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Membrane distal cytokine binding domain of LIFR interacts with soluble CNTFR in vitro

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Abstract Ciliary neurotrophic factor (CNTF) is a member of the gp130 family of cytokines. The functional receptor complex of CNTF is composed of the CNTF receptor α (CNTFR), gp130 and the leukemia inhibitory factor receptor (LIFR). Three regions on CNTF have been identified as binding sites for its receptors. The ligand–receptor interactions are mediated through the cytokine binding domains (CBDs) and/or the immunoglobulin-like domains of the receptors. However, in the case of CNTF, the precise nature of the protein–protein contacts in the signaling complex has not yet been resolved. In this study, we provide the first demonstration that the membrane distal CBD (CBD1) of LIFR associates in vitro with soluble CNTFR in the absence of CNTF. Moreover, purified CBD1 partially blocks CNTF signaling, but not that of interleukin-6 or LIF, in human embryonal carcinoma cell line Ntera/D1 cells. These data raise the possibility that LIFR has the capability to form a ligand-free complex with CNTFR.

Key words: Leukemia inhibitory factor receptor; Ciliary neurotrophic factor receptor; CNTF; LIF; Interleukin-6; Cytokine

1. Introduction

Ciliary neurotrophic factor (CNTF) belongs to the gp130 cytokine family which includes leukemia inhibitory factor (LIF), interleukin-6 (IL-6), interleukin-11 (IL-11), oncostatin M (OSM), cardiotrophin-1 (CT-1) [1,2] and cardiotrophin-like cytokine (CLC) [3,4]. All these cytokines use gp130 as one of their signal-transducing subunits, and CNTF, LIF, OSM, CLC and CT-1 also use the LIF receptor (LIFR) as another signaling molecule in their receptor complexes. IL-6, IL-11, and CNTF must first bind to their specific, non-signaling α-receptors before they can subsequently induce the dimerization of signaling receptor subunits.

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Abbreviations: CNTF, ciliary neurotrophic factor; sCNTFR, soluble CNTF receptor α; LIF, leukemia inhibitory factor; LIFR, LIF receptor β; IL-6, interleukin-6; IL-11, interleukin-11; OSM, oncostatin M; CT-1, cardiotrophin-1; CLC, cardiotrophin-like cytokine; NT-2, human embryonal carcinoma cell line Ntera/D1; CBD, cytokine binding domain; Ig, immunoglobulin; STAT, signal transducer and activator of transcription

CNTF receptor (CNTFR) is anchored to the cell membrane through a glycosylphosphatidylinositol linkage [5]; however, soluble CNTFR (sCNTFR) can also be detected under normal physiological conditions [6]. Association of CNTF with CNTFR leads to the heterodimerization of gp130 and LIFR followed by the activation of pre-associated Janus kinases (JAK1, JAK2, TYK2) [7,8]. Tyrosine-phosphorylated gp130 and LIFR in turn act as docking sites for the signal transducer and activator of transcription (STAT) molecules, such as STAT3 [9]. After tyrosine phosphorylation, STAT3 forms a homodimer, translocates into the nucleus and regulates the expression of CNTF responsive genes.

CNTFR, gp130 and LIFR belong to the class I hematopoietin receptor family, which is characterized by the cytokine binding domain (CBD) formed by two fibronectin type III (FnIII) modules linked by a proline-rich sequence [10]. The N-terminal module of the CBD contains four conserved cysteine residues that form inter-strand disulfide bonds, while the C-terminal module contains the WSXWS sequence conserved in the hematopoietin receptors. The extracellular region of CNTFR contains an N-terminal immunoglobulin (Ig)-like module and a CBD, while that of LIFR contains an N-terminal CBD (CBD1) followed by an Ig-like module, a C-terminal CBD (CBD2) and three membrane-proximal FnIII modules (Fig. 1).

As more data emerged, tripartite receptors were identified for IL-6 and CNTF, suggesting a tetrameric complex, and mutagenesis studies identified three binding sites on the gp130 family cytokines [11]. The crystal structure of CNTF has been solved revealing a four helix bundle and giving the location of the three sites (I, II and III) which are responsible for the binding of CNTFR, gp130 and LIFR [12,13]. However, the precise nature of receptor–receptor interactions in the CNTFR complex remains unclear.

To shed further light on the receptor–receptor interactions involved in CNTF signaling, a variety of domains from LIFR were expressed and assessed for interactions with sCNTFR. We report here that CBD1 of LIFR can bind to sCNTFR in the absence of CNTF. More importantly, purified LIFR CBD1 partially blocked CNTF signaling in human embryonal carcinoma cell line Ntera/D1 (NT-2) cells.

2. Materials and methods

2.1. Cells and reagents

Human NT-2 embryonal carcinoma cells (Stratagene) were routinely grown in Dulbecco's modified Eagle's medium (DMEM), sup-

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plemented with 10% fetal bovine serum. Human CNTF was from R&D Systems. Human LIF was the product of Upstate Biotechnology and IL-6 was from Invitrogen. Polyclonal goat anti-CNTF and -LIFR antibodies were purchased from R&D Systems, while antibodies detecting phospho-tyrosine STAT3 (Tyr 705) and STAT3 were from New England Biolabs. Monoclonal anti-c-myc antibody was the product of Calbiochem.

2.2. Construction of bacterial expression vectors for sCNTFR-myc, CNTF and domains within the extracellular regions of LIFR

cDNA templates encoding the full length human CNTFR and rat CNTF were reverse transcription (RT-) PCR-amplified from mRNAs of NT-2 human embryonal carcinoma cells and rat pheochromocytoma PC12 cells, respectively. Human LIFR cDNA template was RT-PCR-amplified from mRNA of SH-SY5Y human neuroblastoma cells. DNA sequencing was performed using a 310 Genetic Analyzer autosequencer (Applied Biosystems). DNA sequences were checked against the sequences in the GenBank database. The bacterial expression vector for His-tag proteins was based on pET-14b (Novagen) with slight modifications. The *Nco*I site at 580 was changed to an *Nde*I site and the *Nde*I site at 522 was mutated to an *Nco*I site and a new *Not*I site was inserted after the *Bam*HI site at 510. *Nco*I and *Not*I sites were introduced in the 5′- and 3′-primers for PCR amplification of all the constructs. Amplified PCR products were digested with *Nco*I and *Not*I restriction enzymes and subcloned into the modified pET-14b vector. Expressed peptides after N-terminal His-tag labeling were: sCNTFR-myc (M1–I334 followed by a c-myc epitope EQKLSEEDL), full length rat CNTF, CBD1 of LIFR (D52–S239), Ig-like domain of LIFR (P240–N340), CBD2 of LIFR (C341–S523), CBD1-Ig of LIFR (D52–N340), Ig-CBD2 of LIFR (P240–S523), and CBD1-Ig-CBD2 of LIFR (Q45–S523).

2.3. Protein expression, purification and refolding

Escherichia coli bacteria (strain BL21 (DE3)) were transformed with expression vectors containing cDNAs for sCNTFR-myc, rat CNTF and individual domains of LIFR. Transformed bacteria were grown to an A_{600} of approximately 0.2 at 37°C and induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h. For the purification of sCNTFR-myc, rat CNTF, CBD1 of LIFR, inclusion bodies were denatured in 8 M urea, pH 8.0. After binding to a Ni-NTA column (Qiagen), His-tagged peptides were eluted by 8 M urea, pH 4.5. Refolding of sCNTFR-myc was as previously described [13], and refolding of other proteins was achieved by dialysis against refolding buffer (10 mM Tris, pH 8.0, 1 mM reduced glutathione, 0.1 mM oxidized glutathione, 36 h). Refolded proteins were loaded on a Q Sepharose High Performance column (Pharmacia) and eluted with a salt gradient using an Akta explorer 100 FPLC purification system (Pharmacia).

2.4. Enzyme-linked immunosorbent assay (ELISA) for sCNTFR binding

Purified CNTFR-myc was coated on Immulon®2 (Dynatech) ELISA plates at 10 μg/ml. After blocking of non-specific binding with phosphate-buffered saline (PBS) containing 0.25% bovine serum albumin (BSA), purified peptides with serial dilutions in PBS–0.2 μg/μl BSA were applied. For the screening of binding capability of LIFR domains, 10 μg of the supernatants of IPTG-induced bacterial lysates after sonication was added to the wells. After incubation and washing, bound proteins were detected with specific antibodies at 1:2000 dilutions followed by horseradish peroxidase (HRP)-conjugated secondary antibodies with the same dilutions. Peroxidase activity was detected by *o*-phenylenediamine substrate and the orange-colored product was read at 490 nm using a MR 5000 microplate reader (Dynatech).

2.5. Immunoprecipitation and Western blotting for CBD1-sCNTFR pull down assay

200 ng of purified CBD1 was incubated with 200 ng of purified sCNTFR-myc, 1 μg anti-myc antibody and 10 μg BSA in 200 μl PBS overnight at 4°C. 10 μl kappa-lock (Zymed) beads were added. After 2 h incubation, beads were washed with PBS and 20 μl 2× sample buffer for SDS–PAGE was added. The boiled supernatants were collected, separated by SDS–PAGE and blotted onto nitrocellulose membranes. After blocking in TBST containing 5% milk powder, membranes were incubated with primary antibodies in 1:1000 dilutions at 25°C for 1 h. Membranes were incubated with HRP-conjugated secondary antibodies with 1:2000 dilutions for another 1 h after washing with TBST. Proteins were detected by chemiluminescence using an ECL kit (Amersham) following the manufacturer's instructions.

2.6. STAT3 phosphorylation assay

NT-2 cells were starved for 4 h in the serum-free DMEM culture medium, without antibiotics, but containing different concentrations of purified CBD1 peptide, before stimulation with cytokines. After starvation, 50 ng/ml cytokines were applied and cells were incubated at 37°C for 15 min. Cells were lysed in RIPA buffer with protease inhibitors (PBS with 0.1% SDS, 1% NP40, 0.5% deoxycholic acid, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml antipain, 5 mM benzamidine, 1 mM sodium orthovanadate, 10 μg/ml soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride, Sigma) and insoluble material was pelleted. Protein concentrations of supernatants were assayed by the Bradford method (Bio-Rad). 50 μg of total cell lysates was subjected to SDS–PAGE followed by Western blotting using an anti-phospho-tyrosine STAT3 antibody. After stripping of membranes using buffer containing 0.1 M β-mercaptoethanol, 0.0625 M Tris, pH 6.7 and 2% SDS, an anti-STAT3 antibody was used for the detection of total STAT3 expression. Band intensity was analyzed using the Eagle Eye II Still Video System (Stratagene).

3. Results

3.1. Screening of putative binding modules within extracellular regions of LIFR with sCNTFR

To assess the binding capacity of sCNTFR to the individual domains within the extracellular parts of LIFR in the absence of ligand, we set up an ELISA system for the rapid screening of the receptor domains (Fig. 2). sCNTFR was tagged with a c-myc epitope at the carboxyl-terminus, and a

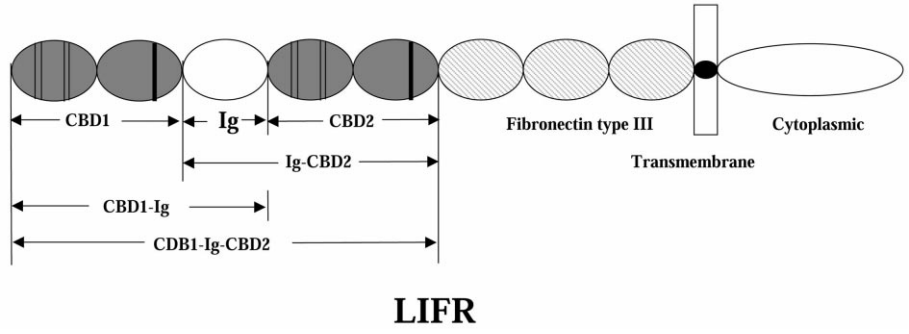

Fig. 1. Schematic representation of the LIFR domain mutants that were used in this study. CBD1, Ig, CBD2, CBD1-Ig, Ig-CBD2 and CBD1-Ig-CBD2 regions of LIFR were expressed as His-tagged peptides in *E. coli* as described in Section 2. The conserved cysteines and the WSXWS motif in the CBDs of LIFR are shown as vertical thin lines and black bars, respectively.

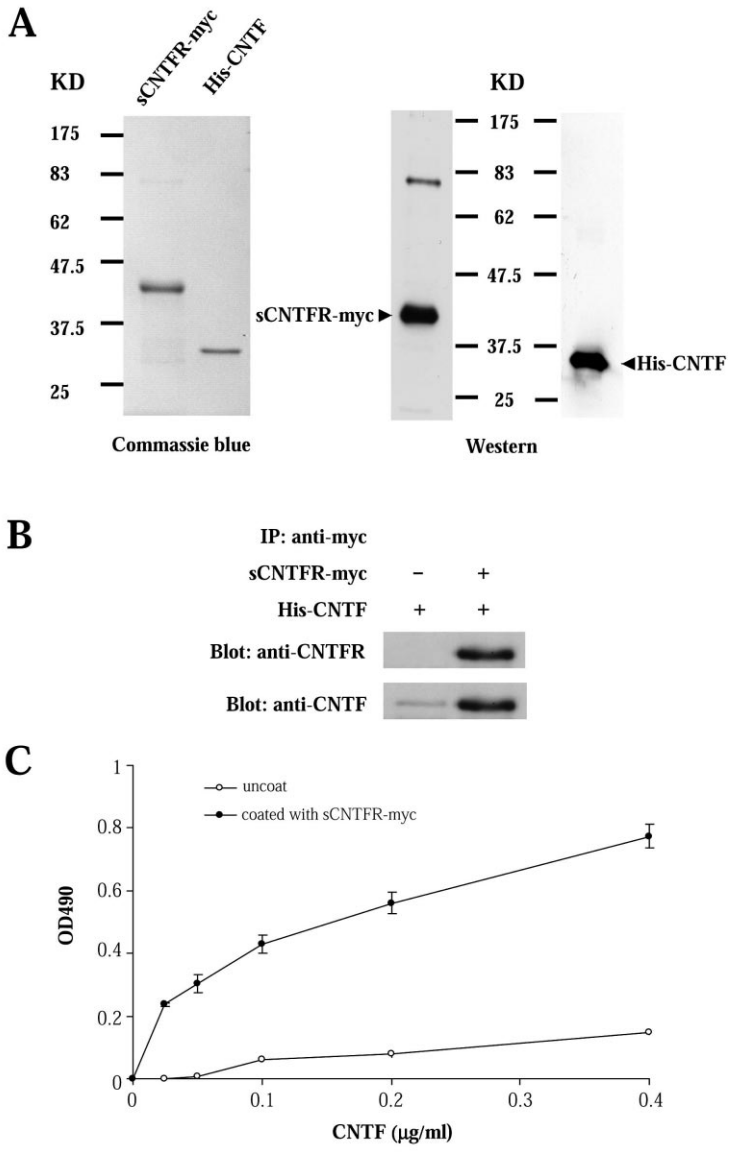

Fig. 2. Establishment of an ELISA system for screening binding partners of CNTFR. sCNTFR-myc and His-CNTF were purified to more than 90% homogeneity as shown by SDS–PAGE followed by Coomassie blue staining and Western blot (A). sCNTFR-myc could be immunoprecipitated with an anti-myc antibody and retained the capability to bind to His-CNTF (B). Binding of His-CNTF to the ELISA plate coated with sCNTFR-myc (10 μg/ml) was specific and dose dependent (C). Results shown represent the mean ± S.D. of a typical experiment, $n = 4$.

polyhistidine epitope at the N-terminus for ease of purification (Fig. 2A). Purified sCNTFR-myc retained the capacity to bind to purified His-CNTF as shown by immunoprecipitation (Fig. 2B). Purified sCNTFR-myc (10 μg/ml) was directly coated onto an ELISA plate and as low as 0.025 μg/ml CNTF could be detected using this system (Fig. 2C).

We screened for the putative binding modules within the extracellular domains of LIFR using sCNTFR. The individual domain constructs included CBD1, Ig, CBD2, CBD1-Ig, Ig-CBD2 and CBD1-Ig-CBD2 of LIFR. These domains were expressed as N-terminal His-tagged peptides in *E. coli*. After IPTG induction followed by sonication, supernatants of bacterial lysates with equal amounts of total protein were subjected to Western blotting and ELISA assays. While most of the peptides could be detected by Western blot in both the supernatants (Fig. 3A) and pellets (data not shown) after sonication, the CBD1-Ig module of LIFR could only be detected in the pellets (data not shown). ELISA screening showed that the CBD1 and CBD1-Ig-CBD2 of LIFR could bind to sCNTFR (Fig. 3B). These results strongly suggested that the CBD1 of LIFR was responsible for the binding with sCNTFR.

3.2. *In vitro binding of sCNTFR with CBD1 of LIFR*

To further confirm the binding capacity of sCNTFR with CBD1 of LIFR, we performed an in vitro pull down assay. LIFR CBD1 was expressed as a His-tagged protein, refolded and purified as described under Section 2 (Fig. 4A). The far-UV circular dichroism spectrum of purified CBD1 was consistent with the published spectra of peptides with a similar secondary structure [14,15] (data not shown). LIFR CBD1 could be pulled down by sCNTFR-myc with an anti-myc antibody (Fig. 4B). As low as 0.125 μg/ml of LIFR CBD1 could be detected using ELISA (Fig. 4C) with sensitivity comparable to that of CNTF binding.

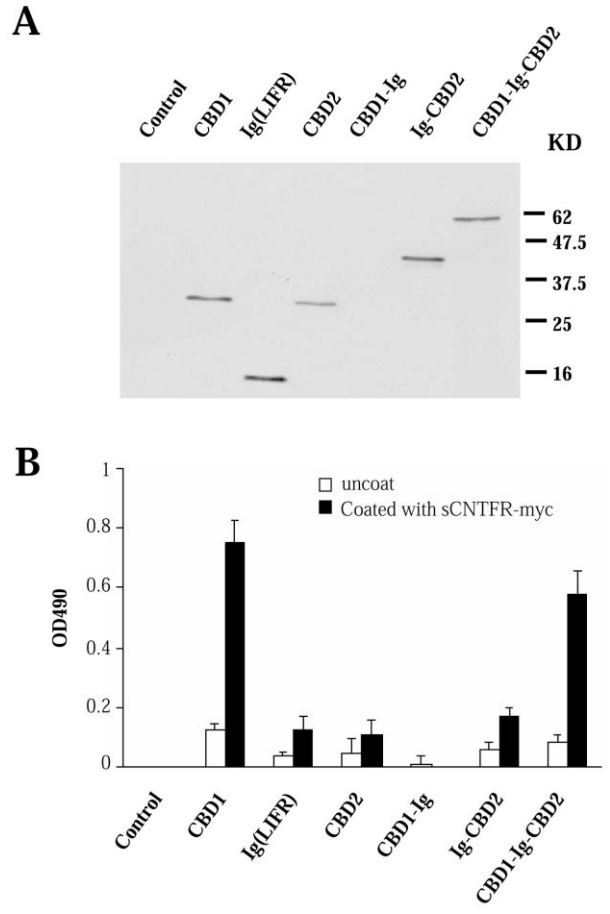

Fig. 3. ELISA screening for putative binding partners, from within LIFR, of sCNTFR. Individual mutants were expressed in BL21 (DE3) bacterial host cells. Cells were lysed by sonication after IPTG induction. Supernatants of the lysates were collected and subjected to SDS–PAGE followed by Western blot analysis using polyclonal anti-LIFR antibody (A). The His-tagged CBD1-Ig of LIFR could not be expressed as soluble protein. CBD1 and CBD1-Ig-CBD2 of LIFR (B) were found to bind strongly to sCNTFR-myc-coated plates. Bars show the mean ± S.D., $n = 3$.

3.3. Blocking of CNTF signaling in NT-2 cells by CBD1 of LIFR

As CBD1 of LIFR could bind to sCNTFR in vitro, we investigated whether this peptide could serve as an antagonist to block CNTF signaling in vivo. We chose NT-2 human embryonal carcinoma cells [16] as the assay system, as these cells had endogenous expression of CNTFR, LIFR and gp130 (Ip, N.Y. et al., unpublished observation). NT-2 cells could respond to CNTF stimulation by rapid STAT3 tyrosine phosphorylation. We first examined the in vivo interaction between CNTFR and LIFR in NT-2 cells. Immunoprecipitation of total NT-2 cell lysate with an anti-CNTFR antibody could pull down LIFR in the presence or absence of exogenous CNTF (Fig. 5A). Addition of LIFR CBD1 (1 μg/ml) could partially block CNTF signaling by ~50% (Fig. 5B,C). IL-6 and LIF signaling were not inhibited, but were enhanced in the presence of LIFR CBD1 (Fig. 5B,C).

4. Discussion

Previous study [17] has suggested CNTF forms a hexameric signaling complex. To explain this complex, a model was proposed whereby the Ig-like domain of gp130 bridged two growth hormone style trimers of specific receptor, ligand and the CBD of gp130 [18]. However, a tetrameric model has been proposed which postulates that the hexamer is an inactive state of the receptor complex, which forms at high concentrations from the active tetramer induced by ligand binding [19]. As LIFR is highly modular (Fig. 1), the different modules in this receptor are likely to have varying roles in the interaction with CNTF and CNTFR. To examine the importance of different receptor regions of LIFR in the CNTF signaling complex, we constructed a series of peptides containing all the combinations of LIFR domains likely to be involved in the CNTFR–LIFR interaction.

A sCNTFR molecule with a c-myc epitope was used as a

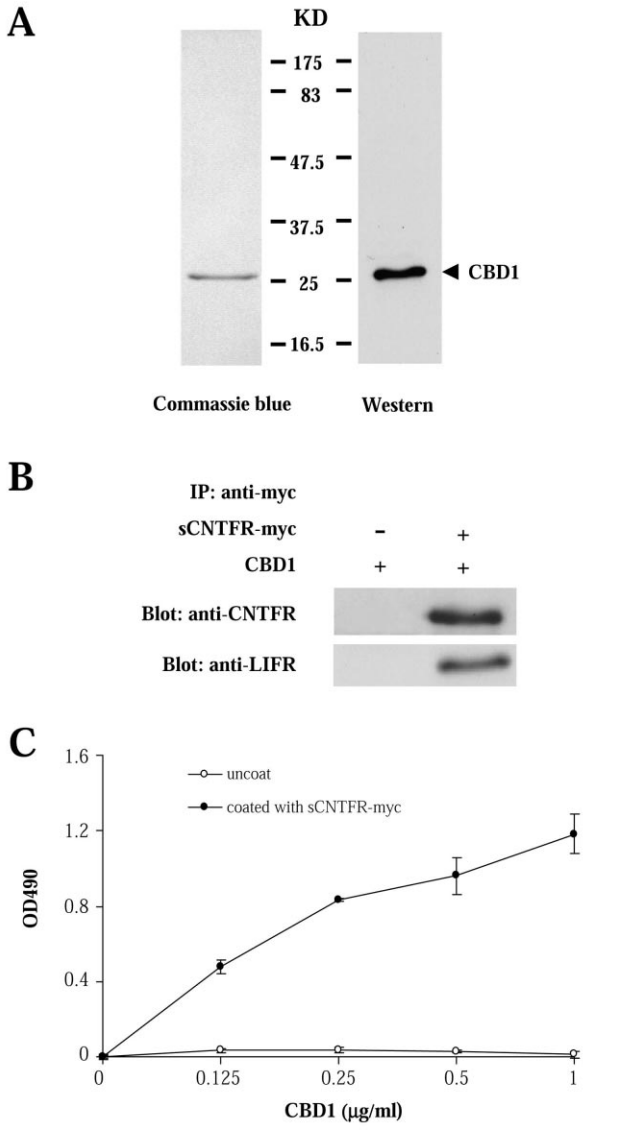

Fig. 4. In vitro binding of CBD1 of LIFR with sCNTFR-myc. CBD1 of LIFR was expressed as a His-tagged peptide and purified to more than 90% homogeneity as shown by SDS–PAGE followed by Coomassie blue staining and Western blotting using polyclonal anti-LIFR antibodies (A). sCNTFR-myc could pull down the LIFR CBD1 by immunoprecipitation with an anti-myc antibody (B). The binding of the LIFR CBD1 to an sCNTFR-myc-coated ELISA plate was dose dependent (C). Results shown represent the mean ± S.D. of a typical experiment, $n = 4$.

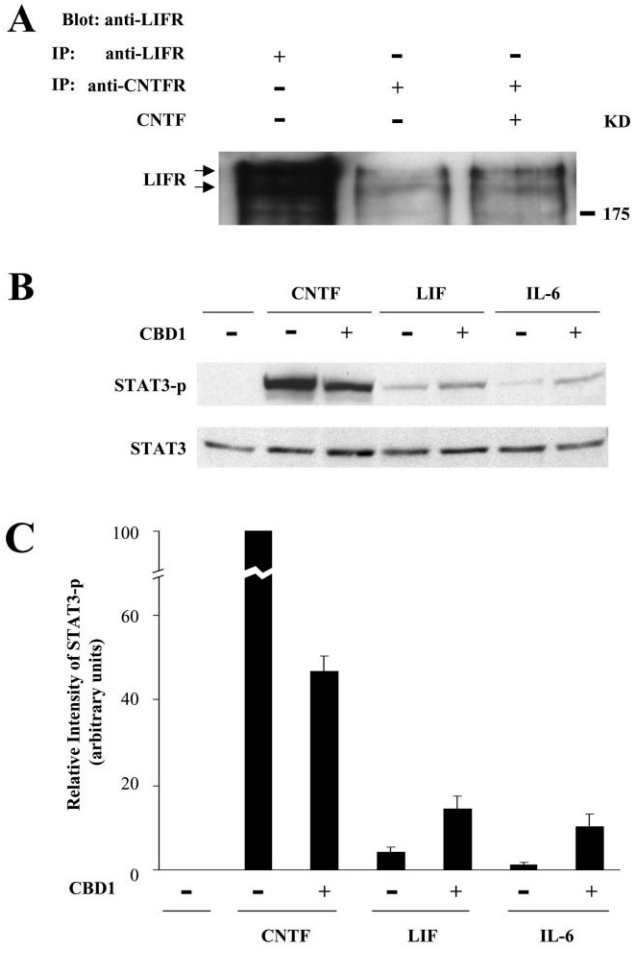

Fig. 5. Blocking of CNTF signaling in NT-2 cells by CBD1 of LIFR. Cellular CNTFR could associate with LIFR on the surface of NT-2 cells (A). Total cell lysates were collected and immunoprecipitated with an anti-CNTFR antibody. Western blot was then probed with an anti-LIFR antibody. The LIFR CBD1 (1 μg/ml) could block CNTF-induced STAT3 tyrosine phosphorylation in NT-2 cells by ~50% (B). LIF and IL-6 signaling were not inhibited, but were enhanced in the presence of LIFR CBD1. Quantitation of relative band intensity was shown as the mean ± S.E.M. of three representative experiments (C). The values are normalized to that observed with CNTF treatment (100). Similar results were obtained by using different batches of purified peptide.

potential binding partner for the LIFR peptides. The correct folding of sCNTFR was confirmed by its ability to bind to CNTF as shown by immunoprecipitation and ELISA assays. Of the five LIFR peptides examined only the peptides containing CBD1 showed binding to sCNTFR, indicating that CBD1 is likely to be the domain involved in the CNTFR–LIFR interaction. Consequently, the LIFR CBD1 was selected for further testing. It is possible that the peptides that did not bind to sCNTFR were misfolded. However, in a study of domains of LIFR [20], CBD2 of LIFR could be recovered and recognized by an anti-CBD2 antibody while CBD1 or CBD1-Ig could not be expressed. These data suggest that proper expression and folding of LIFR CBD2 may be more readily achieved than that of CBD1.

To further examine the role of the LIFR CBD1 in the interaction with sCNTFR, the peptide was purified and assayed by immunoprecipitation and ELISA. This confirmed the binding of this peptide to the sCNTFR in the absence of CNTF. Having established that a ligand-free interaction can exist in vitro between sCNTFR and the LIFR CBD1, we decided to test this peptide in vivo. NT-2 embryonal carcinoma cells which respond to CNTF stimulation by STAT3 tyrosine phosphorylation were used to assess the interaction of the CBD1 with CNTFR. It was seen that the CNTF-induced phosphorylation of STAT3 was reduced by ~50% in the presence of CBD1 of LIFR. This suggests that the soluble CBD1 binds to the same site on CNTFR as the cellular LIFR, blocking its association with CNTFR, and preventing the formation of the signal-transducing complex. Interestingly, the immunoprecipitation assay showed that cellular LIFR and CNTFR can form a complex in quiescent NT-2 cells without the addition of exogenous CNTF, raising the possibility that CNTFR could also interact with LIFR in the absence of CNTF in vivo. As we could not rule out the presence of a trace amount of endogenous ligands in NT-2 cells, further studies will be necessary to explore this interesting observation.

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