Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells

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SUMMARY

Ciliary neurotrophic factor was discovered based on its ability to support the survival of ciliary neurons, and is now known to act on a variety of neuronal and glial populations. Two distant relatives of ciliary neurotrophic factor, leukemia inhibitory factor and oncostatin M, mimic ciliary neurotrophic factor with respect to its actions on cells of the nervous system. In contrast to ciliary neurotrophic factor, leukemia inhibitory factor and oncostatin M also display a broad array of actions on cells outside of the nervous system. The overlapping activities of leukemia inhibitory factor, oncostatin M and ciliary neurotrophic factor can be attributed to shared receptor components. The specificity of ciliary neurotrophic factor for cells of the nervous system results from the restricted expression of the alpha component of the ciliary neurotrophic factor receptor complex, which is required to convert a functional leukemia inhibitory factor/oncostatin M receptor complex into a ciliary neurotrophic factor receptor complex. The recent observation that the alpha component of the ciliary neurotrophic factor receptor complex is expressed by very early neuronal precursors suggested that ciliary neurotrophic factor may act on even earlier precursors, particularly on cells previously thought to be targets for leukemia inhibitory factor action. Here we show the first example of ciliary neurotrophic factor responsiveness in cells residing outside of the nervous system by demonstrating that embryonic stem cells express a functional ciliary neurotrophic factor receptor complex, and that ciliary neurotrophic factor is similar to leukemia inhibitory factor in its ability to maintain the pluripotentiality of these cells.

Key words: leukemia inhibitory factor, ciliary neurotrophic factor, embryonic stem cells, oncostatin M, gp130

INTRODUCTION

Ciliary neurotrophic factor (CNTF), originally characterized for its ability to support the survival of parasympathetic neurons from chick ciliary ganglion (Adler et al., 1979; Lin et al., 1989; Stöckli et al., 1989), also supports the survival of sensory neurons, preganglionic sympathetic spinal cord neurons, retinal neurons, hippocampal neurons and motor neurons (Skaper and Varon, 1986; Blottner et al., 1989; Ernsberger et al., 1989; Hoffmann, 1988; Arakawa et al., 1990; Sendtner et al., 1990; Ip et al., 1991; Oppenheim et al., 1991; Martinou et al., 1992; Sendtner et al., 1992). In addition, CNTF induces the cholinergic differentiation of sympathetic neurons (Ernsberger et al., 1989; Saadat et al., 1989), the astrocytic differentiation of glial progenitors (Lillien et al., 1988; Hughes et al., 1988), and the maturation and survival of oligodendrocytes (Louis et al., 1993). It has recently been shown that CNTF is distantly related to members of a cytokine subfamily that includes leukemia inhibitory factor (LIF), oncostatin M (OSM), granulocyte colony-stimulating factor (G-CSF), and interleukin-6 (IL-6) (Bazan, 1991). While LIF and OSM mimic CNTF in its actions on cells of the nervous system (Bruce et al., 1992; Gearing and Bruce, 1992; Hall and Rao, 1992; Ip et al., 1992; Martinou et al., 1992; Rao et al., 1992a,b; Richards et al., 1992; Kalberg et al., 1993; Ludlam and Kessler, 1993; N.Y. Ip, G.D. Yancopoulos, unpublished data), LIF and OSM also display a broad array of actions outside of the nervous system (Chang and Gough, 1992; Bruce et al., 1992; Gearing and Bruce, 1992; Richards et al., 1992). The overlap in CNTF, LIF and OSM actions is explained by the recent finding that these cytokines share two of their receptor components (Gearing and Bruce, 1992; Gearing et al., 1991, 1992; Baumann et al., 1993; Ip et al., 1992; Liu et al., 1992; Davis et al., 1993a,b; Stahl et al., 1993): gp130, the IL-6 signal transducing receptor component (Taga et al., 1989; Hibi et al., 1990), and LIFR, originally cloned as a LIF-binding protein (Gearing et al., 1991). In contrast to LIF and OSM, however, CNTF requires a third receptor component, CNTFR (Davis et al., 1991), which converts

a functional LIF/OSM receptor (i.e., LIFR and gp130) into a functional CNTF receptor complex (Ip et al., 1992, 1993; Davis et al., 1993a,b; Stahl et al., 1993). CNTFR is almost exclusively expressed within the nervous system, and this restricted expression is apparently responsible for limiting CNTF to its actions on neuronal and glial cells (Davis et al., 1991; Ip et al., 1993). The finding that the CNTF receptor complex includes a functional LIF receptor raised the possibility that some cells previously found to be LIF-responsive may in actuality be physiologic targets for CNTF. Most such cells would be expected to reside within the nervous system, due to the restricted distribution of CNTFR. However, the recent observation that CNTFR is expressed by very early neuronal precursors (Ip et al., 1993) suggested that CNTF may act even earlier in development on cells residing outside of the nervous system. Here we explore the possibility that embryonic stem cells express not only LIF receptors, but a CNTF receptor complex, and that CNTF can maintain the pluripotentiality of these cells.

MATERIALS AND METHODS

Cell culture

The 129/Sv//Ev-derived ES cell line, CCE, used in this study was maintained in culture as described (Robertson, 1987). In brief, ES cells were grown on a feeder layer of STO fibroblast cells (growth-arrested with mitomycin C, Sigma) in Dulbecco's modified Eagle's media (DME, Irvine Scientific) containing 10% FBS (Hyclone), 0.1 mM -mercaptoethanol, L-glutamine (292 mg/ml), penicillin (100 U/ml) and streptomycin sulfate (100 mg/ml). The STO feeder cells were then eliminated by passing the ES cells twice onto gelatin-coated plates (0.1% gelatin from porcine skin, Sigma) in media supplemented with LIF (10-20 ng/ml, R & D Systems or Upstate Biotechnology, Inc.); further culture of STO-free ES cells was performed in media containing either LIF (20-50 ng/ml), CNTF (50 ng/ml, Masiakowski et al., 1991) or factor as indicated. Typically, ES cells were plated at a density of 6×10^6 cells per 100 mm dish or 5×10^5 cells per 35 mm dish (high density platings) and passaged every 3-4 days. Low density cultures of ES cells were plated at 500 or 1000 cells per 35 mm plate. The formation of embryoid bodies was according to techniques described previously (Robertson, 1987).

MTT assay

STO-free ES cells were plated onto gelatin-coated 24-well dishes at a density of 1×10^5 cells per well (high-density culture) in the presence of varying concentrations of LIF or CNTF. Cells were passaged (1:8) every 3 days for two passages; 1 day after the second passage, a MTT cell proliferation assay (Promega) was performed on the remaining attached cells according to manufacturer's recommendations. Absorbance at 570 nm was quantitated using a ThermoMax microplate reader (Molecular Devices).

Northern analysis

ES cells (CCE) used for northern analysis were grown on gelatin plates and were free of contaminating STO feeder cells. Total RNA was prepared by the guanidinium thiocyanate method (Chomczynski and Sacci, 1987). 10 µg of total RNA were fractionated on 1% formaldehyde-agarose gels and then transferred to nylon membranes. Blots were hybridized with ^{32}P-labelled cDNA probes for rat CNTFR (800bp *Pst*1 fragment), human LIFR (1.8 kb *Hin*dIII-*Not*I fragment), human *gp130* (3.0 kb *Eco*RI-*Spe*I fragment), human *tis 11* (2.3 kb *Eco*RI fragment) and mouse *fgf-5*

(2.2 kb fragment) (Ip et al, 1992; Davis et al., 1993; Haub and Goldfarb, 1991).

Immunoprecipitation and anti-phosphotyrosine immunoblotting

STO-free ES cells, grown in media supplemented with LIF (20 ng/ml), were washed three times in phosphate-buffered saline (PBS, without calcium and magnesium salts, Irving Scientific), and then starved in RPMI media for 2 hours at 37°C. After the 2 hour starve period, cells were treated with varying concentrations of CNTF, LIF or IL-6 for 5 minutes, washed in PBS containing orthovanadate (1 mM) and solubilized in lysis buffer (PBS with 1% Brij96 (Sigma), 1 mM orthovanadate, 1 mM PMSF and aprotinin (0.14 U/ml)). The lysate was immunoprecipitated with an antibody that recognizes LIFR (Stahl et al., 1993) for 1-3 hours at 4°C and then protein G-Sepharose (1-3 hours, Pharmacia). The beads were washed three times with lysis buffer and then boiled in protein sample buffer. Immunoprecipitated proteins were separated on 7.5% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene difloride membranes, blocked (15 mM Tris, pH 7.5, 200 mM NaCl, 10% BSA) for 3 hours incubated for 1 hour 45 minutes with anti-phosphotyrosine antibody (monoclonal 4G10, Upstate Biotechnology Inc.) at a dilution of 1:4000 in TBST (20 mM Tris, pH 7.6, 150 mM NaCl, 4% bovine serum albumin and 0.1% Tween-20). The blots were then washed 3×10 minutes in TBST. The immunoprecipitated phosphotyrosine proteins were visualized by the ECL reaction according to the manufacturer's instructions (Amersham).

RESULTS

ES cells express functional CNTF receptors

LIF is thus far the only factor known that can maintain ES cells, which are derived from the inner cell mass of a preimplantation mouse blastocyst, in an undifferentiated state capable of contributing to all cell lineages upon reintroduction into the embryo (Smith et al., 1988; Williams et al., 1988; Pease and Williams, 1990). As would be expected based on their LIF-responsiveness, we find that ES cells express both LIFR and gp130 transcripts (Fig. 1A) and can also be maintained by OSM (Fig. 2C). Moreover, two different ES cell lines examined (but not STO cells normally used as feeders for ES cells) also express CNTFR transcripts (Fig. 1A), suggesting that CNTF might indeed act on ES cells. To determine whether ES cells express functional CNTF receptor complexes and if activation of these receptors results in responses similar to those triggered by LIF, we first compared immediate early signaling events induced by LIF and CNTF in ES cells. It is known that LIF and CNTF induce indistinguishable patterns of protein tyrosine phosphorylation and gene activation in responding neuronal cells (Ip et al., 1992); the gene activations include induction of a characteristic immediate early gene, *tis*11. We find that CNTF is equivalent to LIF in inducing the *tis*11 gene in ES cells (Fig. 1B). In contrast, IL-6, a cytokine related to LIF and CNTF, does not induce *tis*11 in ES cells (Fig. 1B). Furthermore, both LIF and CNTF elicit tyrosine phosphorylation of the receptor components, LIFR and gp130, in a similar dose-dependent manner (Fig. 1C). Hemopoietic cells expressing functional LIF receptors in the absence of CNTFR do not display detectable responses to these doses of CNTF (Davis et al., 1993a), suggesting that
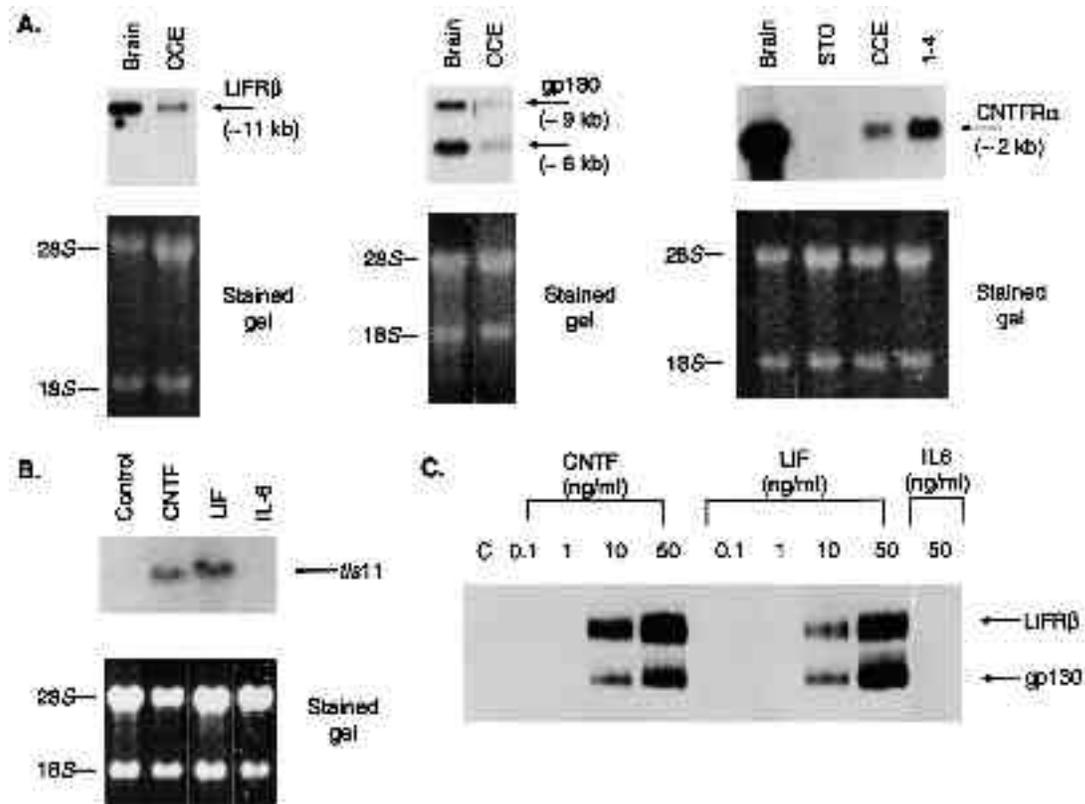
Fig. 1. ES cells have functional CNTF receptors. (A) Expression of LIFR , gp130 and CNTFR mRNAs in ES cells. Two ES cell lines were used, CCE (grown in absence of STO feeder cells) and 1-4 (grown on STO feeder cells, CB1-4 line from Charles Epstein, UCSF). While expression of CNTFR was not detected in mitotically active STO cells (right panels in A), LIFR and gp130 mRNAs were detected in STO cells (data not shown). Adult rat brain RNA (Brain) is shown for comparison. Ethidium bromide-stained gels are included to indicate RNA-loading quantities. (B) The immediate early response gene, *tis*11, is induced following treatment of ES cells with LIF or CNTF, but not with IL-6. ES cells (in the absence of STO cells) were incubated for 2 hours in serum-free defined media and then treated with either LIF (50 ng/ml), CNTF (50 ng/ml), IL-6 (50 ng/ml) or no factor (control) for 45 minutes. The ethidium bromide-stained gel indicates RNA-loading quantities. (C) LIF and CNTF induce tyrosine phosphorylation of LIFR and gp130 in ES cells. After a 2 hour starve period, ES cells (in the absence of STO cells) were treated with the indicated concentrations of CNTF, LIF, IL-6 or no factor (C) for 5 minutes, and then immunoprecipitated and immunoblotted as described in Materials and Methods.

CNTF is not functioning through LIFR and gp130 alone in ES cells. In addition, the similar magnitude of the responses elicited by CNTF and LIF indicates that functional CNTF receptors are found on most (if not all) of the ES cells bearing LIF receptors, and not only on a restricted subpopulation of differentiated ES cells.

CNTF maintains undifferentiated state of ES cells

Our findings that ES cells express functional CNTF receptors, together with previous data indicating that CNTF and LIF share common receptor subunits and signalling pathways (Ip et al., 1992, 1993; Davis et al., 1993a,b; Stahl et al., 1993), suggested that CNTF might be equivalent to LIF in maintaining ES cells in a pluripotential state. Stem-cell morphology was initially used to compare potential effects of CNTF with those of LIF. In low-density cultures in which growth of ES cells as single-cell-derived clones was examined, both LIF and CNTF could maintain the undifferentiated state (characterized by discretely bordered tight clumps of small cells lacking discernible intercellular boundaries) of a substantial proportion of those clones (Fig.

2A, left and middle panels, and Fig. 2B); in contrast, only differentiated clones (characterized by individual, large, flat cells) could be observed in the absence of LIF or CNTF (Fig. 2A, right panel, and Fig. 2B). In high density cultures ES cells could be maintained by either CNTF or LIF for at least 18 passages (>54 days) with no apparent change in their growth rate or morphology. In contrast, ES cells grown in the absence of these factors for even two passages (7 days) displayed substantial cellular differentiation. Consistent with these observations in high density cultures, a direct comparison after three passages at high density reveals an increased number of cells in the presence of either LIF or CNTF as compared to in the absence of these factors (Fig. 2D).

To compare further the extent of differentiation in ES cells maintained in LIF or CNTF we utilized expression of fibroblast growth factor-5 (*fgf-5*) as a sensitive marker of ES cell differentiation. While undifferentiated ES cells and their in vivo correlate, the blastocyst inner cell mass, show no detectable *fgf-5* transcripts, early postimplantation development and differentiation is marked by a burst of epiblastic
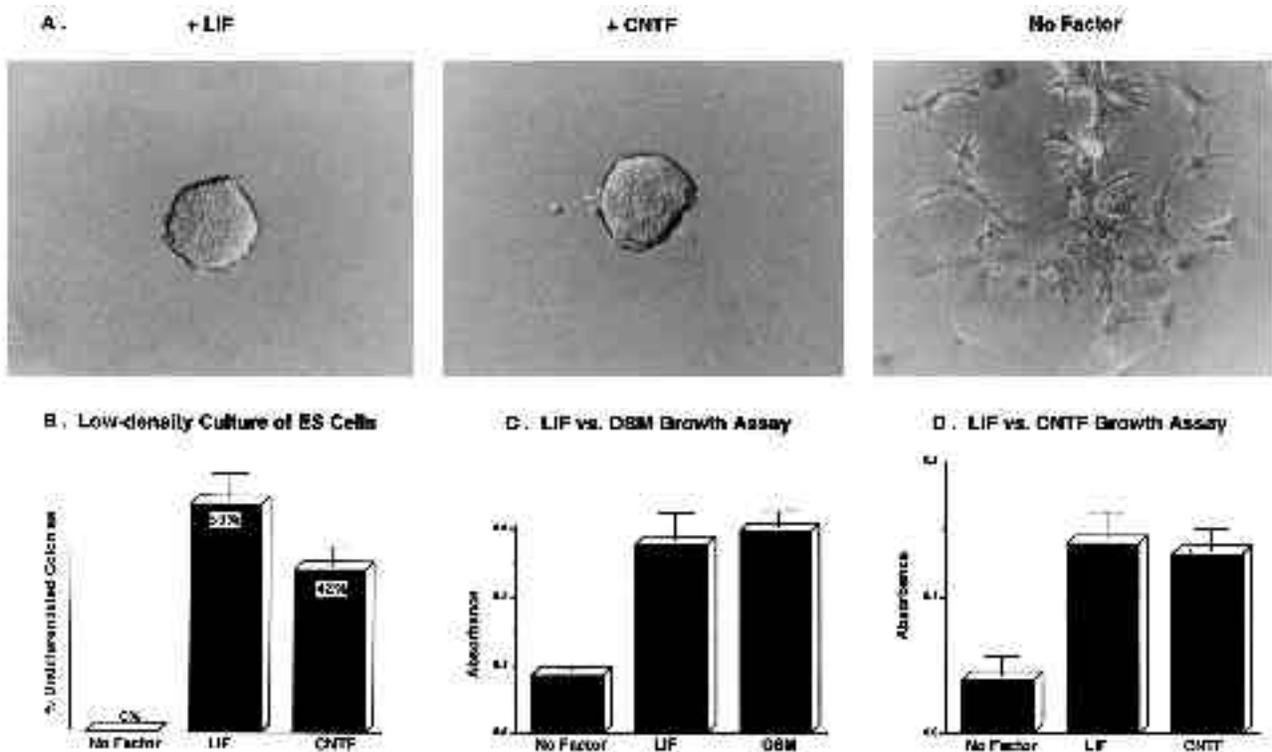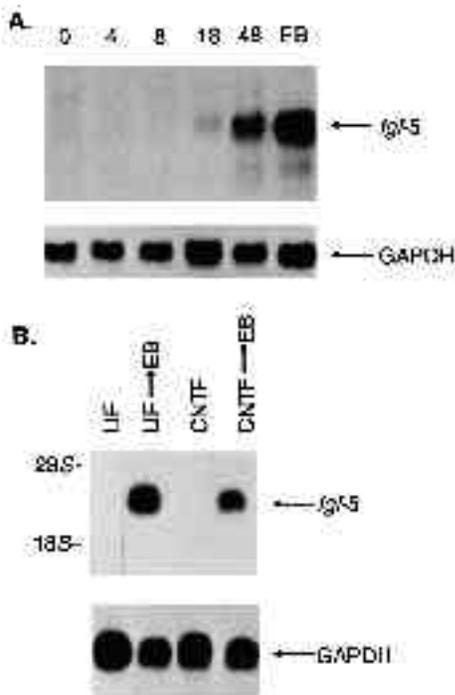
Fig. 2. CNTF is capable of maintaining stem cell morphology. (A,B) A substantial proportion of single cell-derived ES cell colonies retain the characteristic stem cell morphology in low-density cultures when grown in the presence of either LIF or CNTF, while culture without LIF or CNTF resulted only in differentiated colonies (typical colonies under the different conditions depicted in A, with data graphically summarized in B); ES cells used in these experiments were adapted to STO-free conditions for six passages (approximately 24 days) in the presence of either LIF (50 ng/ml) or CNTF (50 ng/ml) prior to plating at single cell cloning density (500-1000 cells per 35 mm plate) in the presence of LIF, CNTF or no factor. (C,D) ES cells cultured for three passages (9 days, high-density cultures) in the presence of LIF (50 ng/ml), OSM (50 ng/ml) or CNTF (50 ng/ml) showed equivalent increases in cell number as compared to ES cells cultured with no factor; cell number was assessed by an MTT assay (see Materials and Methods).
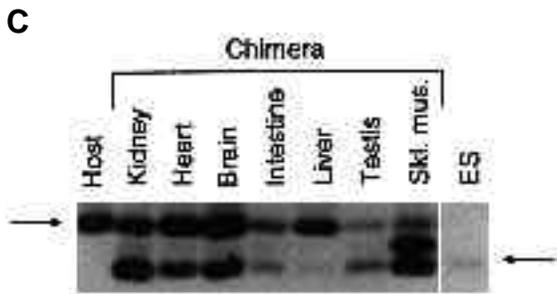

fgf-5 expression (Hébert et al., 1991; Haub and Goldfarb, 1991); this burst of *fgf*-5 expression is mimicked in vitro during the differentiation of suspension cultures of ES cells into embryoid bodies (Haub and Goldfarb, 1991; Hébert et al., 1991; Shen and Leder, 1992; Fig. 3B, compare lanes 1 and 2). We find that depriving even substratum-attached ES cells of LIF results in the induction of *fgf*-5 transcripts within 18-48 hours (Fig. 3A), well before the development of obvious morphological differentiation. Maintenance of ES cells in LIF or CNTF (for at least six passages, >24 days) prevents induction of *fgf*-5 (Fig. 3B, lane 1 and 3). Furthermore, ES cells maintained in CNTF retain the ability, upon removal of CNTF, to express *fgf*-5 and differentiate into embryoid bodies (Fig. 3B, lane 4). Altogether, the morphological studies and the *fgf*-5 analyses suggest that CNTF

Fig. 3. LIF- and CNTF-maintained ES cells do not express *fgf*-5. (A) Time course showing the rapidly induced expression of *fgf*-5 mRNA in ES cells grown in the absence of LIF or CNTF. (B) *fgf*-5 mRNA is not detectable in ES cells maintained for six passages (24 days) in LIF or CNTF; however, these cells are capable of expressing *fgf*-5 and forming embryoid bodies (EB) when grown in suspension in the absence of LIF or CNTF. As a control for the amount of RNA loaded, the filters were probed with the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

A

CNTF   LIF

B

	Pubs Born	# of Chimeras	# Male (%)	Extent Chimerism (%)
CNTF	36	23	19 (83%)	Male (92, 95, 90, 85, 50, 70, 70, 60, 60, 50, 50, 30, 30, 30, 75, 35, 25, 15, 5) Female (35, 10, 10, 10)
LIF	26	20	11 (55%)	Male (90, 80, 60, 40, 25, 15, 15, 10, 10, 5, 5) Female (54, 40, 20, 20, 15, 10, 10, 10, 5)

C

Chimera

Host, Kidney, Heart, Brain, Intestine, Liver, Testis, Skl. mus.   ES

Fig. 4. ES cells maintained in CNTF contribute to the formation of highly chimeric mice. The 129/Sv//Ev XY ES cell line (CCE) used in this study was derived from an agouti mouse. These ES cells, maintained for 6-10 passages in CNTF or LIF in in the absence of STO feeder cells, were then injected into C57Bl/6 blastocysts, which would normally form black mice. The injected embryos were then transferred into pseudopregnant females ((C57Bl/6×CBA)F$_1$, Jackson Laboratory) and allowed to develop to term. (A) Chimeras derived from ES cells maintained with either CNTF or LIF are depicted adjacent to a non-chimeric pup. (B) Record of chimeric mice resulting from ES cells maintained in CNTF or LIF. (C) Glucose phosphate isomerase analysis (GPI) of tissues from a chimeric mouse generated using ES cells maintained in CNTF. The GPI isoforms present in the host blastocysts (C57/Bl/6) and ES cells are indicated (arrows). Skeletal muscle, a syncitial tissue, shows heterodimerization of the two isoforms.

maintains ES cells in an undifferentiated state similar to that maintained by LIF, and that these cells retain the ability to appropriately differentiate upon removal of the CNTF.

CNTF-maintained ES cells can be used to generate chimeric mice

As a final demonstration that CNTF maintains the pluripotentiality of ES cells, we have been able to utilize CNTF-maintained ES cells (in the absence of STO feeder cells) to generate highly chimeric mice by blastocyst microinjection, with a preponderance of males observed among the chimeric offspring (Fig. 4A-C). This distortion in the normal ratio of male to female mice among the chimeras is one indicator of good chimera formation, suggesting high localized incorporation of Y-chromosome-bearing ES cells into the genital ridge. We find that the extent of chimerism obtained with CNTF-maintained ES cells was comparable to that obtained using ES cells maintained with LIF and that the male-to-female ratio distortion was even slightly higher (Fig. 4A,B). However, the extent of chimerism obtained with ES cells maintained with either CNTF or LIF in the absence of STO feeder cells was somewhat less than that obtained using cells maintained on STO feeder cells (data not shown). In addition, ES cells maintained for 6-10 passages in the presence of either CNTF or LIF (but in the absence of STO feeder cells) could contribute to chimeras in which >80% of the coat color was ES-cell derived (Fig. 4A,B) whereas higher passages (18 passages) of either CNTF or LIF-maintained ES cells could contribute less to the coat color of resulting chimeras (20-30% ES-derived).

Since ES cells and the host blastocyst express different isoforms of glucose phosphate isomerase (GPI), GPI analysis of tissues from chimeric animals was used to demonstrate ES cell contribution to various cell lineages. GPI analyses verified that CNTF-maintained ES cells could contribute to all cell lineages examined (Fig. 4C). Finally, chimeric mice generated from CNTF-maintained ES cells exhibited no decrease in vitality nor increase in tumor incidence as compared to control animals.

DISCUSSION

We have found that CNTF is similar to LIF, its distant structural relative, in maintaining the pluripotentiality of ES cells. This equivalency is entirely consistent with the finding that CNTFR, which is expressed in ES cells, converts a LIF receptor (i.e., gp130 and LIFR) into a functional CNTF

receptor (Ip et al., 1992, 1993; Davis et al., 1993a,b; Stahl et al., 1993; Gearing et al., 1993). The LIF/CNTF cytokine subfamily also includes OSM, IL-6 and G-CSF (Rose and Bruce, 1991; Bazan, 1991). As would be expected based on its ability to bind the LIF receptor complex in the absence of additional receptor components (Gearing and Bruce, 1992; Gearing et al., 1992), OSM appears similar to LIF and CNTF in its actions on ES cells. However, ES cells lack responses and required receptor components for IL-6 and G-CSF (Schmitt et al., 1991). Furthermore, ES cells reportedly do not express receptors for any of the other cytokines examined (i.e., receptors for erythropoietin, CSF-1, GM-CSF, interleukin-1, interleukin-3 and interleukin-4; Schmitt et al., 1991).

The rather unique capabilities of CNTF, LIF and OSM to act on ES cells may be of no physiological import, simply providing convenient reagents for the in vitro maintenance of pluripotential stem cells. On the other hand, it seems quite likely that at least one of these factors may influence the maintenance and/or eventual development of pluripotential stem cells during normal embryogenesis. The presence of the entire CNTF receptor complex in ES cells raises the possibility that LIF and OSM responses by these cells simply reflect the inclusion of a functional LIF/OSM receptor within the CNTF receptor complex. In contrast, LIF expression in the uterus coincides with blastocyst implantation (Bhatt et al., 1991) and pregnant mice homozygous for a disrupted LIF gene cannot support blastocyst implantation (Stewart et al., 1992); however, blastocysts lacking a functional LIF gene develop normally in foster mothers (Stewart et al., 1992). The absence of notable developmental defects in these embryos raises the possibility that either OSM (which has not yet been identified in rodents) or CNTF can compensate for lack of LIF during early development.

The actions of CNTF on ES cells provide the first example of CNTF responses in cells outside of the nervous system; recent findings also suggest that CNTF can act directly on skeletal muscle (Helgren et al., unpublished data). Expression of CNTFR by early neuronal progenitors (Ip et al., 1993), together with the findings described here on ES cells, indicate that a factor discovered for its ability to promote the survival of mature neurons may in addition have widespread actions on immature precursor cells and may play a role in the preimplantation stage of embryogenesis. Alternatively, the physiological mediator of CNTF-like actions on pluripotential stem cells and early neuronal progenitors may be a yet undiscovered relative of CNTF that shares the requirement for CNTFR, but also displays appropriate temporal and spatial distributions; the remarkable evolutionary conservation of CNTFR (which exceeds that of any other cytokine receptor) has previously led to the suggestion that it may bind additional members of this growing cytokine family (Ip et al., 1993). Resolution of these issues requires detailed expression studies during embryogenesis, as well as the manipulation of the genes for these cytokines and their receptors in vivo.

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