**Cardiotrophin-1 (CT-1)** is a recently isolated cytokine belonging to the interleukin-6 cytokine family. In the present study we show that CT-1 activates its receptor expressed at the surface of a human neural cell line by recruiting gp130 and gp190/leukemia inhibitory factor receptor β, as shown by analyzing their tyrosine phosphorylation level. Neutralizing antibody directed against gp130 and reconstitution experiments performed in the COS-7 cell line demonstrate that gp130-gp190 heterocomplex formation is essential for CT-1 signaling. Analysis of the subsequent activation events revealed that CT-1 induces and utilizes Jak1-, Jak2-, and Tyk2-associated tyrosine kinases, which are in turn relayed by STAT-3 transcription factor. Cross-linking of iodinated CT-1 to the cell surface led to the identification of a third α component in addition to gp130 and gp190, with an apparent molecular mass of 80 kDa. Removal of N-linked carbohydrates from the protein backbone of the α component resulted in a protein of 45 kDa. 

Our results provide evidence that the CT-1 receptor is composed of a tripartite complex, a situation similar to the high affinity receptor for ciliary neurotrophic factor.

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Interleukin (IL)-6\(^1\) belongs to a growing family of cytokines, which includes leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and IL-11 (Kishimoto et al., 1995). These closely related cytokines share many common biological properties, such as activation of hepatocyte transcription (Baumann et al., 1993), activation of neural proliferation and differentiation (Yamamori et al., 1989), and regulation of hematopoiesis (Leary et al., 1990; Musashi et al., 1991). In addition, LIF, CNTF, and OSM display biological properties in the early stages of embryonic development and allow the in vitro growth of embryonic cell lines in an undifferentiated state (Smith et al., 1988; Conover et al., 1993). IL-6 and IL-11 are also important modulators for the immune response by regulating immunoglobulin secretion (Kishimoto et al., 1995; Yin et al., 1992). The redundancy of their biological properties is in part explained by the shared use of the common signaling protein, gp130, in their multimeric receptors. gp130, initially isolated as an IL-6 receptor signal transducer (Hibi et al., 1990), associates with other receptor components to generate high affinity type receptors for the ligands. This is the case for the gp190/low affinity LIF receptor (Gearing et al., 1991), which associates with gp130 to generate a functional LIF/OSM receptor (Gearing et al., 1992). For the CNTF receptor, a third additional component (CNTF α receptor subunit) (Davis et al., 1991) interacts with the gp130-gp190 heterocomplex to generate a high affinity CNTF receptor (Davis et al., 1993a). The IL-6 receptor is composed of a gp130 homodimer associated with an IL-6-binding chain, gp80 (Yamasaki et al., 1988). More recently, a binding subunit for IL-11 was also isolated (Hilton et al., 1994).

Dimerization of the transducing subunits initiates intracellular signaling by activating members of cytokine receptor-associated tyrosine kinases, referred to as Jakks (reviewed by Ihle et al. (1995)). Both gp130 and gp190/LIF receptor can associate Jak1, Jak2, and Tyk2 (Stahl et al., 1994). The information is next relayed by a family of transcription factors known as STATs (signal transducers and activators of transcription), which are activated in the cytoplasm before translocation to the nucleus (Ihle et al., 1995). IL-6 type cytokines will preferentially activate STAT-1 and STAT-3 (Stahl et al., 1995; Lütteken et al., 1994).

Recently, a new cytokine named cardiotrophin-1 (CT-1) was isolated based on its ability to induce cardiac myocyte hypertrophy (Pennica et al., 1995a; Pennica et al., 1996a). Analysis of its amino acid sequence revealed some homologies with LIF and CNTF, suggesting that it belongs to the IL-6 cytokine family. Since then, additional bioactivities have been ascribed to CT-1, showing that similar to LIF, CT-1 induces the terminal differentiation of the M1 myeloid cell line, the phenotypic switch in rat sympathetic neurons, and inhibits the spontaneous differentiation of embryonic stem cells (Pennica et al., 1995b). Direct binding of CT-1 to gp190/LIF β receptor and the implication of gp130 as a converter protein in the mouse CT-1 receptor were also reported (Pennica et al., 1995b).

In the present study, we have characterized the receptor subunits involved in the signaling of the human CT-1 receptor. We found that gp130 and gp190/LIF receptor associate with a third not yet identified component of 80 kDa, referred to as the CT-1 α receptor subunit (CT-1α), to create a tripartite receptor for the cytokine. Activation of the tripartite CT-1 receptor led to the recruitment of Jak1, Jak2, and Tyk2, which in turn are relayed by STAT-3 to transduce the information inside the cell.
Materials and Methods

Cells and Reagents—The SK-N-MC neuroblastoma, KB epidermoid carcinoma, and COS-7 cell lines from ATCC (Rockville, MD) were routinely grown in RPMI culture medium supplemented with 10% fetal calf serum. For the growth of the multifactor-dependent hematopoietic TF1 and DA1.a cell lines (Kitamura et al., 1989; Gascan et al., 1990), the culture media were supplemented with 1 ng/ml granulocyte-macrophage colony-stimulating factor or granulocyte colony-stimulating factor, respectively. The L-CNTF Rec. cell line was derived by stable transfection of the L929 cell line (ATCC) with a cDNA encoding the human CNTF receptor α subclone in a pCDNA Neo expression vector (In vitrogen, San Diego, Ca). Purified human recombinant LIF (10^6 units/mg) produced in a Chinese hamster ovary cell line, E. coli recombinant granulocyte-macrophage colony-stimulating factor (10^6 units/mg), and IL-6 (2.5 × 10^6 units/mg) were kindly donated by Dr. K. Turner (Genetics Institute, Boston, MA). IL-6 (2 × 10^6 units/mg) and OSM (2 × 10^6 units/mg) were purchased from Peprotech (Canton, MA). Mouse CT-1 was produced as described previously (Pennica et al., 1995a). Rat CNTF was produced as a GST fusion protein by using pGEX-4T2 gene fusion vector from Pharmacia (Uppsala, Sweden), before being cleaved with thrombin. B-T6, B-P4, B-T2, and B-R3 anti-gp130 mAbs were described in detail elsewhere (Chevalier et al., 1986). Polyclonal antibodies raised against gp130 or gp190 were bought from R & D System (Oxon, United Kingdom). Rabbit anti-Tyk2 and antibodies recognizing the carboxy-terminal sequence of gp190 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit antibodies directed against Jak1, Jak2, and Tyk3 and 4G10 anti-phosphotyrosine mAb were from UBI (Lake Placid, NY). mAbs recognizing STAT-1 and STAT-3 were obtained from Transduction Laboratories (Lexington, KY).

Bioassays—Cytokines were added to 10^6 TF1 or DA1.a myeloid cells in triplicate in the assay. Cells were incubated for 72 h before being pulsed with 0.5 μCi of [3H]thymidine for the last 4 h of the culture (Fournier et al., 1994). The KB carcinoma cell line was plated in triplicate in 96-well plates at a concentration of 50 × 10^3 cells/well in 150 μl of culture medium containing serial dilutions of tested cytokines. After 48 h, the supernatants were harvested, and their IL-6 content was determined by enzyme-linked immunosorbent assay (Thoma et al., 1994).

cDNAs and COS-7 Cell Transfection—COS-7 cells were transfected by using the DEAE-dextran method as described previously (Robledo et al., 1996). After a 48-h culture period, expression and function of the transfected proteins was studied. The cDNA encoding the human gp190/LIF receptor β was in pCMX and was a kind gift of Dr. George D. Yancopoulos (Davis et al., 1991). The cDNA encoding the human gp130 transducer was cloned by polymerase chain reaction from the U266 cell line according to the published sequences and subcloned in the pCD vector (Robledo et al., 1996).

Tyrosine Phosphorylation Analysis—After stimulation, cells were lysed in 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, proteinase inhibitors (1 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), and 1% Triton X-100, Nonidet P-40, or Brij 96, depending on the experiments (Fournier et al., 1994). After pelleting insoluble material and after protein standardization, the supernatants were immunoprecipitated overnight. The complexes were then isolated with beads coupled to protein A, submitted to SDS-PAGE, and transferred onto an Immobilon membrane (Millipore, Bedord, MA). The membranes were subsequently incubated with the relevant primary antibody before being incubated with the appropriate secondary antibody labeled with peroxidase for 60 min. The reaction was visualized on an x-ray film by using the ECL reagent (Amersham, Les Ulis, France) according to the manufacturer’s instructions. In some experiments the membranes were stripped with 0.1% HCl/glucose 6.5, for 1 h and neutralized in 1 mM Tris-HCl, pH 7.6, before reblotting.

Radiolabeling, Binding, and Cross-linking Experiments—CT-1, LIF, and CNTF were iodinated by the two-phase method as described previously (Robledo et al., 1995). Rats were killed by cervical dislocation and a 200-fold excess of unlabeled cytokine. After a 2-h incubation with agitation at 4 °C, cells were washed extensively and incubated for an additional 45 min at 20 °C in phosphate-buffered saline, 1 mM MgCl2, 1 mM BS3 (Fierce), 1 mM EG3 (Fierce), pH 8.3. After washes, cells were lysed in lysis buffer containing 1% Brij 96 as described above. Insoluble material was pelleted, and the supernatant was analyzed by SDS-PAGE and autoradiography. In some experiments cross-linked products were immunoprecipitated with anti-gp130 or anti-gp190 antibodies before electrophoresis. For the γ-linked polysaccharide analysis, cross-linked material or CT-1 was incubated overnight at 37 °C in the presence of 1 unit of endoglycosidase F (Boehringer, Mannheim, Germany) per mg of protein in 10 mM phosphate buffer, 0.5 M SDS, 25 mM EDTA, 0.5% 2-β-mercaptoethanol, 0.5% Nonidet P-40, pH 6, before being submitted to SDS-PAGE and autoradiography.

Results

gp130 and gp190/LIF β Receptor Are Essential Signaling Proteins for the CT-1 Receptor—In the present study we have analyzed the tyrosine phosphorylation events observed in response to CT-1. The SK-N-MC neuroblastoma cell line was used for these experiments, since tyrosine phosphorylation was readily detectable in these cells. Treatment of SK-N-MC cells with CT-1 resulted in the induction of gp130 tyrosine phosphorylation and of an additional protein with a molecular mass of 190–210 kDa co-precipitating with gp130 (Fig. 1A). A similar pattern was observed after treating the cells with OSM (or LIF), suggesting that the associated protein was gp190/LIF receptor. This was confirmed by reblotting the filter with an antibody directed against gp190. A blocking antibody to gp130 was sufficient to prevent the induction of tyrosine phosphorylation in both gp130 and gp190 subunit receptors, further indicating that gp130-gp190 heterodimer formation was required to induce the phosphorylation of both receptor components (Fig. 1B). In addition, a slight decrease in gp130 electrophoresis mobility was observed upon CT-1 activation, which was probably a result of the transducer activation as reported previously for IL-3β receptor (Liu et al., 1994). To determine whether gp130 and gp190 were sufficient to generate a functional receptor for CT-1, reconstitution experiments using cDNAs encoding these subunits were carried out in the COS-7 cell line (Fig. 1C). Individual expression of gp130 or gp190 did not allow the induction of tyrosine phosphorylation of these components by CT-1. In contrast, receptor activation was readily detectable when both subunits were co-expressed in the COS-7 cells, indicating that gp130-gp190 heterocomplex formation was essential for CT-1 signaling.

CT-1 Signaling Pathway Involves Jak1, Jak2, Tyk2 Tyrosine Kinases, and STAT-3 Transcription Factor—Receptor activation of the IL-6/LIF family of cytokines results in immediate phosphorylation of the transduction subunits by the Jak family members. Homo- or heterodimerization of the gp130 signal transducer was shown to induce activation and recruitment of Jak1, Jak2, and Tyk2 (Stahl et al., 1994). This led us to investigate whether the Jak-Tyk kinases are involved in signaling initiated by CT-1. Fig. 2, A–C show that tyrosine phosphorylation of Jak1-, Jak2-, and Tyk2-signaling proteins is induced after treatment of SK-N-MC cells by CT-1. Tyk2 phosphorylation observed in response to CT-1 is weaker than the signals obtained with the related members of the family (Guschin et al., 1995). Downstream signaling events were further analyzed by using the transcription factors known as STATs, which are tyrosine-phosphorylated at the cytoplasmic level before being translocated to the nucleus. As shown in Fig. 2, D–E, stimulation of the SK-N-MC cells with CT-1 elicits activation of STAT-3 protein. We failed to detect an induction of tyrosine
phosphorylation in STAT-1 after CT-1 activation (Fig. 2D), sustaining the notion of a specific recruitment of STAT-3 by CT-1.

Moreover, immunoprecipitation of the gp130 receptor allowed the co-precipitation of the LIF receptor but also of some additional tyrosine-phosphorylated proteins with molecular masses of 110–130 kDa and in the range of 80–90 kDa (Fig. 3A). Immunostaining of the blots with antibodies recognizing the Jak family members demonstrated that the Jak1 and Jak2 kinases could be co-precipitated with gp130, and were associated to gp130 in the absence of ligand. Tyk2 did not directly contact gp130 even after induction of gp130-gp190 heterodimerization (Fig. 3, D–F). Interestingly, the STAT-1 signaling protein was preassociated with gp130, but no variation in its detection or activation level could be observed after CT-1 treatment of the cells. In contrast, CT-1 stimulation induced a strong association of STAT-3 to the gp130-gp190 heterocomplex, but the detected, associated form of STAT-3 was weakly tyrosine-phosphorylated (Fig. 3, A and C), which is in striking contrast with the status of STAT-3 directly immunoprecipitated from the cytoplasmic extract (Fig. 2E). This indicates that STAT-3 was immediately released from the transducer receptors after activation.

CT-1 Cell Surface Binding—Scatchard analysis of saturation binding data was carried out in the SK-N-MC cell line, using concentrations of CT-1 ranging from 15 pm to 5 nm. Binding of 125I-CT-1 was specific and saturable and displayed high and low affinity sites in the SK-N-MC cell line (Fig. 4A). A biphasic Scatchard plot was observed with K_d values of 400–500 pm and 4 nM, indicating approximately 1,100–1,500 and 6,000 sites per cell, respectively. To assess the binding capacity of the gp130 and gp190 components, reconstitution experiments were performed in the COS-7 cell line. gp190-transfected cells bound both radiolabeled CT-1 and LIF with an affinity constant of about 10^{-9} M (Table 1). In contrast, the expression of gp130 alone led to a receptor that failed to bind CT-1 in an efficient way, indicating that the contribution of gp130 to CT-1 binding was not essential. Co-expression of gp130 and gp190 receptor subunits in COS-7 cells gave rise to high affinity receptors for both CT-1 and LIF (K_d = 200–470 pm) (Table 1 and Fig. 4B), in agreement with the recruitment of the two receptor subunits to generate a functional response as observed in Fig. 1C. We further analyzed the potential cross-competitions between CT-1 and the other members of the IL-6 family of cytokines toward their receptors. The experiments were performed on the SK-N-MC cell line, which expresses functional high affinity receptors for all described cytokines belonging to this group, as shown by Scatchard analysis and tyrosine phosphorylation assays of gp130 (Fig. 5A) (Chevalier et al., 1996). Cells were incubated in the presence of 1 nM radiolabeled CT-1 and a 200-fold excess of the putative competitors. Fig. 5B shows that bound radioactivity was displaced by an exogeneous addition of cold CT-1 and also by LIF and OSM. Cytokines using a homodimer of gp130 in their transducing machinery, such as IL-6, IL-11, or granulocyte-macrophage colony-stimulating factor, known to use a different receptor complex, did not interfere with CT-1 binding. As to the effect of CNTF on the binding of CT-1 to its receptor, only a slight displacement of the radiolabeled protein was observed. Reciprocal experiments conducted with 125I-CNTF confirmed that CNTF and CT-1 did not cross-compete or cross-competed only in a very marginal way, whereas competition between LIF and CNTF was observed as reported previously (Rohleod et al., 1996) (Fig. 5C).

Identification of a Third Component Involved in the Formation of CT-1 Receptor—We next employed cross-linking of iodinated CT-1 to the SK-N-MC cell surface to determine whether the cytokine directly contacted gp130 and gp190. Three labeled bands were detected corresponding to the molecular masses of 190–210 kDa, 130–150 kDa, and 80 kDa (Fig. 6A). The identity of two higher molecular mass bands was confirmed by immunoprecipitating the cross-linked products with antibodies raised against either gp190 or gp130. Similar patterns were obtained, demonstrating that gp190 and gp130 were components of the cross-linked products (Fig. 6B). In addition, gp130, gp190, and gp80 remained associated, showing that these proteins tightly interacted to generate a tripartite complex. The observed bands were competed by an excess of unlabeled CT-1 or LIF, which is consistent with the results...
obtained in the radioreceptor experiments (Fig. 5A). Similar experiments using radiolabeled LIF were also carried out, and using these experimental procedures a single band that corresponded to gp190 (190–210 kDa) and was competed by an excess of LIF or CT-1 was observed (data not shown).

To rule out the possibility of the 80-kDa band representing a degradation product of the gp130 or gp190 components, the experiments were performed in the presence of protease inhibitors. In additional experiments, incubation times with the cross-linking agents were increased up to 2 h, and an aliquot of the samples was incubated at 37 °C for 24 h before being electrophoresed and compared with the conventional preparations. No variation in the ratio of the three cross-linked components could be observed, showing that gp80 did not result from a proteolytic fragmentation of gp130 or gp190 (data not shown).

The 80-kDa subunit of the CT-1 receptor was further characterized by analyzing its carbohydrate content after removing the N-linked sugars from the protein backbone. A preliminary experiment indicated that N-linked polysaccharides account for 2–3 kDa of the CT-1 molecular mass (Fig. 6C, right part), which is in agreement with the presence of a single potential N-glycosylation site in the CT-1 sequence (Pennica et al., 1995a). Treatment of the 125I-CT-1 cross-linked receptor subunits with endoglycosidase F led, as expected, to a faster electrophoretic mobility of the products. Nevertheless, only a slight shift in gp190/LIF receptor molecular weight was detected, although 20 potential N-glycosylation sites are present in its amino acid sequence (Gearing et al., 1991). This result suggests that either the enzyme digestion was incomplete or that electrophoresis resolution was not sufficient to fully discern a molecular weight shift for large proteins. After treatment of gp130 with endoglycosidase F, a molecular mass of 100–110 kDa was observed as previously reported (Taga et al., 1989). Interestingly, the polysaccharide moiety of the CT-1 receptor α subunit represented about 35 kDa, leading to an N-deglycosylated protein with an apparent molecular mass of 45 kDa. The COS-7 cell line expresses functional high affinity binding sites for CT-1, when cotransfected with cDNAs encoding gp130 and gp190 (Fig. 4B and Table 1). In order to determine whether the 80-kDa component was present in the COS-7 cell line, cross-linking experiments were conducted in the COS-7 cells trans-
fecting with the appropriate cDNAs. In the nontransfected cells or in COS-7 cells transfected with a cDNA encoding gp130, a slight cross-linking of CT-1 to endogeneous LIFRβ could be observed in some instances (Fig. 7A). Introduction of gp190 allowed a clear detection of CT-1 associated to LIFRβ. In COS-7 cells co-transfected with cDNAs encoding gp130 and gp190, the three components could be cross-linked, indicating that the 80-kDa subunit is endogenously present but that expression of both transducing receptor components was required to detect a CT-1RIα cross-linking. After immunoprecipitation with antibodies directed against gp130 or gp190, the cross-linked products remained associated, indicating that the three receptor components tightly interacted in the presence of the ligand (Fig. 7B). These results could explain the presence of high affinity binding sites for CT-1 observed in the COS-7 cells transfected with both cDNAs (Table I).

**CT-1Rα Is Different from the α Components Involved in the Formation of IL-6, IL-11, or CNTF Receptors**—To evaluate any relationship between CT-1Rα and the previously described α components for IL-6, IL-11, and CNTF receptors, a series of experiments were carried out (Fig. 8). CT-1 failed to bind CNTF α receptor expressed in a fibroblast cell line, whereas 125I-CNTF bound to its α receptor component expressed in the same cell line (Fig. 8A and B). The IL-6-sensitive hematopoietic TF1 cell line was also found to proliferate in response to CT-1 and expressed its tripartite receptor. B-R6 mAb recognized the gp80/IL-6 receptor and inhibited its association to gp130 but not IL-6 binding (Fourcin et al., 1994). CT-1Rα was different from the gp80/IL-6 receptor, since B-R6 mAb only abrogated the response of the TF1 cell line to IL-6 but not to CT-1 (Fig. 8C). Since the CT-1 tripartite receptor is present in the KB epidermoid cell line (data not shown), we compared the induction of IL-6 secretion by culturing this line in the presence of CT-1 or IL-11. CT-1 induced IL-6 secretion, but IL-11 remained without effect, indicating that the IL-11 receptor α subunit was not an essential component for the CT-1 receptor (Fig. 8D). Collectively, these results indicate that CT-1Rα is different from the α components involved in the formation of the IL-6, IL-11, or CNTF receptors.

**CT-1Rα Is Required to Have a Fully Functional CT-1 Response**—Scatchard analysis of CT-1 binding to different cell types led to the identification of the murine hematopoietic Da1.a cell line (Gascan et al., 1990), which bound CT-1 only with a low affinity binding component (Kd = 5–7 nm) (Fig. 9A). In contrast, the Da1.a cell line displayed high affinity binding sites for both human and mouse LIF (Kd = 40–50 μM) and strongly proliferated in response to LIF, indicating the cell surface expression of the gp130-LIFRβ heterocomplex (Gascan et al. 1990) (Fig. 9C and data not shown).

Cross-linking of iodinated murine CT-1 to the surface of the Da1.a cells allowed the detection of a single product with an apparent molecular mass of 170 kDa, corresponding to the murine form of LIFRβ (Fig. 9B). Compared with the CT-1 cross-linking experiments carried out with the SK-N-MC cell line, a 10-fold higher Da1.a cell number was used, and the exposure times were lengthened from 4 to 21 days, further indicating that CT-1Rα was probably not expressed in the Da1.a cell line.

Analysis of tyrosine phosphorylation events in the Da1.a cells in response to CT-1, LIF, or OSM did not allow the detection of a gp130 or LIFRβ activation. We recently reported a similar situation for the TF1 cell line, which proliferated in the presence of the IL-6 type cytokines, but where the use of 10^9 cells only allowed the detection of a liminar activation of the gp190-gp130 complex (Chevalier et al., 1996). These results are in agreement with the observations showing that tyrosine phosphorylation of a Δ65 gp130 truncated mutant was not required

![Table I](image)

| Transfected cDNA | CT-1 | LIF |
|------------------|------|-----|
|                  | Kd   | Site number/cell | Kd   | Site number/cell |
| None             | ND   | ND            | ND   | ND            |
| gp190            | 2900–5000 | 35196–42176 | 1500 | 6100         |
| gp130            | ND        | ND            | ≥6000 | ND          |
| gp130 + gp190    | 208–477  | 1347–6990   | 70   | 6310         |

**Fig. 4.** Scatchard analysis of CT-1 binding. A, binding experiments were performed by incubating SK-N-MC cells (3–5 × 10^6) in the presence of increasing concentrations of radiolabeled CT-1. After a 2-h incubation period at 4°C, bound radioactivity was separated from the unbound fraction by centrifugation. The specific binding component was obtained by subtracting from the total bound radioactivity the values obtained in the presence of a 100-fold excess of cold ligand. The affinity-binding constants and site numbers were obtained by Scatchard transformation of the values. B, COS-7 cells were co-transfected with cDNAs encoding gp130 and gp190. 48 h later, CT-1 binding to the transfected cells (5–10^6 cells) was monitored as described in A.
for proliferation (Murakami et al., 1991). To further analyze the functional response of the DA1.a cell line we have studied the proliferation of the cells in response to CT-1 and LIF. Fig. 9C shows that in the KB cells, expressing the tripartite CT-1 receptor complex, a 4-fold decrease in the sensitivity of the response to the cytokines was observed (LIF EC50 = 5 pg/ml; CT-1 EC50 = 330 pg/ml). In contrast, the DA1.a cell line was 200-fold less sensitive to CT-1 compared with LIF (LIF EC50 = 5 pg/ml; CT-1 EC50 = 1,000 pg/ml).

Together, these results indicated an apparent lack of CT-1Rα expression in the DA1.a cell line, resulting in a low affinity binding of the cytokine to the cell surface and a proliferative response observed only in the presence of high concentrations of CT-1. This situation is very similar to the one we observed previously for the hematopoietic TF1 cell line in response to CNTF (Davis et al., 1993b).

**DISCUSSION**

Cardiotrophin-1 was named and isolated based on its ability to induce cardiac myocyte hypertrophy in vitro (Pennica et al., 1995a; Pennica et al., 1996a). Analysis of its amino acid sequence showed some similarities with the members of the IL-6 family of cytokines, in particular with LIF and CNTF. The availability of purified recombinant CT-1 allowed the characterization of the mouse CT-1 receptor. Involvement of the common signal-transducing component, gp130, and LIF receptor in the CT-1 receptor was demonstrated (Pennica et al., 1995b). In the present study we have characterized the human CT-1 receptor and its signaling machinery. Furthermore, reconstitution experiments and cross-linking of the radiolabeled cytokine to its receptor revealed that the CT-1 receptor is a tripartite complex, a situation comparable with that of the high affinity CNTF receptor.

Tyrosine phosphorylation analysis of CT-1 receptor components and reconstitution experiments carried out in the COS-7 cell line led to the conclusion that gp130 associates with the gp190/LIF receptor to create a functional receptor able to transduce CT-1 signaling inside the cell. Transfection experiments revealed that human gp190 is a major binding component for CT-1 as observed for the mouse receptor (Pennica et al., 1995b), whereas gp130 behaves more like a convertor protein, a situation reminiscent of the high affinity LIF receptor, where LIF preferentially contacts gp190 before recruiting the gp130-
transducing chain (Gearing et al., 1992). By using a neutralizing antibody directed against gp130 and performing COS-7 transfection experiments, we show that formation of a gp130-gp190 heterocomplex is essential for CT-1 signaling.

Analysis of the subsequent activation events indicates that CT-1 binding to gp130-gp190 transducing complex leads to the activation of Jak1, Jak2, and Tyk2 kinases, as reported for the other members of the IL-6 family (Stahl et al., 1994; Lütticken et al., 1994; Guschin et al., 1995). The information is next relayed to the nucleus by the STAT-3 transcriptional activator, whose association to the transducing complex is strongly enhanced by the binding of CT-1, before being released after tyrosine phosphorylation in the first 10 min following contact with the cytokine. We failed to detect an implication of STAT-1 in CT-1 signaling, which might represent a difference with the IL-6 pathway or could also be the result of tissue variations.

Several recent studies have reinforced the notion that STAT-3 is a major transcriptional factor phosphorylated in response to gp130-gp190 complex activation and that STAT-1 recruitment seems more restricted to IL-6 stimulation (Boulton et al., 1995; Zhang et al., 1995; Guschin et al., 1995). Very recently, STAT-1 gene inactivation in the mouse has underlined its essential implication in mediating the antiviral properties of interferons (Meraz et al., 1996; Durbin et al., 1996). No evidence for an alteration of the physiological responses dependent upon the IL-6 cytokine family was detected in the STAT-1-deficient mice.

CT-1 binding experiments defined high affinity and low affinity binding sites. Since gp190 behaves as a major binding component for CT-1, we can hypothesize that relative expression levels of each receptor component might affect the apparent affinity of CT-1 for the cell surface, as previously observed for LIF or CNTF receptors (Robledo et al., 1996). Cross-linking of CT-1 to the SK-N-MC cell line led to the identification of a

FIG. 7. CT-1 receptor α component is endogenously present in the COS-7 cell line. A, 3 × 10^6 COS-7 cells were transfected as indicated with the cDNAs encoding gp130 and gp190 or with the empty pCD vector. Cells were incubated with 10 ng of radiolabeled CT-1 and a 100-fold excess of the indicated factors for 2 h, followed by a 45-min incubation in the presence of 1 mM EGS and BS3 cross-linking agents. Brij 98 lysates were submitted to SDS-PAGE and autoradiographed for 4 days. B, samples were prepared as in A, but lysates were immunoprecipitated by using a polyclonal antibody raised against gp190 or with the B-T6 anti-gp130 mAb before SDS-PAGE and autoradiography.

FIG. 8. CT-1 receptor α component is different from the α components of related receptors. A, 3.5 × 10^6 SK-N-MC cells or L-cells stably transfected with a cDNA encoding the CNTF receptor α component were incubated in triplicate in the presence of 1 nM radiolabeled CT-1 and a 100-fold excess of unlabeled competitors for 2 h at 4 °C. Bound radioactivity was separated from the unbound fraction by centrifugation on an oil cushion, and the specific binding component was measured in a γ counter. B, the experiment was performed as in A, except that 125I-CNTF was used instead of radiolabeled CT-1, and 0.5 × 10^6 cells per condition were used. C, 10^5 TF1 cells were incubated in triplicate in the presence of the indicated combinations of CT-1 (20 ng/ml), IL-6 (20 ng/ml), B-R6 anti-IL-6 Rec. mAb (50 μg/ml), or IgG1 control immunoglobulin (50 μg/ml). After 72 h, 0.5 μCi of [3H]thymidine was added to the wells for the last 4 h of the culture, and the incorporated radioactivity was determined. D, 50 × 10^5 KB epidermoid carcinoma cells bearing the tripartite CT-1 receptor were incubated in the presence of 20 ng/ml CT-1 or IL-11. 48 h later, supernatants were harvested, and the amount of IL-6 synthesized was determined by enzyme-linked immunosorbent assay.
third entity with an apparent molecular mass of 80 kDa. Reconstitution experiments carried out in the COS-7 cells suggest that the 80-kDa product/CT1-Rα interacted very weakly, or even did not directly interact with CT-1 when expressed alone. In contrast, in the presence of the cross-linked ligand, the tripartite CT-1 receptor components can be co-precipitated with an antibody raised against either gp130 or gp190. This result indicates that the CT-1 receptor subunits, when activated, can tightly interact together, as reported also in the case of the tripartite CNTF receptor (Stahl et al., 1993).

Interestingly, cross-linked CT-1 could be displaced from gp80 subunit by adding excess unlabeled LIF, suggesting that gp80 might be involved in the formation of the LIF receptor as well. Experiments by using the DA1.a LIF-sensitive cell line indicate that this latest cell line bound CT-1 with a low affinity ($K_d > 5 \text{ nM}$) and bound LIF with a high affinity ($K_d = 40–50 \text{ pM}$). We failed to observe an expression of CT-1Rα on the DA1.a cell surface. Cross-linking experiments only allow the detection of a CT-1 binding to LIFRβ, a situation similar to that reported previously for the M1 myeloid cell line (Pennica et al., 1995b).

In addition, the DA1.a cell line appears to be 200-fold less sensitive to CT-1 compared with LIF. This suggests that the gp80 subunit might not be required for the formation of high affinity LIF receptor and that the identified gp80 component could represent an α chain conferring an increased sensitivity and specificity to CT-1. A parallel can be made with the TF1 cell line, which did not express CNTF receptor α and only responded to the addition of high CNTF concentrations to the cultures (Davis et al., 1993b). Interestingly, the addition of soluble CNTF receptor α to the cultures could increase the sensitivity of the cells to CNTF to reach a level identical to the one observed in the presence of LIF. We can hypothesize that a similar complementation mechanism might occur for the DA1.a cells and CT-1 if a soluble form of CT-1Rα could be found.

Members of the IL-6 cytokine family have been shown to modulate the phenotype and survival of neuronal cells. The ability of CT-1 to induce chick ciliary neuron survival was, however, 1000-fold less potent than the response observed to CNTF (Pennica et al., 1995b). This result might be explained by a lack of CT-1Rα expression in these neurons. On the other hand, CNTF receptor α gene inactivation experiments led to a profound anomaly in motoneuron development, whereas CNTF-deficient mice displayed only mild effects on behavior and physiology (DeChiara et al., 1995; Masu et al., 1993). These studies support the idea that an alternate ligand for the CNTF receptor α exists. We have investigated the possibility of CT-1 being a new ligand for CNTF receptor α. The fact that cells transfected with a cDNA encoding this subunit failed to bind CT-1 and that soluble CNTF receptor α did not potentiate the CT-1 response suggests the notion that CT-1 is not an alternate ligand for the CNTF receptor α component. Two other α-specific binding components, interacting with IL-6 and IL-11, have also been identified. The use of an antibody decoupling IL-6 receptor/gp130 interaction and the absence of response of the CT-1-sensitive KB cell line to IL-11 indicate that CT-1Rα is different from the previously identified α subunits.

A very recent paper from Pennica et al. (1996b) demonstrated that the survival of motoneurons to CT-1 involves, in addition to gp130 and LIFRβ, a third additional component sensitive to a phospholipase C treatment. This result strongly suggests that CT-1Rα links to the membrane through the GPI motif.

The observed situation for the tripartite CT-1 receptor is similar to the one described for the CNTF high affinity receptor (Davies et al., 1993). In this case, CNTF and its associated α component contacts gp130. These initial interactions are followed by gp190 recruitment for signaling. For the CT-1 receptor, a mirror image is observed, where CT-1 probably contacts a third entity similar complementation mechanism might occur for the DA1.a cell line. A parallel can be made with the TF1 cell line, which did not express CNTF receptor α and only responded to the addition of high CNTF concentrations to the cultures (Davis et al., 1993b). Interestingly, the addition of soluble CNTF receptor α to the cultures could increase the sensitivity of the cells to CNTF to reach a level identical to the one observed in the presence of LIF. We can hypothesize that a similar complementation mechanism might occur for the DA1.a cells and CT-1 if a soluble form of CT-1Rα could be found.

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