medium; Day 3, the cells were split into 10-cm dishes at 1:3, 1:10, and 1:30 dilutions and cultured in 5% serum–DMEM containing geneticin. The medium was replaced every 3 to 4 days, and pools of G-418–resistant colonies (either 8 or >100 colonies for each infection) were prepared.
15. Exponentially growing monolayers of NRK^{neo}, NRK^{cyA}, NRK^{cyE}, and NRK^{cdk2} cells were trypsinized and seeded on uncoated or agar-coated 150-mm dishes (2×10^6 cells in DMEM containing growth factors). Duplicate cultures of each transfectant were incubated for 48 hours and collected. One set of cells was extracted for RNA blot hybridizations with equal amounts of ribosomal RNA (rRNA). The other set was extracted in 0.1 ml of nonreducing SDS sample buffer. Protein concentrations were estimated by SDS–gel electrophoresis and silver staining; normalized portions of each sample were subjected to the immunoblot analysis.
16. Adherent, subconfluent cultures of NRK^{neo}, NRK^{cyA}, NRK^{cyE}, and NRK^{cdk2} cells were prepared as in (*17*). After an overnight incubation, [^{3}H]thymidine (1 μCi/ml) was incubated with each culture for 24 hours. Cells were fixed with 5% trichloroacetic acid (TCA), and TCA-insoluble radioactivity was isolated. [^{3}H]thymidine incorporation varied by no more than 20% between the four cell lines. Standard mitogen assays showed that the interval from G_0 through S phase was also similar in NRK^{neo} and NRK^{cyA} cells.
17. Exponentially growing monolayers of NRK^{neo}, NRK^{cyA}, NRK^{cyE}, and NRK^{cdk2} cells (4×10^4 cells per 35-mm dish) were incubated in suspension with growth factors and [^{3}H]thymidine (*2*). Incorporation of radiolabel into DNA was determined during the last 24 hours of a 2-day incubation.
18. NRK^{neo} and NRK^{cyA} cells synchronized in G_0 were suspended (2×10^4 in 2 ml of DMEM containing growth factors) and added to 35-mm dishes coated with either 100 μg of type I collagen (adherent cultures) or agar (nonadherent cultures). S phase progression was monitored by incubation of the growth factor–supplemented cultures with [^{3}H]thymidine (1 μCi/ml) for the 2-hour periods shown in Fig. 3A. DNA synthesis over a 3-day period was determined similarly except that the incubation with [^{3}H]thymidine was for three consecutive 24-hour periods.
19. Cyclin A–associated kinase activity in extracts of adherent and nonadherent NRK cells (2.5×10^6) was determined similarly to the procedure described in (*2, 12*) except that (i) the extraction buffer consisted of 10 mM sodium phosphate buffer (pH 7.0), 0.25 M NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 2 mM EDTA, 0.1 mM sodium orthovanadate, 50 mM sodium fluoride, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 1 mM phenylmethylsulfonyl fluoride; (ii) a 100-fold dilution of antiserum to cyclin A was used; and (iii) the immunoprecipitates were suspended in 30 μl of kinase buffer [J. Pines and T. Hunter, *Cell* **58**, 833 (1989)] containing 50 μM adenosine triphosphate (ATP), 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), and 4 μg of histone H1.
20. The analysis in soft agar was done as described (*2*) except that 35-mm dishes were coated with 1 ml of 0.5% agar and top layers contained 2 ml of 0.3% agar with 10^4 cells. The cultures were incubated for 14 days, during which time the majority of cells in the NRK^{cyA} pool formed colonies in soft agar as did at least 80% of the cells from the isolated NRK^{cyA} clone.
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29. We thank P. Nurse, J. Pines, B. Weinstein, and T. Nishimoto for reagents. T.G. was supported by the National Cancer Center and M.O. was supported by the Uehara memorial foundation (Japan) and the Leukemia Society of America. J.R. is a Lucille P. Markey scholar in the biomedical sciences. R.A. was an established scientist of the American Heart Association (NYC Affiliate). Supported by grants from the NIH and by the Lucille P. Markey Charitable Trust to J.R. and by NIH grant GM48224 to R.A..

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Characterization of a Pathway for Ciliary Neurotrophic Factor Signaling to the Nucleus

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Components of a signaling pathway that couples the ciliary neurotrophic factor (CNTF) receptor to induction of transcription were identified. CNTF stimulated the tyrosine phosphorylation of p91, a protein implicated in interferon signaling pathways, and of two proteins that are distinct but related to p91. Tyrosine-phosphorylated p91 translocated to the nucleus, where p91 and p91-related proteins bound to a DNA sequence found in promoters of genes responsive to CNTF. This DNA sequence, when inserted upstream of a reporter gene, conferred a transcriptional response to CNTF. A pathway that transduces interferon signals may therefore have a more general function in the propagation of responses to certain neurotrophic factors.

Ciliary neurotrophic factor enhances the survival and differentiation of distinct populations of neurons and glia (*1, 2*). It binds to the α component of its receptor and then sequentially associates with two structurally related β signal-transducing receptor components, gp130 and the leukemia inhibitory factor receptor β (LIFRβ), whose heterodimerization apparently transduces a signal across the membrane (*3, 4*). Activation of the receptors for CNTF and its related cytokines (*5*) results in phosphorylation of intracellular proteins on tyrosine and induction of transcription of immediate early genes (*6, 7*). However, the mechanisms by which signaling proceeds from the membrane to the nucleus remain almost completely unknown.

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A CNTF signaling pathway might share components of the signaling pathways for interferon alpha (IFN-α) and gamma (IFN-γ) (*8–10*). The CNTF receptor is related in structure to the IFN receptors (*11*). Protein tyrosine phosphorylation is required for signaling by CNTF and IFNs (*6, 9, 10*). An important DNA promoter site in genes activated by interleukin-6 (IL-6) (*12*) bears similarity to the IFN-γ–activated site (GAS) (*13*), which mediates transcriptional induction of IFN-γ–responsive genes. These observations and the finding that the CNTF and IL-6 receptors share the signal-transducing subunit gp130 (*5, 6*) suggest that the pathways that propagate the CNTF, IL-6, and IFN signals to the nucleus might also be similar.

A 91-kD protein (p91) becomes tyrosine-phosphorylated in cells exposed to IFN-α or IFN-γ (*9, 10*). Phosphorylated p91 then translocates to the nucleus where it participates in the transcriptional activation of interferon-responsive genes. It is believed that IFN-γ induces the binding of a p91 dimer to the GAS (*10*), and IFN-α causes the association of p91 with two newly tyrosine-phosphorylated 84- and 113-kD pro-

teins (p84 and p113) (9) and with a 48-kD protein to form the complex IFN-stimulated gene factor 3 (ISGF3), which binds a distinct promoter site upstream of IFN-α–responsive genes (*14, 15*).

We investigated if a protein related to p91 is expressed in the cell line SK-N-MC, which is a human neuroblastoma cell line that expresses CNTF receptors (*3*). CNTF induces transcription of the c-*fos* gene (*3*) in SK-N-MC cells, indicating that the intracellular mechanisms that transduce the CNTF signal from the receptor to the nucleus are intact. Antibodies to the COOH-terminus of p91 (anti-p91C), which recognize HeLa cell p91 but not p84 (*10*), immunoprecipitated a 91-kD protein from extracts of SK-N-MC cells labeled with [^{35}S]methionine (*16*). This protein comigrated with p91 immunoprecipitated from HeLa cells with anti-p91C (Fig. 1A). Gel slices containing 91-kD protein from SK-N-MC cells and HeLa cells were subjected to partial digestion with V8 protease (*17*). Analysis of the resulting peptides revealed an identical pattern for the two proteins, indicating that the 91-kD proteins in HeLa and SK-N-MC cells are closely related or identical (Fig. 1B).

To determine whether p91 from SK-N-MC cells becomes modified upon exposure to CNTF, we performed protein immunoblotting (*18*) with antibodies to p91 that recognize p91 and p84 (anti-p91,84) (*10*). In extracts from untreated SK-N-MC cells, anti-p91,84 recognized p91 and p84. However, in extracts obtained from cells treated with CNTF (100 ng/ml), another band that migrated more slowly than p91 was detected (Fig. 2A). This band comigrated with the newly phosphorylated p91 detected in extracts of HeLa cells treated with IFN-α (Fig. 2A), suggesting that CNTF induces a similar modification of p91. The kinetics of the CNTF-induced modification of p91 were rapid and transient. Modification of p91 was detected within 1 min (*19*), was maximal within 15 min, and disappeared by 60 min (Fig. 2A). The induction of the p91 modification occurred with as little as 1 ng of CNTF per milliliter of medium (*19*), which is within the physiological range of CNTF response (*20*). In SK-N-MC cells treated with IFN-γ for 18 hours, the amount of p91 was increased, and the CNTF-stimulated modification of p91 was more easily detected (Fig. 2B).

To investigate the nature of the CNTF-induced modification of p91 in SK-N-MC cells, we did phosphotyrosine immunoblotting of immunoprecipitated p91 (*16, 18*). In anti-p91C immunoprecipitates of extracts from untreated SK-N-MC cells, antibodies to phosphotyrosine did not recognize any proteins within the 91-kD range. However, in anti-p91C immunoprecipitates of CNTF-treated cells, a phosphotyrosine-containing protein was detected that migrated with the same mobility as modified p91 (Fig. 2C). The amounts of p91 in untreated and CNTF-stimulated cells were similar as assessed by anti-p91,84 immunoblots (Fig. 2A) and anti-p91C immunoprecipitates of cells labeled with [^{35}S]methionine (*19*). Taken together, these data suggest that the CNTF-induced modification of p91 results from phosphorylation of this protein on tyrosine residues.

To confirm that p91 becomes phosphorylated on tyrosine when SK-N-MC cells are exposed to CNTF, we labeled these cells with [^{32}P]orthophosphate and immunoprecipitated them with anti-p91C (*16*). Although phosphorylated p91 was immunoprecipitated from untreated cells, CNTF caused a fourfold increase in the incorporation of ^{32}P into the immunoprecipitated protein, which was accompanied by the appearance of a band of slower electrophoretic mobility (Fig. 3A). The immunoprecipitated p91 bands were excised and subjected to phosphoamino acid analysis (*21*). The p91 from

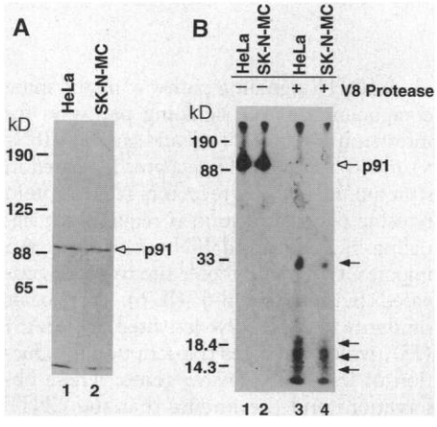

Fig. 1. Expression of p91 in SK-N-MC cells. (**A**) HeLa and SK-N-MC cells were incubated with [^{35}S]methionine (0.167 mCi/ml) in methionine-free DMEM (Dulbecco modified Eagle's medium) with dialyzed fetal bovine serum (FBS, 10%). Lysates from HeLa or SK-N-MC cells were immunoprecipitated with anti-p91C antibodies (*16*). Washed immunoprecipitates were separated by polyacrylamide gel electrophoresis (PAGE). (**B**) The p91 bands from (A) were excised and subjected to V8 protease digestion (100 ng of protease for 30 min at room temperature) followed by PAGE (12%). p91 was untreated (−) or incubated (+) with V8. Open arrow points to undigested p91. Solid arrows point to digested p91 peptides.

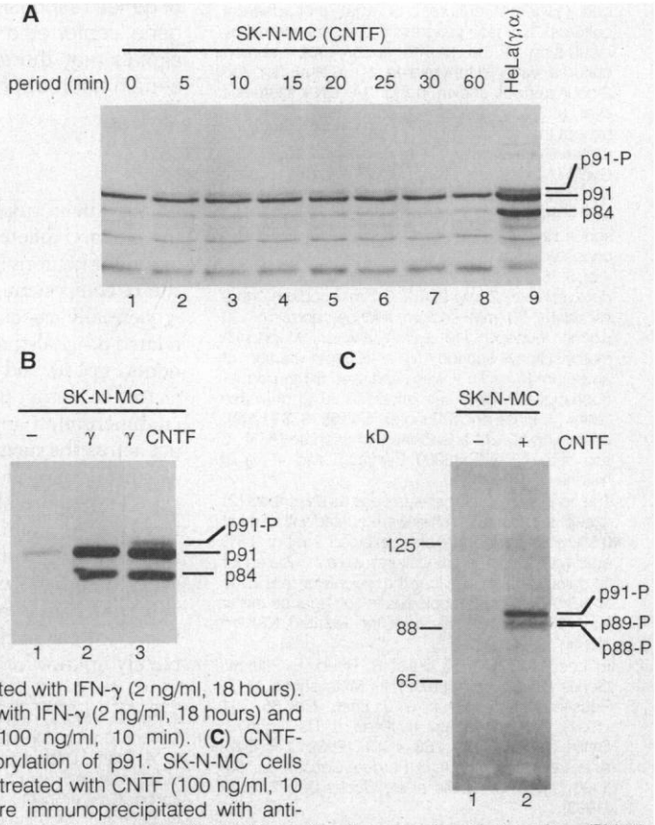

Fig. 2. CNTF-induced tyrosine phosphorylation of p91 in SK-N-MC cells. (**A**) SK-N-MC cells were treated for the indicated periods. Cell lysates were separated by PAGE and immunoblotted with anti-p91,84 antibodies (*18*). Antibody binding was detected with a secondary antibody conjugated to alkaline phosphatase. Modified p91 indicated by p91-P. Lane 1, untreated SK-N-MC cells. Lanes 2–8, SK-N-MC cells treated with CNTF (100 ng/ml). Lane 9, HeLa cells exposed to IFN-γ (2 ng/ml, 18 hours) and then IFN-α (1000 U/ml, 20 min). (**B**) CNTF-induced modification of p91 in SK-N-MC cells pretreated with IFN-γ. Lane 1, untreated SK-N-MC cells. Lane 2, cells treated with IFN-γ (2 ng/ml, 18 hours). Lane 3, cells were treated with IFN-γ (2 ng/ml, 18 hours) and then exposed to CNTF (100 ng/ml, 10 min). (**C**) CNTF-induced tyrosine phosphorylation of p91. SK-N-MC cells were untreated (lane 1) or treated with CNTF (100 ng/ml, 10 min) (lane 2). Lysates were immunoprecipitated with anti-p91C, separated by PAGE, and immunoblotted with antibodies to phosphotyrosine (PY20) [International Chemical and Nuclear (ICN), Irvine, California] and 4G10 [United Biotechnology, Inc. (UBI), Lake Placid, New York], each diluted 1:2000. Antibody binding was detected by enhanced chemiluminescence [ECL (Amersham)] with a secondary antibody conjugated to horseradish peroxidase.

untreated cells contained only phosphoserine, whereas p91 from stimulated cells contained phosphotyrosine in addition to phosphoserine (Fig. 3B). Taken together, these experiments indicate that CNTF induces the rapid tyrosine phosphorylation of p91 in SK-N-MC cells.

In contrast to IFN-α, which stimulates the tyrosine phosphorylation of p84 and p113 in addition to p91 (9) (Fig. 3C) and promotes the formation of the protein complex ISGF3α, CNTF failed to stimulate the phosphorylation of p113 or to induce formation of the ISGF3α complex (Fig. 3C). Instead, the induction of p91 tyrosine phosphorylation that occurred upon exposure of cells to CNTF was accompanied by the tyrosine phosphorylation of two proteins related to p91, p88 and p89 (Figs. 2C and 3D). These proteins are likely to be members of a family of related transcription factors, inasmuch as two antibodies that recognized distinct regions of p91 also recognize p88 and p89 in immunoprecipitation and phosphotyrosine immunoblotting experiments. Tyrosine phosphorylation of p88 and p89 was not detected in HeLa cells treated with IFN-α (*19*) and has not been detected in fibroblast FS2 cells treated with IFN-γ. The intracellular mechanisms by which the CNTF signal is propagated may therefore be partly distinct from the IFN-γ and IFN-α signaling pathways.

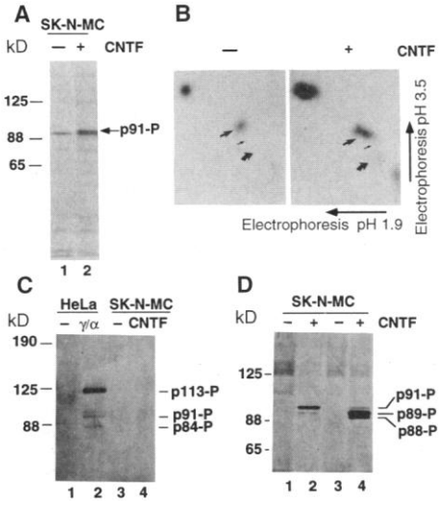

Fig. 3. CNTF-induced tyrosine phosphorylation of p91 and the related proteins p88 and p89. (**A**) SK-N-MC cells were labeled with [^{32}P]orthophosphate (ICN, 1.25 mCi/ml). Cells were untreated (lane 1) or treated with CNTF (100 ng/ml, 10 min) (lane 2). Lysates were immunoprecipitated with anti-p91C and separated by PAGE, transferred to polyvinylidene difluoride membrane, and analyzed by autoradiography. (**B**) Phosphoamino acid analysis (*21*) of immunoprecipitated p91. The p91 bands from (A) were excised from polyvinylidene difluoride membrane and hydrolyzed in 5.7 N HCl. Hydrolysates were analyzed on a thin-layer cellulose plate by two-dimensional electrophoresis. Position of phosphoamino acids was determined by ninhydrin staining. Large arrow, phosphoserine; small arrow, phosphothreonine; curved arrow, phosphotyrosine. (**C**) Lack of effect of CNTF on p113. Lysates were immunoprecipitated with anti-p113, separated by PAGE, and immunoblotted with antibodies to phosphotyrosine. Antibody binding was detected with ECL. Lane 1, untreated HeLa cells. Lane 2, HeLa cells incubated with IFN-γ (2 ng/ml, 18 hours) and then IFN-α (1000 U/ml, 20 min). Lane 3, untreated SK-N-MC cells. Lane 4, SK-N-MC cells treated with CNTF (100 ng/ml, 10 min). CNTF failed to induce tyrosine phosphorylation of p113 in SK-N-MC cells that had been treated with IFN-γ for 18 hours (*19*). (**D**) CNTF-induced tyrosine phosphorylation of p91 and the related proteins p88 and p89. Lysates of untreated (−) and CNTF-treated (+) SK-N-MC cells were immunoprecipitated with anti-p91C antibodies (lanes 1 and 2) or with anti-p91N antibodies (lanes 3 and 4), and immunoblotted with antibodies to phosphotyrosine. Antibody binding was detected by ECL. CNTF induced tyrosine phosphorylation of p88 and p89 with as little as 1 ng/ml (*19*), which is within the physiological range of CNTF response (*20*).

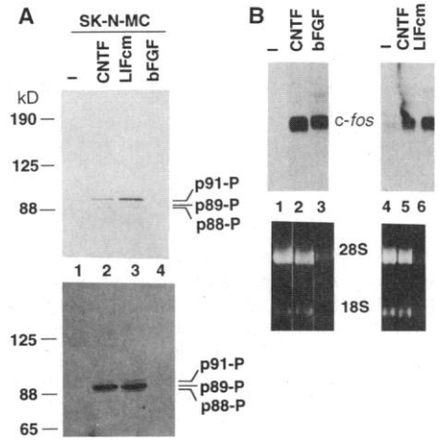

Fig. 4. Specificity of tyrosine phosphorylation of p91 and the p91-related proteins in SK-N-MC cells. (**A**) Anti-p91C (upper panel) and anti-p91N (lower panel) immunoprecipitates of SK-N-MC cell lysates were analyzed by anti-phosphotyrosine immunoblotting. Antibody binding was detected with alkaline phosphatase–conjugated secondary antibody (upper panel) or horseradish peroxidase–conjugated secondary antibody (lower panel). Cells were untreated or were stimulated with CNTF (100 ng/ml, 15 min), LIF-conditioned medium (LIFcm, 1%, 15 min), or bFGF (Collaborative Research) (25 ng/ml, 15 min). (**B**) Induction of c-*fos* expression in SK-N-MC cells in response to CNTF, LIFcm, and bFGF. RNA was isolated from cells following lysis with guanidinium thiocyanate (*33*) and was analyzed by Northern blotting (*37*). Cells were untreated or were stimulated with CNTF (50 ng/ml) for 45 min (lane 2) or 30 min (lane 5), bFGF (25 ng/ml, 30 min), or LIFcm (1%, 30 min). Upper panel shows autoradiograph of blot probed with c-*fos*. Lower panel shows corresponding ribosomal RNA bands from Northern gel.

Tyrosine phosphorylation of p91 and the related proteins was also stimulated in SK-N-MC cells by activation of the LIF receptor (Fig. 4A). However, exposure of SK-N-MC cells to basic fibroblast growth factor (bFGF) was ineffective at inducing tyrosine phosphorylation of p91 or the related proteins (Fig. 4A). SK-N-MC cells express functional FGF receptors because bFGF stimulated c-*fos* transcription in these cells. CNTF, LIF-conditioned medium, and bFGF all activated transcription of c-*fos* within minutes of treatment (Fig. 4B). Nerve growth factor was also ineffective in stimulating tyrosine phosphorylation of p91 or the related proteins in the pheochromocytoma cell line PC12 (*19*). Therefore, CNTF and LIF may be distinct among the neurotrophic factors in their ability to stimulate the tyrosine phosphorylation of p91 and the p91-related proteins.

To determine whether CNTF-induced phosphorylation of p91 is correlated with functional activation of this protein, we investigated the effect of CNTF on subcellular localization of p91 and its DNA binding activity. Immunofluorescence staining (*22*) with anti-p91C revealed that p91 was localized diffusely within the cytoplasm and to a lesser extent in the nucleus of untreated SK-N-MC cells. However, after treatment with CNTF, p91 was detected predominantly in the nucleus in about 85% of the cells (Fig. 5A). In extracts obtained from SK-N-MC cells treated with IFN-γ for 18 hours and then treated briefly with CNTF, the ratio of the phosphorylated form of p91 to the unmodified form was greater in the nuclear extracts than in whole-cell extracts (Fig. 5B). In addition, the overall amount of p91 in the nuclear extract from CNTF-treated cells was greater than that in the nuclear extract from untreated cells (Fig. 5B). When SK-N-MC cells were fractionated into cytoplasmic and nuclear components, a low amount of p91 was detected in the nuclear fraction before CNTF treatment. After CNTF treatment, tyrosine-phosphorylated p91 was detected in the nuclear fraction in an estimated 4 to 1 ratio relative to the cytoplasmic fraction. Thus, the amount of p91 increased in the nuclear

Table 1. DNA sequences resembling the CNTF-RE core in promoters of CNTF-responsive genes.

Gene	Sequence (*31*)
Human c-*fos*	**TTCC**CGTC**AA**
Mouse c-*fos*	**TTCC**CGTC**AA**
Mouse *tis11*	**TTCC**TAAG**AA**
Rat *junB**	**TTCC**GGG**AA**
Rat *SOD1**	**TTCC**TTG**AA**
CNTF-RE (core)	**TTCC**CCG**AA**

*Potential CNTF-responsive genes: *junB* responds to LIF (*7*); for *SOD1*, see text.

fraction as a result of the appearance of the tyrosine-phosphorylated p91 in this fraction (*19*). Taken together, these experiments indicate that CNTF treatment induces p91 translocation to the nucleus.

We examined the effect of treating cells with CNTF on the DNA binding properties of p91. Tyrosine-phosphorylated p91 recognizes the DNA sequence 5′ TTCCNNNAA 3′, termed GAS (*10, 13*). We used a DNA mobility-shift assay (*23*) to test nuclear extracts prepared from SK-N-MC cells for the presence of a factor that interacted with a GAS-related sequence, which we have termed a CNTF response element (CNTF-RE). When this sequence was incubated with nuclear extracts from SK-N-MC cells treated with IFN-γ and then briefly exposed to CNTF, DNA protein complex I was detected. This complex was not detected in the absence of CNTF treatment. Complex I formation was effectively competed by the inclusion of an excess of unlabeled CNTF-RE or GAS sequence in the reaction mix (Fig. 5C). Complex I comigrated on a nondenaturing polyacrylamide gel with a gamma-activated factor (GAF)–DNA complex detected when nuclear extracts of U937 cells treated with IFN-γ were incubated with the CNTF-RE (Fig. 5C). Complex I formation was prevented by addition to the reaction mix of either of two antibodies to p91, anti-p91C or antibodies to the NH_2-terminus of p91 (anti-p91N). Taken together, these data indicate that complex I is composed primarily of p91.

We also tested nuclear extracts from SK-N-MC cells not treated with IFN-γ for the ability to form DNA complexes with the CNTF-RE sequence. In this case, CNTF treatment of SK-N-MC cells induced the formation of complex I and of complexes II and III, which migrated more slowly than complex I (Fig. 5C). Complexes II and III were detected more readily in nuclear extracts from naïve SK-N-MC cells exposed to CNTF than in extracts of SK-N-MC cells treated first with IFN-γ and then with CNTF. This may result from the differential ability of IFN-γ treatment to enhance the amount of p91 relative to that of the related proteins. Like complex I, complex II contains p91, as its formation was also prevented by anti-p91C and anti-p91N (*19*). Complex III does not appear to contain p91 because anti-p91C failed to prevent complex III formation. However, complex III appears to contain one or more p91-related proteins because its formation was effectively inhibited by the addition of anti-p91N to the binding reaction (*19*). Because anti-p91N effectively binds p88 and p89 (Fig. 3D), a likely possibility is that the proteins present in complex III are p88 and p89. The finding that CNTF induced the binding of not only p91 but also p91-related proteins to the CNTF-RE revealed a distinguishing feature of the CNTF signaling pathway.

The p91 and related proteins might be key regulators of changes in gene expression that are triggered by CNTF. DNA sequence elements similar to the consensus DNA sequence required for p91 binding are present upstream of a number of genes that can be induced by CNTF (Table 1). Therefore, the ability of p91 binding sites to confer CNTF responsiveness to a nonresponsive reporter gene was examined. Cultures of SK-N-MC cells were transfected (*24, 25*) with a reporter gene containing nucleotides −71 to +109 of the mouse c-*fos* gene fused

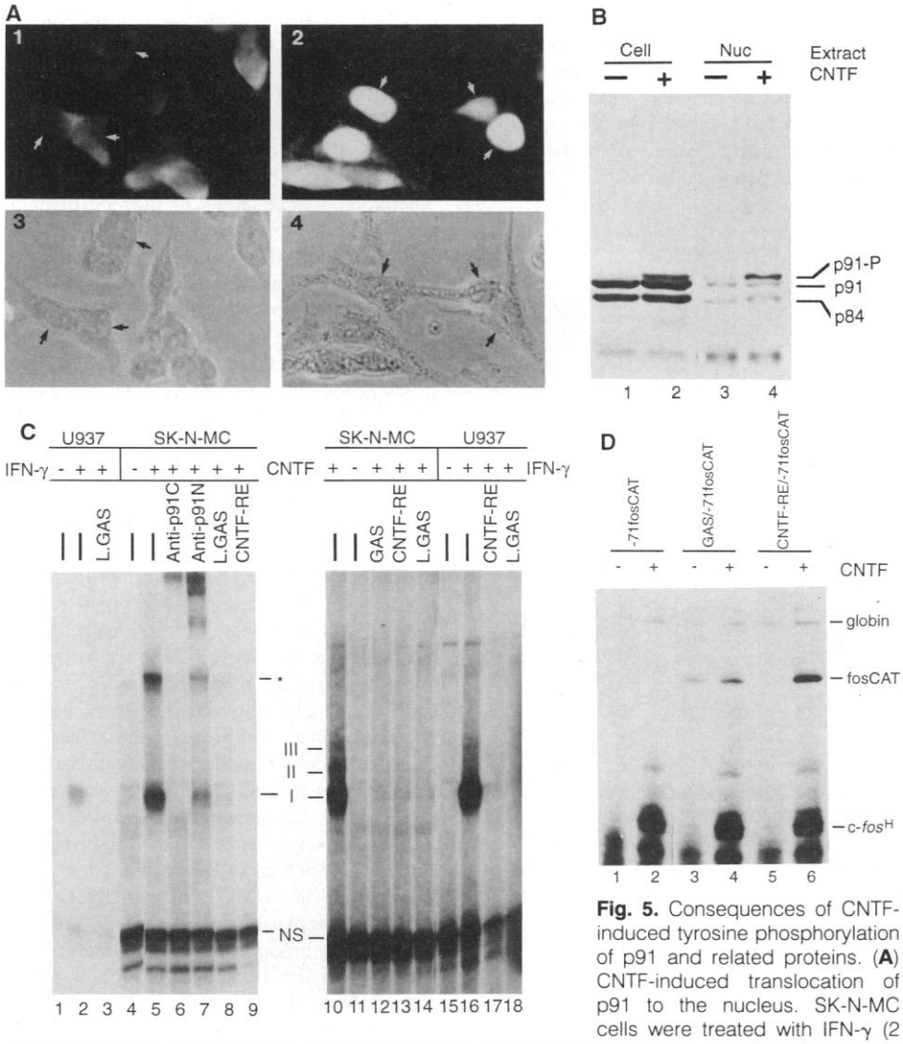

Fig. 5. Consequences of CNTF-induced tyrosine phosphorylation of p91 and related proteins. (**A**) CNTF-induced translocation of p91 to the nucleus. SK-N-MC cells were treated with IFN-γ (2 ng/ml, 18 hours) followed by no addition (panels 1 and 3) or addition of CNTF (100 ng/ml, 10 min; panel 2 and 4). We localized p91 by immunofluorescence (*22*) with anti-p91C (panels 1 and 2). Phase-contrast images of the fields used for immunofluorescence are shown in panels 3 and 4. Arrows point to nuclei. (**B**) Inducibly phosphorylated p91 is localized in the nucleus in CNTF treated SK-N-MC cells. Whole-cell extracts (lanes 1 and 2) or nuclear extracts (*23*) (lanes 3 and 4) from SK-N-MC cells that were treated with IFN-γ (2 ng/ml, 18 hours) followed by no addition (−) or addition (+) of CNTF (100 ng/ml) were immunoblotted with anti-p91,84. (**C**) CNTF-induced DNA-binding complexes (I, II, and III) that contain p91 and related proteins (*36*). CNTF-RE oligonucleotide was incubated with extracts prepared from U937 cells or SK-N-MC cells that were treated with IFN-γ (2 ng/ml, 18 hours, lanes 4–9) or that were not treated with IFN-γ (lanes 10–14). U937 cells were treated with tetradecanoyl phorbol acetate (5 nM, 48 hours) without (−) or with (+) subsequent addition of IFN-γ (2 ng/ml). SK-N-MC cells were treated (+ or −) with CNTF (100 ng/ml, 10 min). Reaction mixtures also included excess (50 times the amount of labeled probe) of unlabeled GAS (*12*) (GAS), Ly6E GAS (*35*) (L.GAS), or CNTF-RE (*35*). DNA binding reactions were also done in the presence of anti-p91C and anti-p91N. The asterisk indicates a slowly migrating complex that is believed to contain p91. A nonspecific complex is indicated (NS). (**D**) Induction of gene expression by CNTF is mediated by a CNTF-RE (*38*). A ribonuclease protection assay (*25*) was used to measure expression of the transfected human α globin gene, fosCAT fusion gene, and endogenous human c-*fos* (c-fos^H) after transfection of SK-N-MC cells with the indicated plasmids. RNA was isolated (*33*) from unstimulated (−) and CNTF-treated (+) cells (100 ng/ml, 50 min).

to the bacterial chloramphenicol acetyltransferase gene (−71fosCAT). To assess transfection efficiency, we cotransfected a human α-globin gene, driven by the simian virus 40 early promoter. Expression of the transfected genes and the endogenous human c-*fos* gene was determined by a ribonuclease protection assay (*25*). After normalization to the amount of α-globin message, the −71fosCAT gene was found to be expressed at the same low amount both before and after CNTF treatment. However, exposure to CNTF resulted in a twofold induction of the construct GAS/−71fosCAT, in which two copies of a palindromic GAS site were inserted immediately upstream of the −71fosCAT gene. Two copies of another DNA sequence that binds p91 were particularly effective at conferring a CNTF response when inserted upstream of the −71fosCAT gene, resulting in an 8.5-fold induction of transcription. Because this p91-binding DNA sequence is very effective in conferring CNTF responsiveness, we have termed it a CNTF-RE. Taken together with the protein phosphorylation and DNA binding studies described above, these gene expression experiments suggest that in untreated SK-N-MC cells, the CNTF-RE is not occupied, and that CNTF treatment induces the tyrosine phosphorylation of p91 and the p91-related proteins, with subsequent translocation and binding of these proteins to the CNTF-RE. This then leads to activation of transcription of genes containing this DNA element within their promoters.

The tyrosine kinases that link the CNTF receptor to p91 and the p91-related proteins are not known. The protein kinase Tyk-2 has been implicated in IFN-α stimulation of p91 tyrosine phosphorylation (*26*). CNTF stimulates the activity of Tyk-2 and its two family members, JAK1 and JAK2 (*27, 28*), each of which appears to be physically associated with the CNTF receptor before their activation (*29*). Once activated, one or more of these tyrosine kinases may then directly or indirectly trigger phosphorylation of p91 and the p91-related proteins. Once phosphorylated, p91 may mediate the cellular responses to a variety of different agents (*30*), and specificity may be conferred to the CNTF pathway by the p91-related proteins, which may interact with p91.

The identification of CNTF-RE sequences within the promoters of neuronal genes may allow identification of CNTF-responsive genes that encode proteins that mediate the biological effects of CNTF in the nervous system. For example, the promoter of a superoxide dismutase gene, the rat *SOD1* gene (*31*), contains a sequence similar to the CNTF-RE. Loss of function mutations of *SOD1* occur in familial amyotrophic lateral sclerosis (*32*), a dominantly inherited degenerative motor neuron disease. Thus, one mechanism by which CNTF may protect motor neurons from degeneration could be through enhanced expression of *SOD1*.

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36. The DNA binding assays were carried out as described in (*35*). Extracts were incubated with [^{32}P]-labeled double-stranded CNTF-RE oligonucleotide (*35*) containing the sequence AGCCTGAT**TTCCCCGAA**ATGACGGC (core sequence shown in bold). For competition analysis, the CNTF-RE, a GAS sequence (L.GAS), ATAT**TTCCTGTAA**GT, from the *Ly6E* gene (*35*), or the near-palindromic GAS sequence (GAS), **TTCCGGGAA** (*12*), was used. SK-N-MC cells were treated with IFN-γ for 18 hours (Fig. 5C, lanes 4–9) to induce the expression of p91. It is not known whether IFN-γ induces tyrosine phosphorylation of p91 in SK-N-MC cells. If this occurs with brief IFN-γ exposure, prolonged treatment with IFN-γ and subsequent exposure to CNTF may lead to downregulation of this response, as it does in other cell types. This would explain the absence of specific complex formation in extracts from SK-N-MC cells treated with IFN-γ for 18 hours followed by no CNTF treatment (Fig. 5C, lane 4). The lanes in Fig. 5C are not shown in their entirety because they were run with the free probe off the gel in order to obtain better resolution of the protein-DNA complexes.
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38. Plasmid −71fosCAT contains nucleotides −71 to +109 of the mouse c-*fos* gene fused to the bacterial chloramphenicol acetyltransferase gene (*34*). This construct does not contain the c-*fos* promoter sequence that resembles the CNTF-RE core (Table 1). Plasmid GAS/−71fosCAT contains two tandem copies of the sequence **TTCCGGGAA** (*12*) inserted with SalI overhangs into the SalI site of the plasmid −71fosCAT. This GAS sequence was chosen because it is a near-perfect palindrome that conforms to the consensus sequence for GAS (*12*). Specific protein-DNA complexes are formed effectively with this GAS sequence in extracts from HepG2 cells treated with the CNTF-related cytokine IL-6 (*12*). CNTF-RE/−71fosCAT contains two tandem copies of the CNTF-RE sequence (*36*) inserted into the SalI site of the plasmid −71fosCAT.

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