The N-terminal cytokine binding domain of LIFR is required for CNTF binding and signaling

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Received 13 June 2005; accepted 22 June 2005

Available online 18 July 2005

Edited by Masayuki Miyasaka

Abstract **Ciliary neurotrophic factor (CNTF) forms a functional receptor complex containing the CNTF receptor, gp130, and the leukemia inhibitory factor receptor (LIFR). However, the nature and stoichiometry of the receptor-mediated interactions in this complex have not yet been fully resolved. We show here that signaling by CNTF, but not by LIF or oncostatin M (OSM), was abolished in cells overexpressing a LIFR mutant with the N-terminal cytokine binding domain deleted. Our results illustrate molecular differences between the CNTF active receptor complex and those of LIF and OSM and provide further support for the hexameric model of the CNTF receptor complex.**

Keywords: Ciliary neurotrophic factor; Leukemia inhibitory factor; Oncostatin M; Neuropoietic cytokine; Interleukin-6; gp130

1. Introduction

Ciliary neurotrophic factor (CNTF) is a member of the gp130, or Interleukin (IL)-6, family of helical cytokines. It is implicated in the survival of neuronal cells [1] and the formation of neuronal sprouts [2], and may have a role in the treatment of amyotrophic lateral sclerosis [3]. Other findings show that CNTF is a potential agent in the treatment of diabetes and obesity [4] and a protective factor in demyelinating central nervous system disease [5].

The gp130 family cytokines, CNTF, leukemia inhibitory factor (LIF), IL-6, IL-11, oncostatin M (OSM), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC) and neuropoietin, all bind either a homodimer of gp130 or a heterodimer of gp130 and the LIF receptor (LIFR) to transduce a signal [6–8]. CNTF, LIF, OSM, CLC and CT-1 use LIFR, although OSM may use the OSM receptor (OSMR) instead. Moreover, IL-6, IL-11, and CNTF must first bind to their specific, non-signaling receptors (IL-6R, IL-11R and CNTFR) before binding their signaling receptors [6]. All these receptors belong to the class I hematopoietin receptor family, which is characterized by the cytokine binding domain (CBD). The extracellular region of CNTFR contains an N-terminal immunoglobulin (Ig)-like module and a CBD, while that of gp130 is similar but followed by three fibronectin type III (FnIII) domains. LIFR is similar to gp130 but possesses an extra, N-terminal, copy of a CBD. The N-terminal (or membrane distal) LIFR CBD is designated CBD1, while the other is known as CBD2.

Elucidation of the molecular details of active gp130 cytokine receptor complexes has been a challenge due to the multiple components and binding sites involved and the fact that either one or two ligand molecules may be involved in the complex. The IL-6 receptor complex was first proposed to be a hexameric complex consisting of two IL-6, IL-6R, and gp130 molecules each [9,10] although a tetrameric complex was also postulated [11]. Subsequent studies further suggested, and later showed, that the IL-6 complex formed by the association of two trimers of IL-6, IL-6R, and gp130, linked by the Ig-like domains of gp130 [12–14].

Unlike IL-6, CNTF recruits a heterodimer (LIFR and gp130) of signaling receptors so that the interactions in its receptor complex will be different from those of the IL-6 complex. While CNTF was reported to have a hexameric receptor complex based on immuno-precipitation studies [15], a tetrameric complex has also been proposed [11]. The precise nature of the protein–protein interactions in the CNTF receptor complex remains elusive. Modeling studies [13,16] suggested that the LIFR CBD1 was involved in an IL-6-like hexameric complex, binding at site II of one of the CNTF molecules in the hexamer while the gp130 CBD bound at site II of the other CNTF molecule.

Our recent findings have revealed the solution structure of the BC domain (the C-terminal FnIII domain of the CBD) of CNTFR which is expected to be involved in receptor–receptor interactions with the equivalent regions of LIFR and gp130 [17,18]. We have also demonstrated that CNTFR and either the CBD of gp130 or the LIFR CBD1 can form dimers in the absence of CNTF in vitro [18,19] and that this dimerisation is affected by mutations in the BC domains of CNTFR, gp130 and LIFR CBD1 [18]. We further showed that the LIFR CBD1 could block signaling by CNTF in NT-2 cells, yet signaling by LIF and IL-6 was not reduced [19]. These findings give support to the hexameric model of the CNTF receptor complex which predicts the interaction of these domains.

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0014-5793/$30.00
doi:10.1016/j.febslet.2005.06.061

To map the contact domains of gp130 and LIFR to CNTF, LIF, and OSM in cytokine-LFR-gp130 complexes, we have studied the binding of each cytokine with glutathione *S*-transferase (GST) fusion peptides of the CBD1, Ig-like domain, and CBD2 of LIFR, and of the Ig-like domain and CBD of gp130 in vitro. The ability of mutants of LIFR, which either lack the CBD1 (ΔCBD1) or contain point mutations within CBD1, to transduce a signal in vivo and the ability of the receptor peptides to affect cytokine signaling in vivo were also analyzed. We report here that the CBD1 of LIFR binds to CNTF and is required for CNTF signaling. Mutations in the region of the LIFR CBD1 expected to interact with CNTFR were found to affect CNTF signaling. In contrast, LIF and OSM did not bind the LIFR CBD1, nor was their signaling affected by its deletion.

2. Materials and methods

2.1. Plasmid construction, protein expression, refolding and purification for GST-LIFR and GST-gp130 fusion peptides

Human LIFR and gp130 cDNA templates were RT-PCR amplified from mRNA of SH-SY5Y neuroblastoma cells. The DNA was sequenced and evaluated as previously reported [19]. BamHI and *Not*I sites were introduced to the 5′ sense and 3′ antisense primers, the PCR products were digested with *Bam*HI and *Not*I, and ligated into PGEX-6-p1 vector. *Escherichia coli* strain BL21 (DE3) cells were transformed with expression vectors containing cDNA for the individual domains. The peptides expressed were LIFR-CBD1 (49A to 246W), Ig-like domain of LIFR (248P to 333P), LIFR-CBD2 (335T to 533A), Ig-like domain of gp130 (23E to 125P), and gp130-CBD (125P to 321T). The procedures for induction and refolding of recombinant proteins and the expression and purification of CNTFR and CNTF were as described previously [19]. Refolded proteins were purified initially with a glutathione conjugated Sepharose 4B column (Pharmacia) and further purified by a Q Sepharose High Performance column (Pharmacia) (Fig. 1A).

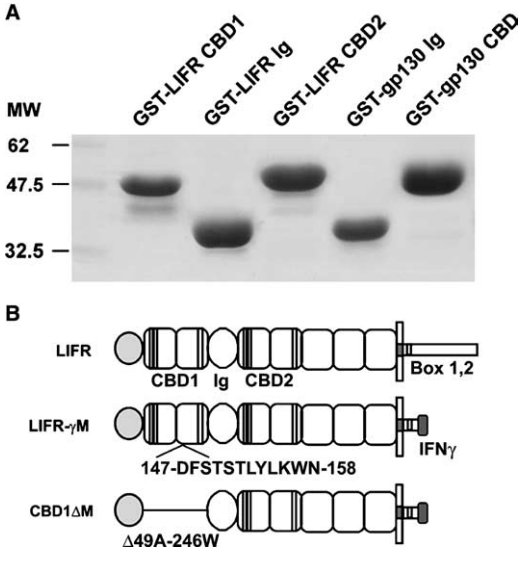

Fig. 1. (A) SDS–PAGE of the GST-fusion peptides of LIFR and gp130. (B) Schematic diagrams of the LIF receptor indicating the location of the interferon-γ sequence downstream of boxes 1 and 2 in the cytoplasmic domain and the locations of the double alanine mutations (DF-M, ST-M, ST′-M, LY-M, LK-M and WN-M) in the BC domain of CBD1 and the deletion of CBD1 (CBD1ΔM).

2.2. GST pull-down assay and Western blot

50–300 ng of human CNTF (R&D Systems), LIF (CHEMICON International), or OSM (R&D Systems) were allowed to incubate with immobilized GST fusion peptides (about 600 ng on 10 μl of glutathione–Sepharose beads) for 30 min at 4 °C. Beads were washed six times with PBS, then proteins on beads were dissolved in 2× SDS sample buffer, separated by 12% SDS–PAGE and blotted onto nitrocellulose membranes. Goat antibodies specific for human CNTF, LIF, OSM, CNTFR, LIFR and gp130 (R&D Systems) at 1:1000 dilution, or for GST (Amersham) at 1:20000 dilution, were used, as appropriate, to identify the proteins.

2.3. Construction of human gp130 and LIFR chimeras

To generate gp130γ or LIFRγ constructs, a YDKPH motif, followed by a stop-codon and a *Not*I site, was introduced into gp130 or LIFR DNA downstream of boxes 1 and 2 [6] via PCR. YDKPH is a sequence motif from the Interferon-γ receptor that, in its tyrosine-phosphorylated form, recruits STAT1 [20]. Either gp130γ or LIFRγ were inserted into expression vector pMT21 or pBluescript II KS (pB) via the *Xho*I and *Not*I sites and the DNA sequences of pMT21-gp130γ, pMT21-LIFRγ, pB-gp130γ and pB-LIFRγ were checked with a 310 Genetic Analyzer autosequencer.

2.4. LIFRγ-CBD1 mutant constructs

LIFRγ mutant constructs [D147A/F148A, S149A/T150A, S151A/T152A, L153A/Y154A, L155A/K156A, and W157A/N158A;18], corresponding to double alanine mutations in the putative AB loop and B strand of the BC domain of LIFR CBD1, were digested with *Bgl*II and *Sty*I. The resulting 210 bp nucleotide sequences containing LIFR alanine mutations were introduced into *Bgl*II/*Sty*I-digested pB-LIFRγ plasmids. The LIFRγ mutants were then subcloned into the pMT21 expression vector via *Xho*I and *Not*I sites after the sequences of the mutations were confirmed. The resulting LIFR CBD1 mutants were designated DF-M, ST-M, ST′-M, LY-M, LK-M and WN-M corresponding to the amino acid pairs from D147 to N158 (Fig. 1B). A pMT21 plasmid containing LIFRΔCBD1, which was generated by PCR based mutagenesis of the LIFR construct, was digested with *Xho*I and *Dra*I and the resulting 430 bp nucleotide fragment was introduced into the *Xho*I and *Dra*I sites of pB-LIFRγ. The LIFRγΔCBD1 was then subcloned into the *Xho*I and *Not*I sites of the pMT21 plasmid and designated as CBD1ΔM (Fig. 1B).

2.5. Transfection of 293 cells

Human 293 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 μg/ml of streptomycin, and 60 μg/ml of penicillin. These cells, which endogenously express CNTFR (Ip, NY unpublished), were transiently transfected with equal total amounts of expression vectors encoding gp130 or gp130γ, LIFR or LIFRγ or the respective mutants, and a luciferase gene reporter construct pGAS (Clontech) by applying the lipofectamine method. Less than 1 ng of the β-galactosidase control vector (Promega) was also added. Cells transfected with the constructs of the luciferase reporter gene, the β-galactosidase control vector, and gp130 and LIFR (wild-type or γ) served as controls. After 48 h, transfected cells were starved in serum-free medium for 3 h, stimulated with 26 ng/ml of the appropriate factor (CNTF, LIF, or OSM), or left unstimulated. Expression of wild-type gp130, wild-type LIFR, gp130γ and LIFRγ and the respective mutants, as well as STAT1 tyrosine phosphorylation of the stimulated cells, was analyzed by SDS–PAGE.

2.6. Reporter gene assay in transfected 293 cells

Luciferase activity, normalized to β-galactosidase activity to correct for transfection efficiency, was measured using the luciferase kit from Promega at 6 h after treatment by the factors.

2.7. Effects of exogenous LIFR and gp130 extracellular domains on cytokine signaling in human NT-2 cells

Cells in a 35 cm dish were starved for 4 h in 1 ml of serum-free DMEM culture medium. The LIFR CBD1, Ig-like domain, and CBD2, and the gp130 CBD and Ig-like domain peptides were generated by removal of the GST tag after digestion with PreScission Protease (Pharmacia). The peptides were added into the culture medium at a final concentration of 40 nM before stimulation with cytokines.

Cell culture medium, with the addition of GST, was used as a control. 50 ng of CNTF, LIF, IL-6, or OSM was then applied and allowed to incubate for 30 min. Cells were lysed in RIPA buffer with protease inhibitors and 25 μg of proteins in the cell lysate was subjected to SDS–PAGE followed by a Western blot using an anti-phospho-tyrosine STAT3 antibody. After stripping of membranes, total STAT3 expression was determined with an anti-STAT3 antibody.

3. Results

The purity of the GST fusion peptides was shown by SDS–PAGE (Fig. 1) and their correct folding and activity could be inferred from the specific protein–protein interactions described below.

3.1. In vitro interaction of CNTF, LIF and OSM with domains of their signaling receptors

We performed an in vitro GST pull down assay to define the domains in the extracellular region of human LIFR which interact with CNTF, LIF and OSM. There are three different regions in LIFR, the N-terminal CBD (CBD1), the Ig-like domain and C-terminal CBD (CBD2), which might interact with these molecules. It was shown that, in the presence of soluble CNTFR, CNTF interacted with the LIFR CBD1 (Fig. 2A) and (more weakly) with the LIFR Ig-like domain (Fig. 2B), but did not interact with CBD2 (Fig. 2B). In the absence of CNTFR a weak interaction of CNTF with the Ig-like domain was observed (Fig. 2B). Both LIF and OSM were found to be able to bind to the Ig-like domain and CBD2, while no distinct binding to CBD1 could be detected in the in vitro pull down assay (Fig. 3).

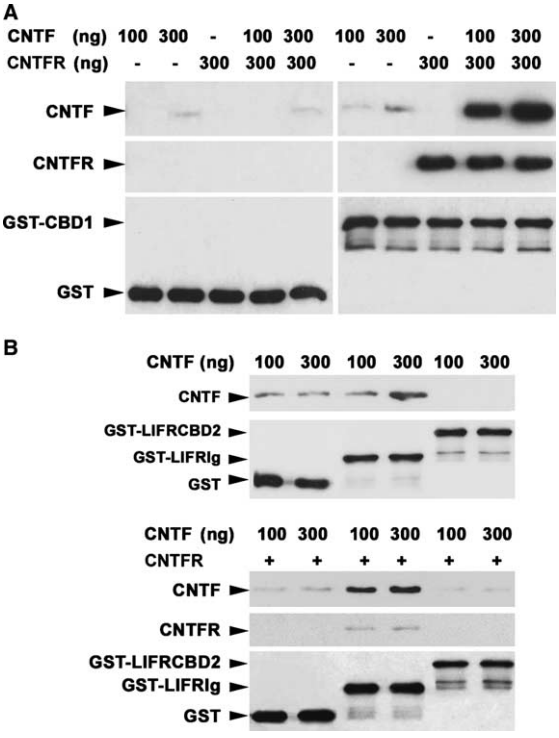

Fig. 2. Binding of GST fusion peptides of LIFR domains to CNTF in the presence or absence of CNTFR. 100 or 300 ng of CNTF was incubated with immobilized GST fusion peptides of the LIFR domains in the presence or absence of CNTFR (200 or 300 ng) for 30 min at 4 °C. After washing, peptides were separated by SDS–PAGE and identified by Western blotting with antibodies specific for GST, CNTF and CNTFR. (A) Interaction of GST and GST-LIFR CBD1 with CNTF in the presence or absence of CNTFR. (B) Interaction of GST, GST-LIFR Ig-like domain and GST-LIFR CBD2 with CNTF in the presence or absence of CNTFR.

The second signaling receptor for CNTF, LIF and OSM is gp130 which has two regions, the Ig-like domain and the CBD, that may interact with the cytokines. In the absence of CNTFR, CNTF did not bind the gp130 Ig-like domain but could bind to the gp130 CBD (Fig. 4A). When CNTFR was present, both the Ig-like domain and CBD bound to CNTF (Fig. 4A). OSM was able to bind to the gp130 Ig-like domain and CBD (Fig. 4B), in contrast to LIF which failed to show any binding to either of these domains (data not shown). However, in the presence of the LIFR Ig-like domain, LIF could bind to the gp130 CBD (Fig. 4C) but not to the gp130 Ig-like domain (data not shown).

3.2. Specific requirement of the LIFR CBD1 for CNTF signaling in vivo

As only CNTF was shown to interact with the LIFR CBD1, its role in CNTF signaling was further investigated by a luciferase reporter system in transfected human 293 cells. To circumvent interference from endogenous LIFR and gp130 activity, and as full length LIFR and gp130 induced only weak luciferase reporter activity in cells (Fig. 5), chimeric constructs (see Section 2) LIFRγ and gp130γ were used. Coexpression of LIFRγ and gp130γ greatly enhanced STAT1-driven luciferase activity in cells treated with CNTF, LIF or OSM (Fig. 5). Deletion of the LIFR CBD1 to create LIFRγΔCBD1 (CBD1ΔM) abolished CNTF induced luciferase activity, while signaling induced by LIF or OSM remained unchanged when compared with that from LIFRγ (Fig. 5).

Our previous report demonstrated the importance of L155/K156 in the putative B-strand of the BC domain of LIFR CBD1 in the ligand free interaction between the LIFR CBD1 and CNTFR [18]. As CNTF can bind to the LIFR CBD1 only in the presence of CNTFR (Fig. 2), we predicted that the L155A/K156A mutation in LIFR could impair CNTF signaling due to the deficient interaction between the LIFR CBD1 and CNTFR. Serial site-directed LIFRγ mutants in the region of CBD1 believed to bind to CNTFR were generated. A luciferase reporter assay showed that the L155A/K156A mutant (LK-M) had the strongest inhibitory effect on CNTF signaling,

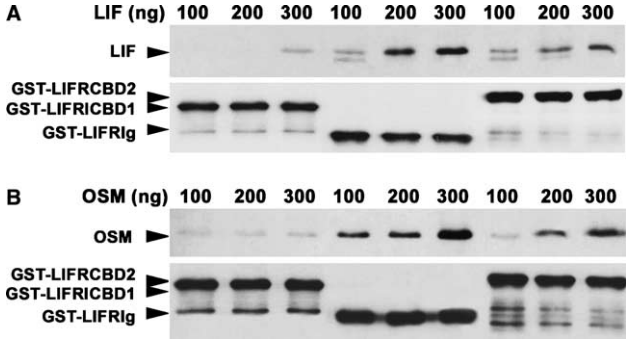

Fig. 3. Binding of GST fusion peptides of LIFR domains to LIF and OSM. 100–300 ng of LIF and OSM was incubated with immobilized GST fusion peptides of the LIFR domains for 30 min at 4 °C. Peptides were separated by SDS–PAGE and identified by Western blotting with antibodies specific for GST, LIF and OSM.

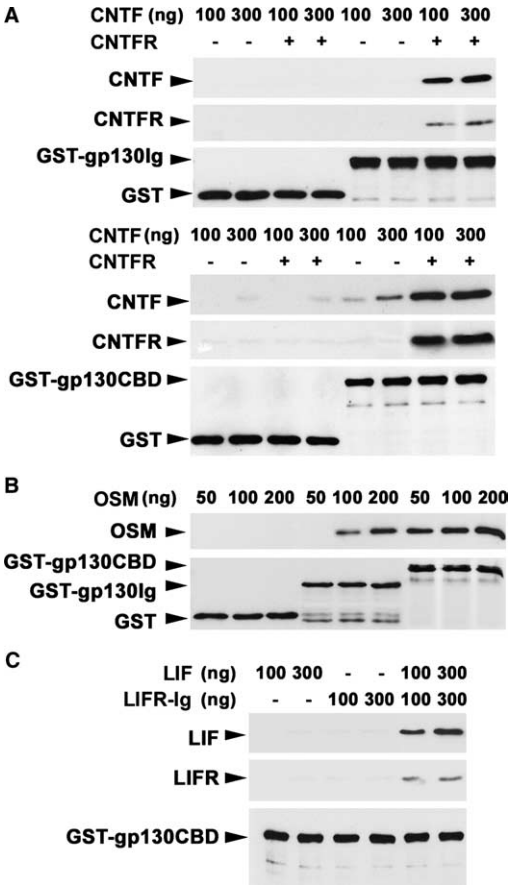

Fig. 4. Binding of GST fusion peptides of gp130 domains to CNTF, LIF and OSM. Proteins were incubated with immobilized GST fusion peptides of the gp130 domains for 30 min at 4 °C. After washing, peptides were separated by SDS–PAGE and identified by Western blotting with antibodies specific for GST, CNTF, LIF, OSM, LIFR and CNTFR. (A) Interaction of 100 or 300 ng of CNTF with the GST fusion peptides of the gp130 domains in the presence or absence of 300 ng CNTFR. (B) Interaction of 50–200 ng of OSM with the GST fusion peptides of the gp130 domains. (C) Interaction of LIF with the GST-gp130 CBD in the presence of the LIFR Ig-like domain. LIF did not interact with the gp130 CBD in the absence of LIFR Ig-like domain, nor with the gp130 Ig-like domain in the presence or absence of LIFR Ig-like domain (data not shown).

while none of the LIFRγ CBD1 mutants had any inhibitory effect on LIF induced luciferase activity when compared with activity induced through wild-type LIFRγ (Fig. 6).

3.3. Effect of the LIFR and gp130 peptides on CNTF, LIF, IL-6 and OSM signaling in vivo

As shown in our earlier work, the LIFR CBD1 could block signaling by CNTF but not that by LIF or OSM in NT-2 cells, which endogenously express CNTFR, LIFR and gp130 [19]. In light of that finding and the in vitro results described above, the LIFR and gp130 peptides were tested for their ability to inhibit signaling by CNTF, LIF, IL-6 and OSM in NT-2 cells in vivo. A representative experiment is shown (Fig. 7A) and the average results of three independent experiments are depicted in Fig. 7B. The LIFR CBD1 blocked signaling by CNTF but not that by LIF, IL-6 or OSM (Fig. 7B). Consistent with the in vitro binding data (Figs. 2 and 3), both the LIFR Ig-like and CBD2 peptides blocked LIF and OSM signaling, however these peptides did not affect IL-6 signaling

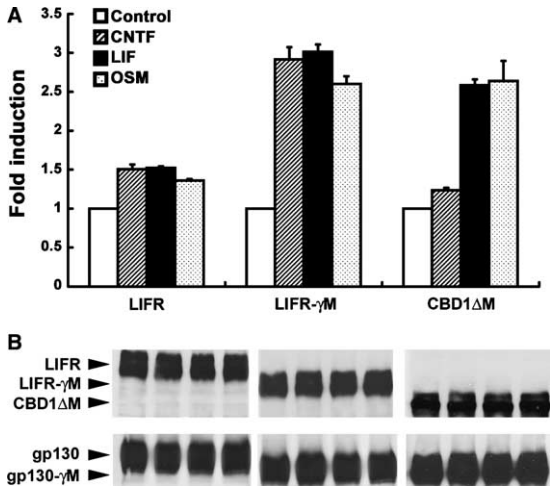

Fig. 5. Specific requirement of the LIFR CBD1 for CNTF signaling. Human 293 cells were transfected with a luciferase reporter construct and with LIFR and gp130 or with chimeric constructs of gp130 (gp130γ) and LIFR with or without CBD1 deleted (LIFR-γM, CBD1ΔM). Cells were then either not stimulated or stimulated with CNTF, LIF or OSM. Upper panel shows the fold induction relative to unstimulated cells. Error bars represent the S.D. of three replicated experiments. Lower panel shows Western blots of the LIFR and gp130 constructs.

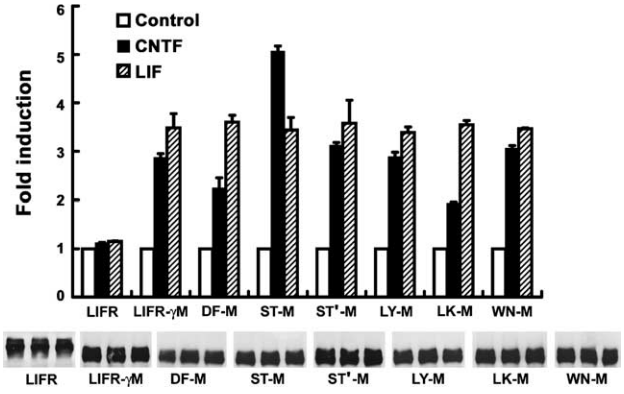

Fig. 6. Effect of LIFR CBD1 mutants on signaling by CNTF and LIF. Human 293 cells were transfected as described in the legend of Fig. 5 with LIFR, LIFR-γM or LIFRγ constructs with consecutive double alanine mutations in CBD1. The double alanine mutations (DF-M, ST-M, ST′-M, LY-M, LK-M and WN-M) spanned the putative AB loop and B strand of the LIFR CBD1 BC domain (LIFR 147-DFSTSTLYLKWN-158) that is likely to be involved in receptor–receptor interactions. ST′-M indicates mutation of the second ST pair (S151/T152) in this sequence. Cells were then either not stimulated or stimulated with CNTF or LIF. Upper panel shows the fold induction relative to unstimulated cells. Error bars represent the S.D. of three replicated experiments. Lower panel shows Western blots of LIFR and the LIFRγ constructs.

(Fig. 7B). The gp130 CBD inhibited signaling by all the cytokines while the gp130 Ig peptide only blocked IL-6 and CNTF signaling (Fig. 7B).

4. Discussion

New roles for CNTF in neuronal diseases, diabetes and obesity have been emerging recently [1–5]. Coupled with the

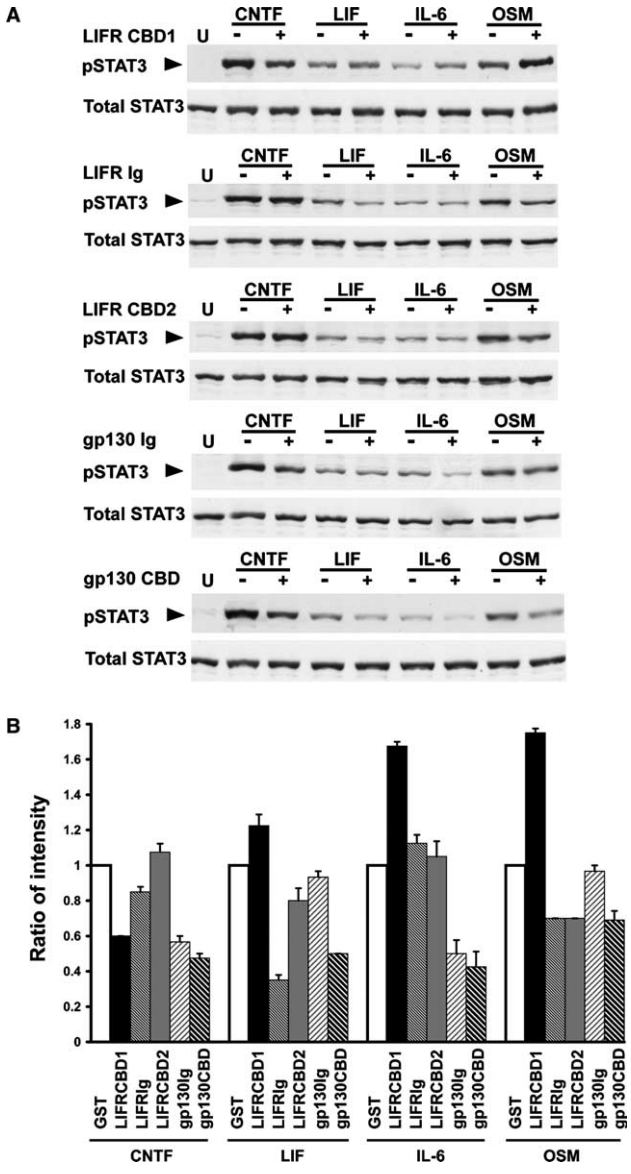

Fig. 7. Effects of exogenous LIFR and gp130 extracellular peptides on CNTF, LIF, IL-6 and OSM signaling. Human NT-2 cells were starved in serum free medium for 4 h before addition of the different peptides. Cells were then treated with different cytokines for 30 min. (A) Western blot analysis of total cell lysates after cytokine treatment. Blots were probed with an anti-phospho-STAT3 or an anti-STAT3 antibody. (B) Statistical analysis of the relative band intensities from the Western blot analyses. Results are the means ± S.D. of three independent experiments.

newly discovered, but long hypothesized, alternate ligands for CNTFR [7,8] knowledge of the interactions among CNTF and its receptors will help in understanding the functions of these molecules and in developing them as therapeutic agents. In this work, we have further elucidated the roles of the regions in LIFR and gp130 in CNTF binding and signaling.

Evidence for the specific and essential role of the LIFR CBD1 for CNTF binding and downstream signaling has been presented. In vitro binding assays showed that the LIFR CBD1 could pull down CNTF in the presence of CNTFR, whereas LIF and OSM did not interact with this peptide. A weaker interaction was observed between CNTF and the LIFR Ig-like domain. However, no interaction was seen between CNTF and the LIFR CBD2. In contrast, both these LIFR receptor domains bound to LIF and OSM, with the LIFR Ig-like domain showing stronger binding. This demonstrated a role for the LIFR CBD1 in CNTF binding and that the interactions of CNTF with LIFR are different from those of LIF and OSM.

The importance of the LIFR CBD1 in formation of the active CNTF receptor complex was demonstrated by in vivo luciferase reporter assays in human 293 cells, which endogenously express CNTFR and were transfected to express LIFR (or its mutants) and gp130. A LIFR mutant with the CBD1 deleted (LIFRγΔCBD1–CBD1ΔM) failed to respond to CNTF stimulation. Structural destabilization of LIFRγΔCBD1 did not occur, since the molecule could still make the appropriate interactions with LIF and OSM as their signaling was not affected. Mutations in the LIFR CBD1 that were found earlier to affect LIFR–CNTFR interactions in vitro [18] also inhibited CNTF signaling in vivo while having no effect on LIF signaling. These results show that the LIFR CBD1 is required for CNTF signaling and support its interaction with CNTF and CNTFR in a manner consistent with that of the proposed hexameric signaling complex [18] where the LIFR CBD1 is suggested to bind CNTF at site II.

Binding to gp130 domains also differed among the cytokines. CNTF bound strongly to both the gp130 Ig-like domain and the CBD. OSM was also able to bind both these modules, although binding to the Ig-like domain was weaker. Neither of the gp130 domains was able to bind to LIF in the absence of LIFR. In the presence of the LIFR Ig-like domain, binding of LIF to the gp130 CBD, but not to the gp130 Ig-like domain, was observed. It has previously been shown that the gp130 Ig-like domain is not required for either LIF or OSM signaling [21]. Our in vitro pull down assays showed no substantial differences in the binding of the gp130 CBD to LIF (in the presence of LIFR), OSM and CNTF.

Binding of LIF to gp130 in the absence of LIFR has been observed previously through cross-linking studies [22] and under crystallization conditions and using isothermal titration calorimetry [23]. However, similar to our results, a LIF-gp130 interaction was not seen under native PAGE and gel-filtration chromatography [24]. Cross-linking showed a 1:1 complex and surface plasmon resonance revealed low affinity binding with a high dissociation rate [24], which is consistent with our inability to observe an interaction between LIF and gp130 in our experiments.

A trimeric complex for LIF and its receptors, LIFR and gp130, has been identified [24]. Several lines of evidence, based on chimeric receptors and site-directed mutagenesis, indicated that the Ig-like domain of LIFR was involved in both LIF and OSM binding and signaling [25–28]. These studies suggested that site III of LIF binds to the LIFR Ig-like domain. Possible roles for the CBD1 and for site I of LIF having a weaker interaction with CBD2 were also proposed [26]. These findings are consistent with our results, except that the LIFR CBD1 does not appear to have a role in LIF binding or signaling.

The Ig-like domain of LIFR was shown here to bind LIF, OSM and CNTF (in the presence or absence of CNTFR) and the gp130 Ig-like domain was shown to bind OSM and CNTF. Earlier, and in this work, it was shown that, despite

its ability to bind OSM, the Ig-like domain of gp130 is not required for OSM signaling [21]. Binding to CNTF by the Ig-like domain of LIFR in the active CNTF receptor complex is incompatible with the role of the LIFR CBD1 shown here. The abolition of CNTF signaling by deletion of the LIFR CBD1 and the effect on signaling of LIFR CBD1 mutants suggest that the LIFR Ig-like domain may not be implicated in CNTF signaling. This was supported by the very slight inhibition of CNTF signaling caused by the LIFR Ig-like domain. It may be that binding of isolated receptor Ig-like domains may reflect a common ability of these domains to bind to site III of the LIFR binding members of gp130 cytokine family through the conserved FXXK motif and not necessarily reflect a functional role.

It was suggested previously that two residues in site III of CNTF may affect LIFR binding [29]. However, structural destabilization of CNTF by these mutations could not be excluded as the individual mutations did not necessarily affect CNTF signaling and the double mutant did not antagonize the biological effects of wild-type CNTF [29]. A mutant of LIF, that could not bind gp130, has been shown to antagonize LIFR dependent signaling by LIF, CT-1, OSM and CNTF [30]. One possible explanation could be that these ligands bound LIFR at the same location and competed with the mutant LIF molecule for that site. However, antagonism of CNTF binding would still be possible if CNTF bound LIFR through a site different from that used by LIF, as proposed here. Binding of LIFR by LIF, at probably the Ig-like domain, may alter the conformation of the receptor and so prevent binding by another molecule. However, a LIF molecule bound to LIFR at the Ig-like domain would, by steric hindrance, be very likely to prevent either other molecules from binding to the other receptor domains at the same time or the formation of the complete receptor complex.

Given the binding interactions of the receptor peptides described above, it would be expected that they would have differing effects on the biological activity of the cytokines. Earlier we showed that the LIFR CBD1 inhibited signaling by CNTF but not that by LIF or IL-6 [19]. In this work that result was replicated and the effect of all the receptor peptides on CNTF, LIF, IL-6 and OSM signaling in vivo was investigated. CNTF signaling was blocked by the LIFR CBD1 and the gp130 Ig-like domain and gp130 CBD. It was not affected by the LIFR CBD2 and a slight effect was seen for the LIFR Ig-like domain. These interactions are consistent with the hexameric complex of CNTF and its receptors [16,18].

Signaling by LIF was inhibited by the LIFR Ig-like domain and the gp130 CBD. Some inhibition from the LIFR CBD2 was observed, however there was no effect from the gp130 Ig-like domain and the LIFR CBD1 did not inhibit LIF signaling. These results are consistent with the trimeric complex and ligand–receptor interactions proposed for LIF [24–28]. Consistent with the interactions seen from the crystal structure of IL-6 with its receptors [14], IL-6 signaling was inhibited by both gp130 peptides but not those of LIFR. OSM signaling was reduced by the LIFR Ig-like and CBD2 domains and the gp130 CBD but not by the gp130 Ig-like domain or the LIFR CBD1, consistent with earlier studies [21,23]. The interactions of the peptides studied here are consistent with the known ligand–receptor interactions of LIF, IL-6 and OSM.

As in our previous study [19], it was found that the LIFR CBD1 actually enhanced signaling from LIF, IL-6 and OSM. One possible reason for this may be due to the ligand free interaction between the LIFR CBD1 or the gp130 CBD and CNTFR reported earlier [18,19]. Binding of the LIFR CBD1 to cellular CNTFR may prevent cellular CNTF from competing for binding to cellular LIFR and gp130, thus making more signaling receptors available to the other cytokines. As a consequence of the greater number of free gp130 molecules, increased responsiveness of NT-2 cells to IL-6 would be expected, while increased availability of both gp130 and LIFR would similarly increase responsiveness to LIF and OSM as we observed.

Our finding of the critical role of the LIFR CBD1 in CNTF signaling is consistent with a hexameric model for the CNTFR complex where the LIFR CBD1 binds CNTF at site II [13,16,18]. CNTF might form two trimers, one with CNTFR and LIFR and the other with CNTFR and gp130. The interaction with LIFR would be through CBD1, consistent with its requirement for CNTF signaling reported here and that of models of the LIFR CBD1 that showed it had a similar electrostatic profile to the gp130 CBD [18]. One possible linkage of two trimers of CNTF, CNTFR and either LIFR or gp130 is through the interaction between the site III of one molecule of CNTF and the Ig-like domain of gp130 as seen in the IL-6 receptor complex [14]. The differential role of the LIFR CBD1 found here may allow the design of agents that can specifically inhibit LIF and OSM signaling while leaving CNTF signaling unaffected, or vice versa.

Acknowledgments: This study was supported by the Research Grants Council of Hong Kong SAR (HKUST 6127/99M) and the Area of Excellence Scheme of the University Grants Committee (AoE/B-15/01). N.Y.I. was a recipient of the Croucher Foundation Senior Research Fellowship.

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