Cross-linking Identifies Leukemia Inhibitory Factor-binding Protein as a Ciliary Neurotrophic Factor Receptor Component*

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Ciliary neurotrophic factor (CNTF) promotes the survival or differentiation of diverse cells within the nervous system (1). CNTF is unrelated to the family of neurotrophic factors exemplified by nerve growth factor, and instead shares features with cytokines such as IL-6, LIF, and G-CSF (2). Expression-based cloning and subsequent analysis of a CNTF-binding protein (CNTFRa) (3, 4) revealed closest homology to one component of the IL-6 receptor (IL-6Ra); both CNTFRa and IL-6Ra exhibit the general characteristics of cytokine binding domains (5). These homologies, coupled with the presence of a glycosylphosphatidylinositol linkage and absence of a cytoplasmic domain on CNTFRa, suggest that functional CNTF receptors would require an additional component analogous to gp130, the signal-transducing subunit of the IL-6 receptor (6).

Candidate CNTF receptor components were discovered through comparative studies of signal transduction following addition of CNTF, LIF, or IL-6 to responsive cell lines (7). A distinct set of proteins called CLIPs (CNTF and LIF-induced phosphoproteins) became rapidly phosphorylated on tyrosine after stimulation by CNTF and LIF. Two of these phosphoproteins, CLIP1 and CLIP2, were transmembrane proteins that could be specifically recovered in a complex with biotinylated CNTF, suggesting that they constituted receptor components (7). CLIP2 has been identified as gp130 using monoclonal antibodies, and we have suggested that CLIP1 is LIFRβ (a protein first identified as a LIF-binding protein; Ref. 8) based on its molecular weight and the identity of the phosphorylation pattern induced by LIF and CNTF. We therefore proposed that a functional CNTF receptor is composed of CNTFRa, gp130, and LIFRβ (7). Since functional LIF receptors appear to require only gp130 and LIFRβ (8, 9), a consequence of this model is that a functional LIF receptor can be converted into a functional CNTF receptor by the presence of CNTFRa (4, 7, 10, 11). Thus in a sense, the LIF signal-transducing machinery, which occurs with widespread distribution, would be appropriate for use by CNTF in the restricted set of cells that also express CNTFRa (11). Recently, we have shown that CNTFRa can function when present in a soluble form (10). The detection of soluble CNTFRa in cerebrospinal fluid, as well as its release from muscle after nerve injury, enhances the possibility that CNTF could act at LIF receptors through the regulated release of soluble CNTFRa in vivo (10).

In order to test our conjecture that CLIP1 is LIFRβ and to determine whether CLIP1 and CLIP2 directly interact with CNTF, we employed specific antisera and cross-linking approaches on cells bearing functional receptors, as well as on COS cells transfected with various combinations of CNTFRa, gp130, and LIFRβ. The results of these experiments confirm that gp130 and LIFRβ are receptor components for CNTF, and along with CNTFRa, appear to directly contact CNTF.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant rat CNTF was prepared as described (12). LIF and anti-phosphotyrosine (4G10) conjugated to agarose were purchased from Upstate Biotechnology, Inc. The human LIFRβ open reading frame was cloned by polymerase chain reaction methodology using primers derived from the published sequence, while the clones for gp130 (6) and CNTFRa (3) were described previously. Antibodies recognizing LIFRβ was raised in rabbits against a COOH-terminal peptide with the sequence KGEDSPKSNWGGSGTFNFKNDN, which was synthesized with an Nα-terminal extension of CGG to facilitate coupling to purified protein derivative of tuberculin (Parke-Davis) using m-maleimidobenzoyl-N-hydroxysuccinimide. Iodination of CNTF was carried out with either Bolton-Hunter reagent (Du Pont-New England Nuclear) or soluble lactoperoxidase and sodium iodide (13); only the latter procedure was used for LIF. 125I-LIF was purified from unincorporated iodine on an Econo-Pac 10DG desalting column.

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§ The abbreviations used are: CNTF, ciliary neurotrophic factor; a-Tyr(P), anti-phosphotyrosine; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; LIF, leukemia inhibitory factor; BSA, bovine serum albumin; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; CNTFR, CNTF receptor; LIFR, LIF receptor.
column (Bio-Rad). 125I-CNTF was purified by chromatography on a Superdex HR-75 column (Pharmacia LKB Biotechnology, Inc.) to allow separation of monomer from dimer; the monomeric fraction was used in all experiments. Specific activities of the iodinated probes ranged from 650 to 2000 Ci/mmol. Iodination of tyrosine was reported not to affect the bioactivity of LIF (14); 125I-CNTF generally showed full retention of bioactivity on ciliary ganglia. IARC-EW-1 (hereafter referred to as EW-1) cells were cultured as previously described (7). Construction of Myc tags on CNTFRα and LIFRP, and transfection of COS cells is described elsewhere.

Cross-linking and Immunoprecipitation—Six-well plates of the indicated cells were incubated with 1 nM iodinated factors for 30 min at room temperature in a buffer consisting of phosphate-buffered saline + 1 mg/ml bovine serum albumin. Where indicated, either 1 μg/ml CNTF (44 nm) or 0.5 μg/ml LIF (22 nm) was added as an unlabeled competitor. Experiments employing precipitation with α-Tyr(P) instead used 1 nM 125I-CNTF or 2 nM 125I-LIF + 100 nM nonradioactive competitor in a 10-min incubation. For cross-linking, plates were placed on ice and incubated for 20 min with 0.2–0.5 mM disuccinimidyl suberate taken from a 100 mM stock freshly made in dimethyl sulfoxide. Following 2 washes with cold TBS (20 mM Tris hydrochloride, pH 7.8, 0.15 M sodium chloride), the cells were lysed in TBSN (TBS containing 1% Nonidet P-40, 1 mM EDTA, 5 μg/ml leupeptin, 0.14 units/ml aprotinin, 1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride) for 15–30 min on ice. After centrifugation for 15 min at top speed in a microcentrifuge, the lysate was either subjected to SDS-PAGE directly or incubated overnight at 4 °C with the indicated antibody, then 1 h in goat anti-IgG conjugated to agars (for α-gp130; Sigma) or protein A-Sepharose (for α-LIFRβ; Pharmacia). The beads were washed 3 times in 1 ml of lysis buffer, then boiled in sample buffer and subjected to SDS-polyacrylamide gel electrophoresis on gels containing 7% acrylamide. The gels were dried before autoradiography.

Anti-phosphotyrosine Blots—Immunoblots probed with α-Tyr(P) were visualized by enhanced chemiluminescence (ECL; Amersham Corp.). Following electrophoresis to polyvinylidene difluoride, blots were blocked with 10% BSA in TBS for 3 h, then incubated for 3 h with the α-Tyr(P) monoclonal 4G10 at a dilution of 1:4000 in a buffer containing TBST (TBS + 0.1% Tween 20) and 4% BSA. Blots were then washed 3 × 10 min in TBST, and incubated for 1 h in goat anti-mouse IgG conjugated to horseradish peroxidase (Promega) diluted 1:20,000 in TBST containing 2.5% BSA. The blots were then washed 3 × 10 min with TBST, 3 × 5 min with TBS + 0.3% Tween 20, followed by a 30-min wash in TBS. The ECL reaction was then carried out according to the manufacturer’s recommendations.

RESULTS

Specific Antisera Verify That CLIP1 Is LIFRβ—Previous experiments revealed that a human Ewing sarcoma (EW-1) cell line gives a robust tyrosine phosphorylation of CLIP1 and CLIP2 in response to either CNTF or LIF. Monoclonal antibodies were used to demonstrate that CLIP2 is gp130, the IL-6 signal transducer (1, 7). In order to test whether CLIP1 corresponds to LIFRβ, we employed polyclonal antisera raised against a carboxyl-terminal peptide of human LIFRβ. This antisera specifically immunoprecipitated CLIP1 and depleted CLIP1 from lysates of EW-1 cells stimulated with either CNTF or LIF (Fig. 1A); recognition was inhibited by the presence of the cognate peptide. Coprecipitation of CLIP2 is not observed in this experiment in which the lysates were heated to 95 °C before incubation with the antisera; coprecipitation of CLIP2 is observed if the heating step is omitted (data not shown). These results demonstrate that LIFRβ corresponds to CLIP1 and becomes tyrosine-phosphorylated in response to CNTF or LIF.

Cross-linking of CNTF and LIF to Endogenous Receptor Components—We next employed cross-linking of iodinated factors to determine whether CNTF directly contacts gp130 and LIFRβ. EW-1 cells were cross-linked to 125I-CNTF or 125I-LIF, followed by immunoprecipitation with antibodies to phosphotyrosine, gp130, or LIFRβ (Fig. 1B). All three antibodies precipitated a common set of radioactive bands after cross-linking to 125I-CNTF, consistent with the formation of a complex containing CNTF together with CNTFRα and the tyrosine-phosphorylated forms of gp130 and LIFRβ. As expected, the apparent molecular weights of two labeled bands (indicated by * and + in Fig. 1B, lane 3) correspond to the predicted for the cross-linked products of 125I-CNTF (22 kDa) with CNTFRα (80 kDa) and LIFRβ/CLIPl (190 kDa). Although the major remaining cross-linked product is larger than would be expected for gp130/LIFRβ (145 kDa) cross-linked to CNTF (165 kDa; ■ in Fig. 1B), experiments using a transfected version of gp130 verify that this product migrates aberrantly (see below). Higher molecular weight species observed in some cases most likely contain multiple receptor components. Bands corresponding to LIFRβ/CLIPl and gp130/LIFRβ2 are also observed cross-linked to 125I-LIF (Fig. 1B, lane 1) and may be equivalent to species previously observed after LIF cross-linking (14). Although the LIF-cross-linked products migrate slightly faster relative to the corresponding products resulting from cross-linking to CNTF, this difference is also observed with transfected versions of these receptor components (see below). These cross-linking results suggest that gp130 and LIFRβ directly contact CNTF in the receptor complex, consistent with their roles as signal-transducing receptor components.

Cross-linking of CNTF and LIF to Transfected Receptor Components—We next turned to a COS cell expression system to explore the combinations of receptor components
required to reconstruct a receptor complex mimicking that found in EW-1 cells. The LIFβ and CNTFRα coding sequences were modified to include a 10-amino acid "tag" that corresponds to a well-defined peptide epitope from c-Myc. This epitope is recognized by monoclonal antibody 9E10, allowing for unequivocal identification of the Myc-tagged proteins (3).

Expression of LIFβ-Myc alone gives good cross-linking to 125I-LIF as expected since this protein was identified as a LIF-binding protein (8), while cross-linking to 125I-CNTF is not observed (Fig. 2, left panel). Expression of gp130 alone gives very faint cross-linking to either factor (Fig. 2, center panel). Coexpression of CNTFRα-Myc with gp130 not only allows for cross-linking of CNTF to CNTFRα (lower band in Fig. 2, right panel) but also increases slightly the amount of 125I-CNTF cross-linked to gp130 (upper band in Fig. 2, right panel); this result is consistent with independent evidence proving that CNTF can form a relatively stable interaction with CNTFRα and gp130 in the absence of LIFβ. The identities of the receptor components in the various cross-linked products were verified by immunoprecipitation with α-gp130 and α-Myc (not shown).

Coexpression of gp130 and LIFβ-Myc in the absence of CNTFRα allowed for prominent cross-linking of 125I-LIF to both gp130 and LIFβ, while no cross-linking to 125I-CNTF was observed (Fig. 3A, left panel). Specific precipitation of the 125I-CNTF-containing species was achieved by α-gp130 or α-Myc (Fig. 3A, right panel). These two cross-linked products reproduce those observed in EW-1 cells cross-linked to 125I-LIF; this result is consistent with the notion that LIFβ and gp130 are sufficient to form a functional LIF receptor complex, while CNTFRα is also required to form a functional CNTF receptor complex (4, 7, 10, 11).

COS cells cotransfected with CNTFRα-Myc, gp130, and LIFβ-Myc now reveal three main products upon cross-linking to 125I-CNTF (Fig. 3B, left panel), reproducing the pattern observed in EW-1 cells. Immunoprecipitation with α-gp130 primarily recovers the 190-kDa product, while α-Myc recovers a complex containing all three products (Fig. 3B). Surprisingly, LIF cross-links to CNTFRα as well as gp130 and LIFβ in these transfected COS cells; as above, the identities of the cross-linked products were confirmed by immunoprecipitation (Fig. 3B). Although LIF cross-linking to CNTFRα is easily observed when all three components are over-expressed in COS cells, a minor product of similar size is also observed in EW-1 cells (Fig. 1B). The products resulting from cross-linking to LIF migrate slightly faster than those resulting from CNTF cross-linking, reproducing the behavior observed in EW-1 cells. These reconstruction experiments show that while LIFβ and gp130 expression are sufficient to observe cross-linking to LIF, coexpression of CNTFRα is required to form a ternary receptor complex and allow cross-linking of CNTF to all three receptor components. This result corroborates our previous findings that physiologic levels of CNTF could not induce signal transduction events in cells bearing gp130 and LIFβ in the absence of CNTFRα (7, 10).

Cross-competition between CNTF and LIF—One prediction of our models for the LIF and CNTF receptors is that the factors should cross-compete for cross-linking to shared receptor components. This has been observed for binding of oncostatin M and LIF (9), as well as between human IL-3 and GM-CSF, which share a receptor component (15). Fig. 3B shows that cotransfection of CNTFRα, gp130, and LIFβ gives rise to a situation in which a 22-fold excess of LIF does not compete for cross-linking to 125I-CNTF, while a similar excess of CNTF does compete for cross-linking of 125I-LIF to all three components. As would be expected, competition of CNTF for 125I-LIF cross-linking is dependent on the expression of CNTFRα (Fig. 3A). The observed inability of LIF to compete for CNTF cross-linking could be explained by the following considerations. 1) CNTF could have a higher relative affinity for its receptor complex, which contains three receptor components and thus may be more stable than the two component complex made with LIF. 2) Insufficient amounts of LIF were used in the competitions; higher concentrations of LIF did in fact effectively compete for cross-linking.

**Fig. 2. Cross-linking of 125I-CNTF and 125I-LIF to "incomplete" receptor combinations.** COS cells were transfected with either LIFβ-Myc, gp130, or gp130 and CNTFRα-Myc as indicated, and subjected to cross-linking after incubation with the indicated factors. Nonradioactive LIF (L) or CNTF (C) was added as competitor where shown. The figure shows an autoradiograph following SDS-PAGE of total lysates from each sample.

**Fig. 3. Cross-linking of 125I-CNTF and 125I-LIF to transfected COS cells.** COS cells were cotransfected with LIFβ-Myc and gp130 either in the absence (A) or presence (B) of CNTFRα-Myc, incubated with the 125I-CNTF or 125I-LIF as indicated, and cross-linked with disuccinimidyl suberate. Either LIF (L) or CNTF (C) was added as a nonradioactive competitor where indicated. The figure shows an autoradiograph following SDS-PAGE of total lysates, or of proteins precipitated by α-gp130 (AM64) or α-Myc (9E10). Lanes marked STD contain 14C-labeled molecular weight markers from BRL.
of CNTF in EW-1 cells (data not shown). 3) Finally, since CNTF cannot compete for LIF binding to LIFRβ in the absence of CNTFRα, competition by CNTF for LIF binding to LIFRβ upon cotransfection of all components (Fig. 3B) implies that both gp130 and CNTFRα must be in excess of LIFRβ in this experiment. Differences in cross-competition between IL-3 and IL-5 resulting from different ratios of receptor component, G-CSF receptor, and the IL-3 receptor implies that both gp130 and CNTFRα are needed for receptor contacts made between CNTFRα and the heterodimeric LIF receptor complex. Other experiments using epitope-tagged versions of gp130, LIFRβ, and CNTFRα expressed in COS cells revealed that complex formation between CNTFRα, gp130, and LIFRβ does not occur in the absence of added CNTF, and also showed that CNTF can form a stable, non-functional complex with CNTFRα and gp130 in the absence of expressed LIFRβ. These experiments demonstrate that addition of CNTF drives complex formation from three separate, previously non-interacting receptor components, which may well proceed in an ordered fashion through intermediates in which CNTF first binds to CNTFRα, followed by gp130, then LIFRβ. Complex formation then presumably initiates the signal transduction process (see below).

A surprising result was the observation that 125I-LIF could cross-link to CNTFRα in the presence of LIFRβ and gp130. CNTFRα is not required to initiate signal transduction by LIF (7, 10), although there is some evidence that the presence of CNTFRα may augment the responsiveness of cells to LIF (10), although there is some evidence that the presence of CNTFRα may augment the responsiveness of cells to LIF (7, 10, 11). The rapid tyrosine phosphorylation of LIFRβ and the presence of the conserved cytoplasmic region mentioned above indicates that LIFRβ is likely to function as a signal-transducing partner to gp130. Thus for CNTF and LIF, heterodimerization due to apposition of gp130 and LIFRβ may be analogous to the homodimerization of the β-type receptors for erythropoietin (19), G-CSF (20), human growth hormone (21, 24), and perhaps IL-6 (4, 7). Thus it is possible that a cytoplasmic tyrosine kinase is non-covalently associated with the cytoplasmic domain of each β receptor, and that either hetero- or homodimerization of two β subunits would allow for kinase transactivation in a manner analogous to receptor tyrosine kinases (22).

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