate sites. The binding of CNTF to sortilin was inhibited by other sortilin ligands and completely abolished by the tridecapeptide NT and a 13-residue peptide covering the C-terminal sequence of CNTF itself. In agreement with this, truncated CNTF missing the C-terminal peptide showed no binding activity. Thus, CNTF interacts with the β-propeller of sortilin via a site close to and possibly incorporating its own carboxy terminus. This is very similar to the binding mode of NT (41), and in fact, preliminary data on the crystal structure of the C-term peptide in complex with sortilin indicate that NT and CNTF may target the very same site within the tunnel of the β-propeller (M. Hansen et al., unpublished data).

CNTF has been reported to bind CNTFRα via residues located in helix A, the AB loop, helix B, and the C-terminal residues of helix D (37); hence, CNTF binds sortilin and CNTFRα via separate binding sites. Nevertheless, CNTFRα completely inhibits the binding of CNTF to s-sortilin, and CNTF is unable to bind both receptors simultaneously. Considering this and the fact that CNTFRα itself does not bind to sortilin, it is highly unlikely that sortilin in any way partakes in the sorting of isolated CNTFRα or the CNTFRα-CNTF complex. Since CNTFRα itself is not an endocytic receptor and the uptake via gp130/LIFRβ concerns only the CNTFRα-CNTF complex, there is no doubt that sortilin may contribute significantly to the uptake and clearance of extracellular CNTF (11, 46, 48). This may also be case with CLC/CLF-1, which binds sortilin with a similar high affinity. However, unlike CNTF, CLC/CLF-1 is secreted via the classical secretory pathway, and it can so far not be excluded that sortilin, once its propeptide has been cleaved, can target and transport this cytokine in Golgi compartments as well as at the cell surface (34, 38).

**Sortilin enhances CNTF signal transduction.** Following the assembly of the trimeric CNTF receptor complex, information is relayed to the nucleus by a number of signaling molecules, including STAT3. The present work demonstrates that sortilin facilitates CNTF signaling as determined by STAT3 phosphorylation as well as by the proliferation of responding cells. Sortilin’s contribution to signaling is clearly less significant than that of CNTFRα, which serves to concentrate CNTF on the

| Ligand | Kd of s-sortilin (nM) |
|--------|----------------------|
| CNTF   | ~25                  |
| CLC/CLF-1 | ~10                 |
| Neuropoietin | ~100        |
| CT-1    | ~200                 |
| LIF     | ~700                 |
| OSM     | No binding           |
| IL-6    | ~700                 |
| IL-11   | Not tested           |

**TABLE 1. Affinity of helical type 1 cytokine binding to immobilized s-sortilin determined by Biacore analysis**
cell surface and at the same time, in complex with CNTF, binds and assembles the gp130/LIFR\textit{H9252}. On the other hand, the effect of sortilin seemingly depends neither on the presence of CNTFR\textit{H9251} nor on the binding of ligand; i.e., enhanced signaling was seen with both wt CNTF and a C-terminally truncated CNTF that does not bind sortilin. Thus, sortilin-mediated signaling appears to be conditioned by only two things: the expression of gp130/LIFR\textit{H9252} and the presence of a ligand that can bind this heterodimer. It follows that sortilin cannot replace CNTFR\textit{H9251} but adds to signal transduction by a separate mechanism (9).

Sortilin may promote signaling by helical type 1 cytokines through an interaction with the LIFR\textit{H9252}. The above-described conclusion is underscored and expanded by our findings for the related cytokines CT-1, LIF, OSM, and IL-6. Thus, sortilin enhanced STAT3 phosphorylation in gp130/LIFR\textit{H9252}-expressing cells responding to CT-1, LIF, and OSM, although all three are independent of CNTFR\textit{H9251} and exhibit little or no binding to sortilin. In contrast, signaling by IL-6 in cells expressing gp130 or gp130/LIFR\textit{H9252} was unaltered by the presence of sortilin.

The latter observation is particularly informative because it strongly indicates that LIFR\textit{H9252}, rather than gp130, is directly implicated in sortilin function. This notion was supported by our SPR analysis of the interaction between s-sortilin and the ectodomains of the two signaling receptor chains. Sortilin was previously shown to bind other transmembrane proteins (29, 35), and whereas gp130 did not bind, LIFR\textit{H9252} bound with medium affinity, and saturating concentrations of neither NT nor CNTF could reduce binding by more than 30%. Soluble sortilin was not seen to facilitate signaling (not shown), yet the results imply that full-length sortilin and LIFR\textit{H9252} may interact on the cell membrane and thereby promote gp130/LIFR\textit{H9252}-mediated signal transduction. In support of this, sortilin and LIFR\textit{H9252} are both found in flotillin-1-containing cell fractions and exhibit distinct colocalization in cells. Thus, it is conceivable that the binding to sortilin may cause, e.g., a conformational change that increases LIFR\textit{H9252}'s affinity for cytokine ligands or, perhaps, even gp130. This implies that the effect of sortilin could be important in tissues with no or low levels of expression of CNTFR\textit{H9251} and under conditions of low concentrations of CNTFR\textit{H9251}-CNTF or CNTF alone in circulation. However, our findings obviously do not allow definitive conclusions. The fact that sortilin facilitated signaling in the absence of its cytoplasmic tail suggests that its effect is related to the ectodomain and/or transmembrane domain and events on the plasma membrane, but different/alternative mechanisms, including receptor translocation, may be involved, and although it seems unlikely, it cannot be entirely excluded that changes due to transfection may play a role.

In conclusion, we demonstrate that sortilin mediates the cellular uptake of CNTF and related helical type 1 cytokines targeting CNTFR\textit{H9251}, in addition to being a facilitator of cytokines that signal through the gp130/LIFR\textit{H9252} heterodimer. The latter function is independent of both CNTFR\textit{H9251} and ligand binding to sortilin and seems to implicate a direct interaction with LIFR\textit{H9252}. Even though the contribution from sortilin is perhaps modest, it is nonetheless clear, and it may implicate...
sortilin in physiological processes in which these cytokines play important roles. Thus, future \textit{in vivo} studies should reveal the potential role of sortilin in the modulation of helical type 1 cytokine function.

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