

Video Article

SorLA and CLC:CLF-1-dependent Downregulation of CNTFR α as Demonstrated by Western Blotting, Inhibition of Lysosomal Enzymes, and Immunocytochemistry

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Abstract

The heterodimeric cytokine Cardiotrophin-like Cytokine:Cytokine-like Factor-1 (CLC:CLF-1) targets the glycosylphosphatidylinositol (GPI)-anchored CNTFR α to form a trimeric complex that subsequently recruits glycoprotein 130/Leukemia Inhibitory Factor Receptor- β (gp130/LIFR β) for signaling. Both CLC and CNTFR α are necessary for signaling but so far CLF-1 has only been known as a putative facilitator of CLC secretion. However, it has recently been shown that CLF-1 contains three binding sites: one for CLC; one for CNTFR α (that may promote assembly of the trimeric complex); and one for the endocytic receptor sorLA. The latter site provides high affinity binding of CLF-1, CLC:CLF-1, as well as the trimeric (CLC:CLF-1:CNTFR α) complex to sorLA, and in sorLA-expressing cells the soluble ligands CLF-1 and CLC:CLF-1 are rapidly taken up and internalized. In cells co-expressing CNTFR α and sorLA, CNTFR α first binds CLC:CLF-1 to form a membrane-associated trimeric complex, but it also connects to sorLA via the free sorLA-binding site in CLF-1. As a result, CNTFR α , which has no capacity for endocytosis on its own, is tugged along and internalized by the sorLA-mediated endocytosis of CLC:CLF-1.

The present protocol describes the experimental procedures used to demonstrate i) the sorLA-mediated and CLC:CLF-1-dependent downregulation of surface-membrane CNTFR α expression; ii) sorLA-mediated endocytosis and lysosomal targeting of CNTFR α ; and iii) the lowered cellular response to CLC:CLF-1-stimulation upon sorLA-mediated downregulation of CNTFR α .

Video Link

The video component of this article can be found at <https://www.jove.com/video/55019/>

Introduction

The CLC and CLF-1 constitute the two subunits of the heterodimeric cytokine CLC:CLF-1¹⁻³. For cellular signal induction, CLC:CLF-1 first targets the CNTFR α , its primary and GPI-anchored receptor, to form a membrane-bound trimeric complex. The CLC:CLF-1:CNTFR α complex subsequently recruits gp130/LIFR β which mediates transmembrane signaling via the Janus Kinase/Signal Transducers and Activators of Transcription 3 (JAK/STAT3) pathway⁴. Mice deficient in any of the three subunits display one and the same phenotype and die within 24 h after birth due to a reduced number of facial neurons and insufficient suckling⁵⁻⁸. Findings in humans likewise underscore the functional coherence of the three subunits. Thus, human mutations (homozygote or compound) causing dysfunction of CLC:CLF-1 or CNTFR α , all result in the so-called cold-induced sweating/Crisponi syndrome, a condition characterized by symptoms such as impaired suckling and swallowing, various dysmorphic features, temperature spikes, and paradoxical and profuse sweating at low temperatures⁹⁻¹¹.

In vitro, the combination of CLC and CNTFR α is both necessary and sufficient for interaction with gp130/LIFR β and the induction of signaling in cells¹². The role of CLF-1 on the other hand is less clear. It is not directly involved in signaling, and has long been regarded as an appendix that mainly serves to facilitate the cellular secretion of CLC¹. However, recent findings show that CLF-1 has additional and more important functions implicating both the signaling and turnover of CLC and CNTFR α . Thus, it appears that CLF-1 contains three independent binding sites: one for its well-known binding to CLC; one that mediates direct binding to the CNTFR α ; and a third (high affinity) site for interaction with the endocytic receptor sorLA. As both CLC and CLF-1 seem to target CNTFR α with a considerable lower affinity than the CLC:CLF-1 complex, it is conceivable that CLF-1 (via its CNTFR α -binding site) promotes the unification of CLC and CNTFR α and thereby facilitates signaling¹³.

CLF-1's interaction with sorLA, the main issue of the present presentation, plays a completely different role. SorLA is one of the five type 1 receptors that constitute the Vps10p-domain receptor family¹⁴. It is expressed in a variety of tissues but in particular in brain and neuronal tissues¹⁵. Similar to the other family members sorLA carries an N-terminal ligand binding Vps10p-domain, but in addition, it also comprises other domain types including ligand-binding elements found in members of the low-density lipoprotein receptor family¹⁵. Its cytoplasmic tail interacts with several adaptor proteins, e.g., adaptor protein-1 and -2, and sorLA conveys efficient endocytosis as well as intracellular sorting and transport of bound proteins¹⁶⁻¹⁸. The Vps10p-domain comprises a large ten-bladed β -propeller^{19,20}, which binds a series of unrelated ligands including CLF-1 (but notably, not CLC)¹³. The binding site in CLF-1 is accessible even after complex formation with CLC and CNTFR α which means that sorLA binds not only free CLF-1, but also CLC:CLF-1 and the trimeric complex CLC:CLF-1:CNTFR α ¹³. SorLA conveys rapid endocytosis of

CLF-1 and CLC:CLF-1, but these are soluble proteins whereas CNTFR α is fixed to the surface membrane by a GPI-anchor. The question is therefore if binding of CLC:CLF-1:CNTFR α allows sorLA to internalize the entire complex and thereby to alter the surface-membrane expression of CNTFR α (and the subsequent cellular susceptibility to CLC:CLF-1 signal induction), and/or the turnover of CNTFR α .

The experiments described in the present report were designed to clarify the following questions:

Does sorLA mediate CLC:CLF-1-dependent downregulation of surface-membrane CNTFR α ? To clarify this, it was initially tried to determine the turnover of CNTFR α (in the absence and presence of sorLA) by means of metabolic labeling and pulse-chase experiments as described in²¹. However, attempts to label CNTFR α using a mixture of [³⁵S] cysteine and [³⁵S] methionine showed poor incorporation of radioactivity. This suggested a very low degree of new-synthesis, which in all probability would be unable to compensate for a sudden and significant loss of receptors. To determine if CNTFR α was downregulated when interconnected via CLC:CLF-1 to sorLA, it was therefore decided simply to measure (using Western blotting) the total cellular pool of CNTFR α before and after exposure to CLC:CLF-1 - and to compare results in cells transfected or not transfected with sorLA.

Does sorLA target CNTFR α to lysosomes? To answer this question, the localization of CNTFR α — internalized via its CLC:CLF-1-mediated binding to sorLA — was examined using immunocytochemistry, and labeling with antibodies directed towards CNTFR α and the late-endosome/lysosome marker Lysosomal-associated Membrane Protein 1 (LAMP-1). The experiment was performed on cells treated as well as untreated with inhibitors of lysosomal enzymes. The idea was of course that if CNTFR α was degraded in lysosomes, cells treated with enzyme-inhibitors would present CNTFR α -staining accumulating in LAMP-1 positive vesicles, whereas untreated cells would show little or no staining for CNTFR α .

Does CNTFR α downregulation lower the cellular response to CLC:CLF-1 stimulation? The trimeric complex consisting of CLC, CLF-1, and CNTFR α signals via the gp130/LIFR β heterodimer using the JAK/STAT3 pathway. Accordingly, the capacity of cells to respond to CLC:CLF-1 signaling upon downregulation of CNTFR α was explored by Western blot detection and quantitation of the phospho-STAT3 (pSTAT3)/total STAT3 level in sorLA transfectants.

Protocol

1. Western Blotting of CNTFR α Lysates

- Express full-length sorLA and Myc-tagged CNTFR α constructs in human embryonic kidney 293 (HEK293) cells using pcDNA3.1/zeo(-) and pcDNA3.1/hyg(-), respectively. Transfect the HEK293 cells with the constructs using a commercial transfection reagent according to manufacturer's protocol. Select stable clones in medium containing 150 μ g/mL Zeocin and/or 500 μ g/mL Hygromycin B Gold¹³.
- Seed equal numbers of HEK293 transfectants expressing either CNTFR α -Myc or co-expressing CNTFR α -Myc/sorLA in two 4-well plates as shown in **Figure 1**. Culture the cells in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin (full medium) in a 5% carbon dioxide incubator at 37 °C.
- Wait until the cells are 50 - 80% confluent.
- Remove the medium from the wells, wash once with Dulbecco Phosphate Buffered Saline (DPBS), and add fresh full medium with or without (control) 10 nM CLC:CLF-1 as indicated in **Figure 1**. Incubate the cells at 37 °C.
- After 0 and 5 h of incubation, recover the medium and store it at 4 °C in 1.5 mL tubes. Then add 1% Triton X-100 lysis buffer (20 mM Tris-HCl, 10 mM EDTA, pH 8.0) supplemented with a proteinase inhibitor cocktail to each well (1 min, 4 °C). Subsequently, transfer the cell lysates to 1.5 mL tubes (4 °C).
- Spin the tubes containing the medium and cell lysate (3,000 x g, 3 min, 4 °C) and transfer the supernatants to 1.5 mL tubes (4 °C). Discard the pellet. Transfer 5 μ L of each cell lysate supernatant to new 1.5 mL tubes — use them to measure the protein content later (step 1.7). Immediately, add nonreducing LDS sample buffer to all the supernatants and vortex the samples.
- Use the 5 μ L aliquots to measure the protein content in the cell lysate supernatants using a commercial protein assay according to manufacturer's protocol.
- Heat the samples at 95 °C for 5 min.
- Subject an equal amount of protein from each sample to reducing SDS-PAGE¹³ and Western blotting¹³ and visualize the content of sorLA, β -actin, and CNTFR α -Myc in the medium and in the cell lysates using rabbit anti-sorLA¹⁶ (5 μ g/mL), mouse anti- β -actin (0.4 μ g/mL), and mouse anti-Myc (1 μ g/mL) antibodies and the appropriate HRP-linked secondary antibodies (1:2,000).
- Quantify the bands by densitometry according to manufacturer's protocol.

2. Immunocytochemistry in Combination with Lysosomal Inhibitors

- Seed HEK293 cells stably transfected with CNTFR α -Myc/sorLA on cover slides in a 4-well plate as shown in **Figure 2**. Culture the cells in full medium as above.
- Wait until cell confluence is 20 - 40%.
- Remove the medium from the wells and add full medium with or without 50 μ g/mL of leupeptin and of pepstatin A (leu/pep) (inhibits lysosomal hydrolases) to the cells as indicated in **Figure 2**. Incubate the cells at 37 °C. The medium must be replaced every 6 h during the following 24 h of incubation.
- After 24 h of incubation, once again replace the medium (with or without leu/pep) and this time add 10 nM CLC:CLF-1. Incubate the cells at 37 °C for 5 h.
- Wash the cells once in PBS and fix them in 4% paraformaldehyde, pH 7 for 15 min at RT.
Caution: Paraformaldehyde is highly toxic, avoid contact with skin, eyes, and mucous. Gloves and safety glasses should be worn and solutions made inside a fume hood.
- Wash the cells once in PBS and permeabilize the cells for 5 min at RT in saponin diluted in PBS (0.5% saponin).
- Dilute goat anti-CNTFR α (1 μ g/mL) (notably not the mouse anti-Myc used for Western blotting) and mouse anti-LAMP-1 (15 μ g/mL) antibodies in 0.5% saponin and incubate the cells with the antibodies for 2 h at RT.

8. Wash the cells 4x (5 min) in 0.5% saponin and incubate the cells with donkey anti-goat Alexa 488- and donkey anti-mouse Alexa 568-conjugated secondary antibodies (6 µg/mL) for 2 h at RT.
9. Wash the cells 4x (5 min) in 0.5% saponin, twice in deionized water (1 min), and mount the cover slides on microscope slides using mounting media.
10. Analyze the antibody-staining of the cells by confocal microscopy using a laser-scanning confocal microscope with a 63X C-apochromat water immersion objective, NA 1.2.

3. Western Blot-detection of the pSTAT3 Level

1. Seed HEK293-sorLA cells (which express endogenous levels of CNTFR α) in a 4-well plate as shown in **Figure 3**. Culture the cells in full medium as above.
2. Wait until the cells are 50 - 80% confluent.
3. Remove the medium from the wells, wash once in PBS, and add fresh full medium with or without 10 nM CLC:CLF-1 to the cells as indicated in **Figure 3**. Incubate the cells at 37 °C for 5 h.
4. Remove the medium, wash the cells twice in unsupplemented DMEM (blank medium; without FBS and antibiotics), and then reincubate the cells for 90 min (37 °C) in blank medium.
5. Once again, remove the medium and add fresh blank medium with or without 5 nM CLC:CLF-1 (to initiate STAT3 phosphorylation) as indicated in **Figure 3**. Incubate for 15 min at 37 °C.
6. Quickly remove the medium and lyse the cells (1 min, 4 °C) by adding 1% Triton X-100 lysis buffer (20 mM Tris-HCl, 10 mM EDTA, pH 8.0) supplemented with a proteinase inhibitor cocktail and a phosphatase inhibitor cocktail to each well. Subsequently, transfer the cell lysates to 1.5 mL tubes (4 °C).
7. Transfer 5 µL of each supernatant to new 1.5 mL tubes — use them to measure the protein content later (step 3.8). Immediately, add nonreducing LDS sample buffer to all the supernatants and vortex the samples.
8. Measure the protein content of the 5 µL aliquots using a commercial protein assay according to manufacturer's protocol.
9. Sonicate each sample at RT until they are no longer viscous (approximately 1 min) and subsequently heat them at 95 °C for 5 min.
10. Subject an equal amount of protein from each sample to SDS-PAGE¹³ and Western blotting¹³ and visualize the content of pSTAT3 and total STAT3 in the cell lysates using rabbit anti-pSTAT3 (1:2000) and mouse anti-STAT3 (1:1,000) antibodies and the appropriate HRP-linked mouse and rabbit secondary antibodies (1:2,000).
11. Quantify the bands by densitometry according to manufacturer's protocol.

Representative Results

Figure 1 shows a schematic representation of the cell-seeding and CLC:CLF-1 incubation in protocol 1. **Figure 2** shows a schematic representation of the cell-seeding and leu/pep and CLC:CLF-1 incubation in protocol 2. **Figure 3** shows a schematic representation of the cell-seeding and CLC:CLF-1 incubation and stimulation in protocol 3. **Figure 4** shows the sorLA-mediated CLC:CLF-1-dependent downregulation of CNTFR α . **Figure 5** shows the sorLA-mediated endocytosis and lysosomal targeting of CNTFR α . **Figure 6** shows the lower response to CLC:CLF-1 stimulation after CNTFR α downregulation.

Note that bands signifying CNTFR α -Myc have similar density at time zero, whereas downregulation of CNTFR α -Myc is demonstrated by the weaker band in sorLA transfectants after 5 h (**Figure 4**). **Figure 5** shows that CNTFR α -Myc has accumulated in LAMP-1 positive vesicles of leu/pep treated cells. In contrast, no accumulation of CNTFR α -Myc is seen in cells not subjected to leu/pep treatment. This demonstrates that CNTFR α -Myc is sorted to lysosomes and degraded. As can be seen in **Figure 6**, the level of pSTAT3 is significantly reduced in pre-stimulated cells as compared to the level in cells that has not been exposed to CLC:CLF-1. This is in accordance with the observed sorLA-mediated downregulation of CNTFR α in the pre-stimulated cells (**Figure 4**).

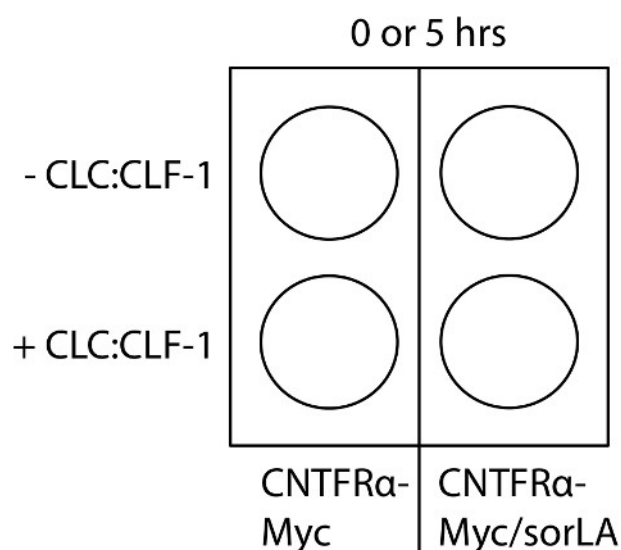


Figure 1: Schematic representation of cell-seeding and CLC:CLF-1 incubation in protocol 1. Seed HEK293-CNTFR α -Myc and HEK293-CNTFR α -Myc/sorLA cells in two 4-well plates (0 and 5 h) and incubate cells with CLC:CLF-1 as indicated. [Please click here to view a larger version of this figure.](#)

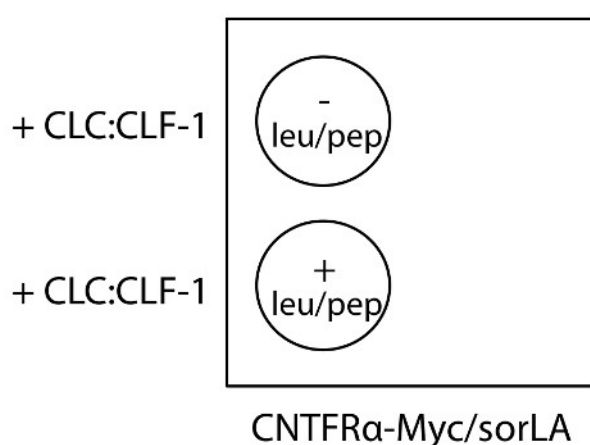


Figure 2: Schematic representation of cell-seeding and leu/pep and CLC:CLF-1 incubation in protocol 2. Seed HEK293-CNTFR α -Myc/sorLA cells on cover slides in a 4-well plate and incubate cells with or without leu/pep and CLC:CLF-1 as indicated. [Please click here to view a larger version of this figure.](#)

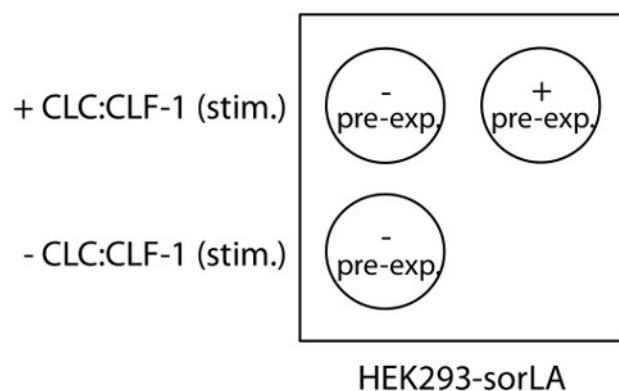


Figure 3: Schematic representation of cell-seeding and CLC:CLF incubation and stimulation in protocol 3. Seed HEK293-sorLA cells in one 4-well plate and incubate cells with or without CLC:CLF-1 (pre-exp.) to downregulate the CNTFR α followed by CLC:CLF-1 stimulation (stim.) to initiate STAT3 phosphorylation as indicated. [Please click here to view a larger version of this figure.](#)

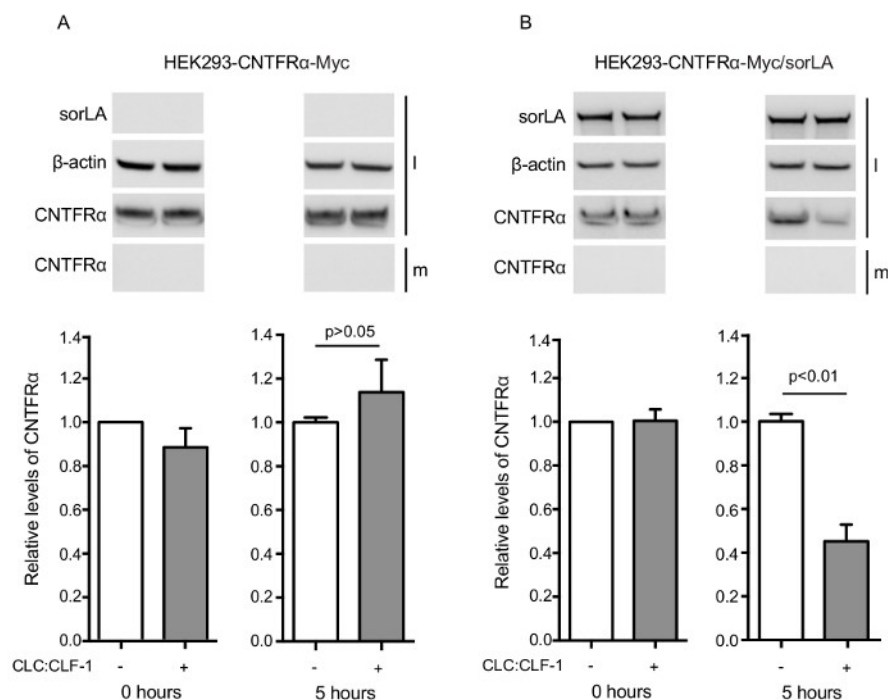


Figure 4: Results showing that SorLA mediates CLC:CLF-1-dependent downregulation of CNTFRα. (A) HEK293-CNTFRα-Myc and (B) HEK293-CNTFRα-Myc/sorLA cells were incubated in the absence (white columns) or presence (grey columns) of 10 nM CLC:CLF-1. After 0 and 5 h, the incubation was stopped and the content of CNTFRα-Myc in the medium (m) and in the cell lysates (l) was detected by Western blotting and quantified by densitometry. The upper panels show Western blot results from representative experiments and the lower panels show the detected levels of CNTFRα-Myc found in the cell lysates. The levels are shown relative to the CNTFRα-Myc level in the single and double transfectants at 0 h. Each column represents mean \pm SEM (n = 3). p-values calculated using t-test. Reproduced after original figure¹³. [Please click here to view a larger version of this figure.](#)

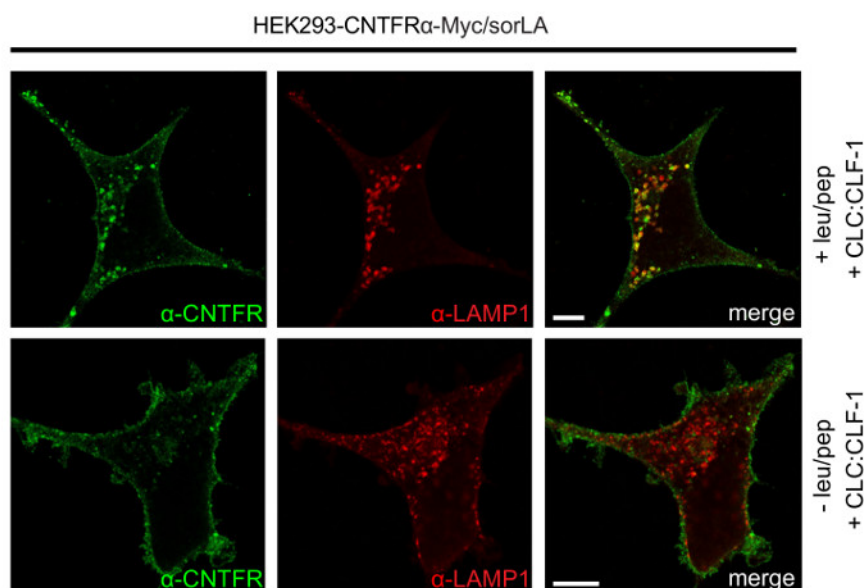


Figure 5: Results showing that SorLA mediates endocytosis and lysosomal targeting of CNTFRα. HEK293-CNTFRα-Myc/sorLA cells were treated with or without leu/pep, incubated with 10 nM CLC:CLF-1 for 5 h, fixed, and finally stained using anti-CNTFRα and anti-LAMP-1 antibodies as described in protocol 2. Scale bars: 5 μ m. Reproduced after original figure¹³. [Please click here to view a larger version of this figure.](#)

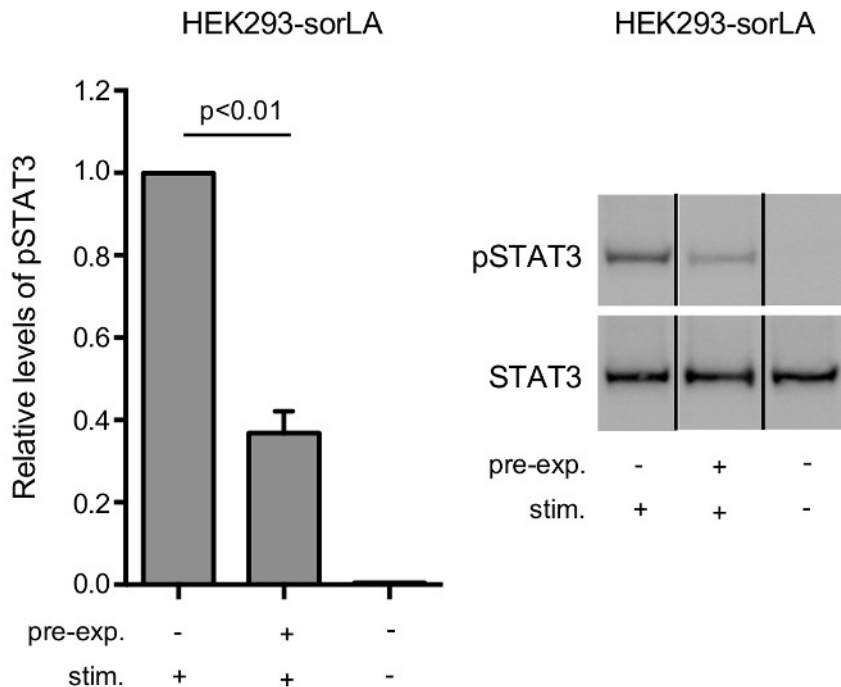


Figure 6: SorLA-mediated downregulation of CNTFR α is accompanied by a lowered cellular response to CLC:CLF-1 stimulation. HEK293-sorLA cells were pre-incubated in the absence or presence of 10 nM CLC:CLF-1 for 5 h (pre-exp.), starved in blank medium for 90 min, and stimulated with 5 nM CLC:CLF-1 for 15 min (stim.). (left) The columns show the relative levels of pSTAT3 in the cells. Each column represents mean \pm SEM ($n = 3$) relative to the pSTAT3 level in cells preincubated in the absence of CLC:CLF-1 but stimulated with CLC:CLF-1. p-value calculated using t-test. (right) The Western blot shows the response of pSTAT3 and STAT3 obtained in a representative experiment. Reproduced after original figure¹³. [Please click here to view a larger version of this figure.](#)

Discussion

The protocol described here can be used specifically to demonstrate the sorLA-mediated CLC:CLF-1-dependent downregulation and lysosomal targeting of CNTFR α , as well as the accompanying weakened response to CLC:CLF-1 stimulation.

The HEK293 cell line is well-suited for this protocol, as they have only a minor endogenous expression of CNTFR α and sorLA, are easy to transfect, express gp130/LIFR β , and have a large cell body, which is suited for immunocytochemistry. However, in theory any cell line with similar properties should work as well.

The protocol has two critical steps. The first concerns step 2.3, in which the leu/pep medium must be replaced approximately every 6 h for 24 h. Failure to do so may result in active lysosomal enzymes and less or no detectable accumulation of protein in the lysosomes. The second critical step (3.4) concerns washing upon pre-incubation with CLC:CLF-1. It is important to remove any unbound ligand, and to allow the cells time to recover (avoiding continued stimulation) in unsupplemented DMEM (blank medium). This will ensure a low background level of pSTAT3 during the subsequent re-stimulation with CLC:CLF-1 (step 3.5).

Notably, **Figure 6** demonstrates that the cellular response (pSTAT3) decreases in parallel with the sorLA-mediated downregulation of CNTFR α . It should be emphasized however, that although a reduced response is in accordance with (and to be expected upon) a reduction of the CNTFR α pool, it does not prove that the low CNTFR α expression alone accounts for the reduced cellular response. Thus, changes — resulting from pre-stimulation or transfection — in any of the functional elements of the signaling pathway upstream to pSTAT3 may have contributed to the altered response.

The basic concept of the protocol is not limited to this particular receptor system. By modifying the protocol (*i.e.* another cell line, other antibodies, other ligands, *etc.*) it can be used to determine the ligand-induced downregulation of other receptors as well — regardless of sorLA's involvement. However, it should be noted that analysis of receptor-downregulation by Western blotting is mainly suited for receptors, *e.g.*, GPI-anchored receptors, which exhibit a slow turnover and a low degree of new synthesis in unstimulated cells. Thus, in receptors showing a high level of new-synthesis, ligand-induced downregulation may be compensated for by synthesis of new receptors. Under these circumstances, the downregulation of the receptor pool can instead be determined by means of metabolic labeling and pulse-chase experiments as described in²¹. Alternatively, iodinated antibodies (preferably Fc-fragments) that do not interfere with receptor binding can be used to "tag" the ectodomain of the receptor, and the expected downregulation can then be determined by radioactive counting of cell pellet and medium.

For logistic reasons we transfected our cells with a CNTFR α construct carrying a Myc-tag, and in the present protocol a mouse anti-Myc antibody was used for Western blot-detection of the receptor. In contrast, a goat anti-CNTFR α antibody was used for immunocytochemistry and double labeling as LAMP-1 was detected by a mouse antibody — and obviously use of two primary mouse antibodies would result in unspecific

(overlapping) staining with secondary antibodies. Note that since the goat anti-CNTFR α also works in Western blotting (not shown), the protocol could easily be modified and applied to cells transfected with untagged CNTFR α .

Finally, it has recently been shown that also the endocytic receptor sortilin binds CLC:CLF-1 with high affinity²². Thus, it is conceivable that sortilin, like sorLA, interconnects to CLC:CNTFR α via CLF-1 and is able to mediate endocytosis and lysosomal targeting of CNTFR α as well. Using sortilin instead of sorLA, the present protocol may be used to clarify this question.

Disclosures

The authors have nothing to disclose.

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