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Intracellular and Extracellular Leukemia Inhibitory Factor Proteins Have Different Cellular Activities That Are Mediated by Distinct Protein Motifs

Bryan P. Haines,* Roger B. Voyle, and Peter D. Rathjen†‡

Department of Biochemistry, University of Adelaide, and †Australian Research Council Special Research Centre for the Molecular Genetics of Development, Adelaide, South Australia 5005, Australia

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Although many growth factors and cytokines have been shown to be localized within the cell and nucleus, the mechanism by which these molecules elicit a biological response is not well understood. The cytokine leukemia inhibitory factor (LIF) provides a tractable experimental system to investigate this problem, because translation of alternatively spliced transcripts results in the production of differentially localized LIF proteins, one secreted from the cell and acting via cell surface receptors and the other localized within the cell. We have used overexpression analysis to demonstrate that extracellular and intracellular LIF proteins can have distinct cellular activities. Intracellular LIF protein is localized to both nucleus and cytoplasm and when overexpressed induces apoptosis that is inhibited by CrmA but not Bcl-2 expression. Mutational analysis revealed that the intracellular activity was independent of receptor interaction and activation and reliant on a conserved leucine-rich motif that was not required for activation of cell surface receptors by extracellular protein. This provides the first report of alternate intracellular and extracellular cytokine activities that result from differential cellular localization of the protein and are mediated by spatially distinct motifs.

INTRODUCTION

Cytokines and growth factors have been extensively studied as extracellular signaling molecules that function via interaction with cell surface receptors; however, there is increasing recognition that some of these molecules can fulfill alternate signaling roles within the cell (Jans and Hassan, 1998). Diverse growth factors and cytokines are retained within intracellular compartments because they are produced as proteins lacking conventional secretion signal sequences (Lin *et al.*, 1989; Rubartelli *et al.*, 1990; Mignatti *et al.*, 1992; Miyamoto *et al.*, 1993; Pennica *et al.*, 1995). Alternatively, secreted cytokines may enter cytosolic and nuclear compartments after receptor-mediated endocytosis (Curtis *et al.*, 1990; Lobie *et al.*, 1994; Jans *et al.*, 1997). Many cytokines and growth factors contain functional nuclear localization sequences (Maher *et al.*, 1989; Jans and Hassan, 1998), and nuclear localization of fibroblast growth factor (FGF)-1 and -2 (Zhan *et al.*, 1992; Arnaud *et al.*, 1999) appears to be of functional significance because it is regulated by cell cycle and differentiation (Baldin *et al.*, 1990; Shiurba *et al.*, 1991). Intracellular FGF-2 can be found complexed with receptors (Maher, 1996), signal transduction molecules (Bonnet *et al.*, 1996), and chromatin (Bouche *et al.*, 1989). FGF-2 can have activating or inhibitory effects on the promoter activity of the *pgk-1, pgk*-2 (Nakanishi *et al.*, 1992), and rRNA genes (Bouche *et al.*, 1989). Nuclear localization of FGF-1 is also necessary for the full elaboration of its mitogenic activity (Imamura *et al.*, 1990; Wiedlocha *et al.*, 1996). Collectively, these observations suggest that the intracellular localization of growth factors and cytokines is likely to be important biologically.

Leukemia inhibitory factor (LIF) was originally described as a secreted glycoprotein belonging to the interleukin 6 (IL-6) family of cytokines produced from a simple three-exon gene (Stahl *et al.*, 1990; Metcalf, 1991). Secreted LIF is known to signal extracellularly by formation of a cell surface receptor complex between the low-affinity LIF receptor and gp130 and initiation of a signal transduction cascade (Hein-

* Present address: Division of Haematology, Hanson Center for Cancer Research, Institute of Medical and Veterinary Science, Adelaide, South Australia 5000, Australia.
‡ Corresponding author. E-mail address: prathjen@biochem.adelaide.edu.au.

Abbreviations used: FGF, fibroblast growth factor; GFP, green fluorescent protein; h, human; IL, interleukin; iLIF, intracellular leukemia inhibitory factor; IRES, internal ribosome entry site; LIF, leukemia inhibitory factor; m, mouse; PBST, PBS containing 0.1% Tween 20.

rich *et al.*, 1998). It was subsequently determined that there is a more complex organization of the LIF gene, conserved among eutherian mammals, which results in the expression of three independently regulated LIF transcripts, LIF-D, LIF-M, and LIF-T, containing alternative first exons spliced to common second and third exons (Haines *et al.*, 1999; Voyle *et al.*, 1999). Although the human (h) and mouse (m) LIF-D transcripts encode only secreted proteins, and secreted proteins can be translated from hLIF-M and mLIF-M (Rathjen *et al.*, 1990b; Voyle *et al.*, 1999), the first exons of hLIF-M, hLIF-T, and the mLIF-T contain no in-frame ATG. This results in initiation of translation at an ATG located in exon 2 and gives rise to an intracellular 17-kDa LIF protein that lacks a secretion signal sequence and is N-terminally truncated by 22 amino acids relative to mature LIF-D.

LIF transcripts are expressed in a temporally and spatially regulated manner by many murine tissues, during both embryogenesis and adult life (Robertson *et al.*, 1993; Haines *et al.*, 1999). Expression of each of the three LIF transcripts is independently regulated. For example, adult brain expresses only mLIF-M, neonatal intestine expresses only mLIF-D (Robertson *et al.*, 1993), and mLIF-T is expressed in the adult liver at levels fourfold higher than in the adult lung (Haines *et al.*, 1999). Expression of relatively high levels of all three LIF transcripts is seen in murine embryonic stem cells, and, generally, in tissues harboring stem and progenitor cell populations, such as bone marrow (Smith *et al.*, 1992; Robertson *et al.*, 1993; Haines *et al.*, 1999). Expression of LIF-D and -M transcripts is up-regulated in response to the proinflammatory cytokines IL-1β and tumor necrosis factor and by signaling molecules active in tissue growth and development, including glucocorticoids, estradiol, FGF-2, and transforming growth factor-β1 (Rathjen *et al.*, 1990a; Smith and Rathjen, 1991; Bamberger *et al.*, 1997). LIF-D and -M expression is also induced during embryonic stem cell differentiation (Rathjen *et al.*, 1990a). Expression of alternate hLIF transcripts is also independently regulated and induced by cytokines and other factors (Rathjen *et al.*, 1990a; Voyle *et al.*, 1999). A consistent hLIF transcription profile is seen in human embryonal carcinoma cell lines, with hLIF-M and -T transcripts being the predominant LIF transcripts (Voyle *et al.*, 1999). These transcripts encode intracellular proteins with potentially cell autonomous actions. In contrast, variable expression of hLIF transcripts was seen in other cultured cell lines of hematopoietic and tumor origin (Voyle *et al.*, 1999). The regulated and independent expression of alternate LIF transcripts suggests that they each serve a distinct and biologically significant function.

In vitro, LIF exhibits a wide range of activities, and LIF knockout mice have a complex, nonlethal phenotype that suggests considerable pleiotropy and some redundancy in LIF function (Piquet-Pellorce *et al.*, 1994). LIF expression is required for endocrine stress responses in the pituitary (Chesnokova *et al.*, 1998), T lymphocyte activation and proliferation of hematopoietic stem and progenitor cells (Escary *et al.*, 1993), recovery of muscle and neuronal tissue from injury (Rao *et al.*, 1993; Kurek *et al.*, 1997), support of motor neuron function (Sendtner *et al.*, 1996), and priming the uterus for embryonic implantation (Stewart *et al.*, 1992). Some of the tissues affected in LIF knockout mice are known to express LIF transcripts (Robertson *et al.*, 1993); however, these defects are poorly understood at the molecular and cellular level, and it is not yet possible to discern whether deficiency of particular LIF transcripts or protein underlies them. Some aspects of the LIF knockout phenotype, including the reduction in size of their hematopoietic stem and progenitor cell populations (Escary *et al.*, 1993), are not recapitulated in the phenotype of LIF receptor knockout mice (Li *et al.*, 1995; Ware *et al.*, 1995). This suggests that these aspects of the LIF knockout phenotype might result from receptor-independent actions of the LIF protein.

Although release of the truncated 17-kDa protein encoded by LIF-T transcripts allows it to signal extracellularly in the conventional manner, it is normally retained intracellularly by overexpressing Cos-1 cells (Haines *et al.*, 1999). This suggested the possibility that the LIF-T-encoded proteins might also be capable of initiating signals within the cell.

Several lines of evidence suggest that IL-6 cytokine family cytokines, including LIF, are capable of signaling intracellularly. Ciliary neurotrophic factor and cardiotrophin 1 are both expressed without a secretory signal sequence, and ciliary neurotrophic factor is also retained intracellularly when overexpressed in Cos cells (Lin *et al.*, 1989; Pennica *et al.*, 1995). Human neutrophils contain intracellular oncostatin M protein, which can be released in response to granulocyte-macrophage colony-stimulating factor (Grenier *et al.*, 1999), and a transcript that potentially encodes an intracellular oncostatin M protein has been identified in mouse bone marrow and spleen (Voyle and Rathjen, 2000). An alternate IL-6 transcript encoding a predominantly intracellular protein has been reported (Kestler *et al.*, 1995), and the ability of antisense oligonucleotides but not neutralizing antibodies to inhibit IL-6-dependent proliferation indicates that IL-6 acts as an intracellular, autocrine cytokine during melanoma progression (Lu and Kerbel, 1993), the tumor necrosis factor response of leukemic hairy cells (Barut *et al.*, 1993), and platelet-derived growth factor-induced proliferation of nontransformed human fibroblasts, vascular smooth muscle cells, and mesangial cells (Roth *et al.*, 1995). Finally, HepG3B cells exhibit a transcriptional response to LIF that is resistant to neutralizing antibodies and thus potentially initiated within the cell (Baumann *et al.*, 1993).

The molecular organization of the LIF gene, in which intracellular and extracellular LIF proteins are translated exclusively from LIF-T and LIF-D transcripts, respectively, provides an experimental approach for separate investigation of intracellular and extracellular LIF action by expression of alternative cDNAs. In this work we demonstrate that intracellular and extracellular LIF proteins can have distinct cellular activities that are mediated by alternate signaling pathways. Intracellular LIF (iLIF) activity was independent of receptor-mediated signaling and required a leucine-rich repeat motif, which was spatially distinct from regions of the LIF protein required for receptor binding and activation.

MATERIALS AND METHODS

Nucleic Acid Manipulations

Expression vectors for mLIF-T (pmLIF-TX; Haines *et al.*, 1999) and mLIF-D (pDR10 [pmLIF-DX]; Rathjen *et al.*, 1990b) cDNAs have been described previously. mLIF DNA sequences are numbered according to the system of Gearing *et al.* (1987).

Mutations of the mLIF cDNA were generated by PCR of plasmid DNA using a mutant primer incorporating a convenient restriction

site for reconstruction of the open reading frame. PCR reactions contained 100 ng of plasmid DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2–3 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 200 mM each dNTP, 20 pmol of each primer, and 1 unit of *Taq* DNA polymerase (Bresatec, Thebarton, Australia) in a final volume of 20 μl. Reactions were cycled at 94°C for 5 s, 50–55°C for 5 s, and 72°C for 60 s for 30 cycles using a capillary thermal cycler (Corbett Research, Sydney, Australia).

LIF-T_K is an mLIF-T cDNA in which the sequence around the ATG initiation codon has been altered from CTCATGAAC to GATATGAAC, to conform with the consensus translational initiation sequence (A/GNNATGGNN) (Kozak, 1989). This cDNA was generated by PCR of pmLIF-T (Haines *et al.*, 1999) with 5′ primer mLIF-T 3640 (5′-ATCATATGAACCAGATCAAG-3′), which hybridizes to the mLIF-T first exon (Haines *et al.*, 1999), and a T3 3′ primer (5′-ATTAACCCTCACTAAAGGGA-3′; Stratagene, La Jolla, CA). PCR products were end filled and blunt cloned into *Eco*RV-digested pBluescript II KS (Stratagene). This vector was digested with *Sal*I and *Nde*I, end filled with Klenow fragment, and religated to produce pmLIF-T_K. pmLIF-T_KX was produced by digestion of pmLIF-T_K with *Xho*I and *Eco*RI and cloning the LIF fragment into *Xho*I–*Eco*RI-cut pXMT2 (Rathjen *et al.*, 1990b).

pmLIF-T_{EXTRA}X directs secretion of the 17-kDa iLIF protein and has been described elsewhere (Haines *et al.*, 1999).

LIF-D_{INTRA} directs intracellular localization of the 20-kDa mature LIF protein that results from proteolysis. This cDNA was produced by PCR of pmLIF-D Ban^- (Haines *et al.*, 1999) using the 5′ primer (5′-ATGAATTCGATATG^{85}AGCCCTCTTCCCAT^{98}-3′) and the 3′ primer (5′-AAGAATTC^{655}AGTCCATGGTACATTCAAGA^{636}-3′). PCR products were digested with *Eco*RI and cloned into *Eco*RI-cut pBluescript II KS producing pmLIF-D_{INTRA}. pmLIF-D_{INTRA}X was generated by digesting pmLIF-D_{INTRA} with *Eco*RI and ligating into *Eco*RI-cut pXMT2.

pmLIF FK-A is a cDNA in which F and K residues required for receptor interaction have been mutated to A. This mutation was produced by PCR of pmLIF-T with 5′ primer T7 (5′-TAATACGACTCACTATAGGGAGA-3′; Stratagene) and 3′ primer FK-A (5′-^{586}TCCCCAGAAGCTGGCAACCCAACTTAGCCCTTTGGGCGGCTTCT^{543}-3′). PCR products were digested with *Sma*I and *Pfl*MI and cloned into *Sma*I–*Pfl*MI-digested pmLIF-T to create pmLIF-T FK-A. pmLIF-D FK-A was produced by digesting pmLIF-T FK-A with *Nco*I and cloning the LIF sequence into *Nco*I digested pDR1 (Rathjen *et al.*, 1990b). Expression vectors pmLIF-D FK-AX and pmLIF-T FK-AX were generated by cloning *Pst*I–*Eco*RI-digested pmLIF-T FK-A and *Eco*RI-digested pmLIF-T FK-A into *Pst*I–*Eco*RI- and *Eco*RI-digested pXMT2, respectively.

pmLIF L2I3-A, pmLIF L2-A, pmLIF L117-A, pmLIF L4-A, pmLIF L5-A, and pmLIF V126-A are cDNAs in which residues implicated in iLIF activity have been mutated to Ala. pmLIF L2I3-A, pmLIF L2-A, and pmLIF L117-A were generated by PCR of pmLIF-T with 3′ primers L2I3-A (5′-^{385}GGTCCCGGGTAGCATTGGTCAGGGAGGCGCTAGCGTATG^{346}-3′), L2-A (5′-^{385}GGTCCCGGGTGATATTGGTCAGGGAGGCGCTAGCGTATGC^{345}-3′), and L117-A (5′-^{386}GGTCCCGGGTGATATTGGTAGCGGA^{361}-3′), respectively, and 5′ primer T7. PCR products were digested with *Sma*I and *Pst*I, and the LIF-T open reading frame was reconstructed by cloning into *Sma*I–*Pst*I-digested pmLIF-T to generate pmLIF-T L2I3-A, pmLIF-T L2-A, and pmLIF-T L117-A. pmLIF L4-A, pmLIF L5-A, and pmLIF V126-A were generated by PCR of pmLIF-T with 5′ primers L4-A (5′-^{374}TCACCCGGGACCAGAAGGTCGCTAACCCC^{402}-3′), L5-A (5′-^{374}TCACCCGGGACCAGAAGGTCCTGAACCCCACTGCCGTGAGCGCCCAGGTC^{423}-3′), and V126-A (5′-^{374}TCACCCGGGACCAGAAGGCCCTG^{396}-3′), respectively, and 3′ primer T3. PCR products were digested with *Sma*I and *Eco*RI, and the LIF-T open reading frame was reconstructed by cloning into *Sma*I–*Eco*RI-digested pmLIF-T to generate pmLIF-T L4-A, pmLIF-T L5-A, and pmLIF-T V126-A. Vectors for expression of mutated LIF-T proteins pmLIF-T L2I3-AX, pmLIF-T L2-AX, pmLIF-T L4-AX, pmLIF-T L5-AX, pmLIF-T L117-AX, and pmLIF-T V126-AX were produced by digesting pmLIF-T L2I3-A, pmLIF-T L2-A, pmLIF-T L4-A, pmLIF-T L5-A, pmLIF-T L117-A, and pmLIF-T V126-A with *Pst*I and *Eco*RI and cloning into *Pst*I–*Eco*RI-digested pXMT2. pmLIF-D L2I3-A, pmLIF-D L2-A, pmLIF-D L4-A, pmLIF-D L5-A, pmLIF-D L117-A, and pmLIF-D V126-A were generated by digesting pmLIF-T L2I3-A, pmLIF-T L2-A, pmLIF-T L4-A, pmLIF-T L5-A, pmLIF-T L117-A, and pmLIF-T V126-A with *Nco*I and cloning the mutated LIF sequence into *Nco*I-digested pmLIF-D. Vectors for the expression of mutated extracellular LIF proteins pmLIF-D L2I3-AX, pmLIF-D L2-AX, pmLIF-D L4-AX, pmLIF-D L5-AX, pmLIF-D L117-AX, and pmLIF-D V126-AX were produced by digesting pmLIF-D L2I3-A, pmLIF-D L2-A, pmLIF-D L4-A, pmLIF-D L5-A, pmLIF-D L117-A, and pmLIF-D V126-A with *Eco*RI and cloning into *Eco*RI-digested pXMT2.

The plasmid pLIF-T L2I3-A GFP directs expression of a green fluorescent protein (GFP)-LIF-T L2I3-A fusion protein. The plasmid was constructed by PCR on pmLIF-T L2I3-AX with primers LIF-T GFP 5′ (5′-CCGGAATTCA^{149}TCATGAACCAGATCAAG^{165}-3′) and LIF-T GFP 3′ (5′-GGCGGATCCCG^{621}GAAGGCCTGGACCAC^{607}-3′), digestion of the PCR product with *Eco*RI and *Bam*HI, and cloning into *Eco*RI–*Bam*HI-digested pEGFP-N1 (Clontech, Cambridge, United Kingdom).

Nonreplicating internal ribosome entry site (IRES) expression vectors were constructed in the expression vector pIRES-βgeo T7T3, which was produced by cloning the IRES-βgeo *Xba*I–*Bam*HI fragment from pIRES-βgeo (Mountford *et al.*, 1994) into *Xba*I–*Bam*HI-digested pT7T3 19U (Amersham Pharmacia Biotech, Uppsala, Sweden). Expression of LIF sequences in these vectors is directed by the adenovirus major late promoter and coupled via an IRES to expression of β-geo. pmLIF-TXIres (LIF-T), pmLIF-DXIres (LIF-D), and pXIres (control, no cDNA) were produced by *Bam*HI–*Afl*II excision of LIF cDNAs and the adenovirus major late promoter from pmLIF-TX, pmLIF-DX, and pXMT2, respectively, and blunt ligation into the end-filled *Sal*I site of pIRES-βgeo T7T3. IRES-based expression vectors pmLIF-T L2I3-AXIres (LIF-T L2I3-A), pmLIF-T L2-AXIres (LIF-T L2-A), pmLIF-T L4-AXIres (LIF-T L4-A), and pmLIF-T L5-AXIres (LIF-T L5-A) were produced by *Bam*HI–*Afl*II excision of LIF cDNAs and the adenovirus major late promoter from the appropriate XMT2 expression vector (pmLIF-T L2I3-AX, pmLIF-T L2-AX, pmLIF-T L4-AX, and pmLIF-T L5-AX, respectively) and blunt ligation into the end-filled *Sal*I site of pIRES-βgeo T7T3.

The CrmA expression vector (pCXN2-CrmA; Niwa *et al.*, 1991) and human Bcl-2 expression vector (pRSV-hBcl-2; Kumar *et al.*, 1994), which contain the SV40 origin of replication, were kindly provided by Dr. Sharad Kumar (Hanson Centre for Cancer Research, Adelaide, Australia).

Transfection of Cos-1 and 293T Cells

Cos-1 (Gluzman, 1981) and 293T (Pear *et al.*, 1993) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) FBS (Life Technologies, Gaithersburg, MD) at 37°C under 5% CO_2. Cos-1 and 293T cells were transfected by electroporation as previously described (Haines *et al.*, 1999) or by lipofection using LipofectAMINE (Life Technologies) according to the manufacturer's instructions. Briefly, cells were seeded at a density of 2×10^5 cells per 35-mm-diameter dish and grown to 70–80% confluence (~18 h). For each transfection a solution containing 1 μg of plasmid DNA and 100 μl of Opti-MEM transfection medium (Life Technologies) was combined with a solution containing 8 μl of LipofectAMINE and 100 μl of gentamicin-free DMEM and incubated at room temperature for 30 min. Gentamicin-free DMEM (0.8 ml) was added to the DNA-LipofectAMINE solution, which was overlaid on cells washed once with 2 ml of gentamicin-free DMEM. Cells were then incubated for 5 h at 37°C in 5% CO_2, after which the transfection solution was replaced with 3 ml of DMEM/FBS. Cotransfection of replicating and nonreplicating LIF expression vectors with CrmA, hBcl-2, and control expression vectors was at a ratio of 1:3.

Staining of Transfected Cells

For detection of LIF protein, cells were transfected by either electroporation or lipofection and cultured in 10-cm diameter plates or 35-mm-diameter wells, respectively, containing a 22 × 22-mm coverslip. Forty-eight to 72 h after transfection coverslips were removed, washed three times with 1× PBS (136 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4), permeabilized in methanol for 2 min, and rehydrated in PBS for 15–30 min. One hundred twenty microliters of a 1:100 dilution of anti-mouse LIF antibody (Haines *et al.*, 1999) in PBS containing 3% (wt/vol) BSA were applied to each coverslip, incubated for 1–3 h at room temperature, and washed three times in PBS containing 0.1% Tween 20 (PBST) for 5 min each wash. One hundred twenty microliters of a 1:30 dilution of sheep anti-rabbit FITC-conjugated antibody (Silenius, Hawthorn, Victoria, Australia) in PBS were applied to each coverslip and incubated at room temperature for 45 min in the dark. Cells were then washed three times in PBST and incubated for 60 s in 1 mg/ml Hoechst 33258 (bisBenzimide; Sigma, St. Louis, MO) in PBS before three more 5-min washes in PBST. Coverslips were mounted in 80% glycerol and viewed using a Zeiss (Thornwood, NY) Axioplan microscope equipped for three-channel fluorescence (Zeiss filter sets II, IX, and XV), and photographed with a Zeiss MC 100 camera attachment using 35-mm Ektachrome 160T film (Eastman Kodak, Rochester, NY).

Confocal laser scanning microscopy was performed using a MRC1000UV laser unit (Bio-Rad, Hercules, CA) in combination with a Diaphot 300 inverted microscope (Nikon, Melville, NY). For FITC fluorescence and enhanced GFP visualization, excitation and emission wavelengths were 488/10 nm and 522/35 nm, respectively. For Hoechst 33258 fluorescence, excitation and emission wavelengths were 363/8 nm and 455/30 nm, respectively. Detection of concanavalin A and Lysotracker was in accordance with the manufacturer's instructions (Molecular Probes, Eugene, OR). Images were acquired in the equatorial plane using the 100× water immersion objective (numerical aperture, 1.4). Overlaid images were prepared using Confocal Assistant 4.0 (Todd Clark Brelje, Department of Cell Biology and Neuroanatomy, University of Minnesota Medical School, Minneapolis, MN).

Costaining of transfected cells with the apoptosis detection kit Apoptag (Oncor, Gaithersburg, MD) was performed according to the manufacturer's instructions. Briefly, transfected cells were treated for Apoptag staining up to the final development step, and then cells were stained for LIF protein and DNA and photographed as described above. The horseradish peroxidase Apoptag staining was then developed, and corresponding fields were photographed as above using bright-field optics.

Costaining of LIF-transfected cells for endoplasmic reticulum was achieved by staining cells with 20 μg/ml concanavalin A Alexa 594 conjugate (Molecular Probes) in PBS for 30 min, followed by two washes with PBS. These cells were then stained for LIF expression as described above.

Localization of lysosomes and acidic vesicles was achieved by labeling live, transfected cells with 50 nM Lysotracker Red DND-99 (Molecular Probes) in DMEM and 10% FCS for 30 min followed by two washes with PBS.

Staining of transfected cells for β-galactosidase activity was carried out 72 h after transfection. Cells were transfected by electroporation and plated into 10-cm-diameter plates as above. Cells were washed three times in PBS and fixed in 0.2% glutaraldehyde in PBS for 5 min. After a further three washes in PBS, cells were incubated in β-galactosidase stain solution [0.45 mM $K_2Fe(CN)_6$, 0.45 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$, and 400 μg/ml 5-bromo-4-chloro-3-indoyl-β-*O*-galactopyranoside] overnight at 37°C.

LIF Protein Analysis

Embryonic stem cell assays for LIF biological activity and Western blot analysis of overexpressed LIF protein were carried out as described previously (Haines *et al.*, 1999).

RESULTS

Alternate Subcellular Localization of Secreted and iLIF Proteins: iLIF Protein Is Localized to the Nucleus and Cytoplasm

The subcellular localization of alternate LIF proteins was examined by immunohistochemical staining of Cos-1 cells 2 d after transfection with vectors directing overexpression of the mLIF-T (pmLIF-TX) and mLIF-D (pmLIF-DX) transcripts, which encode intracellular and extracellular LIF proteins, respectively (Haines *et al.*, 1999). Typical staining patterns for Cos-1 cells transfected with pmLIF-DX as visualized by light and confocal laser scanning microscopy are shown in Figure 1, A and E. High levels of LIF protein were located adjacent to the nucleus with filamentous protein staining extending into the cytoplasm, indicative of localization to the Golgi apparatus and endoplasmic reticulum (Gu *et al.*, 1989; Mullis and Kornfeld, 1994; Bristol *et al.*, 1996). This was consistent with colocalization of cytoplasmic LIF-D protein with concanavalin A (Figure 1, E–G), a marker of the endoplasmic reticulum (Virtanen *et al.*, 1980). No LIF-D-encoded protein could be detected in the nucleus (Figures 1E and 2, D and E) as assessed by Hoechst staining.

Two distinct staining patterns were observed in cells transfected with the LIF-T expression vector pmLIF-TX. Two days after transfection, 54 ± 2% of LIF-staining cells exhibited a rounded, compact cell morphology with uniform protein staining that is discussed later. All other LIF staining cells showed LIF protein localized within the nucleus and cytoplasm (Figure 1, B, D, and H), which was confirmed by colocalization with Hoechst DNA staining (Figure 1C) and confocal laser microscopy (Figure 1D). Costaining with concanavalin A further highlighted iLIF nuclear localization and indicated differences between secreted and intracellular LIF proteins. Cytosolic protein encoded by LIF-T did not always colocalize with the endoplasmic reticulum and was also observed as diffuse staining additional to the Golgi and endoplasmic reticulum (Figure 1, H–J). The subcellular localizations of secreted and intracellular LIF protein therefore differ in that the former is localized in components of the secretory apparatus, whereas the latter is not confined to the endoplasmic reticulum and has a more uniform distribution within the nucleus and cytoplasm.

Equivalent staining patterns were seen in 293T human kidney cells transfected with pmLIF-DX and pmLIF-TX (our unpublished results), indicating that the localization of iLIF protein is not species or cell type specific.

Alternate Cellular Activities of Intracellular and Extracellular LIF Proteins

The cellular effects of LIF overexpression were investigated in time course experiments in Cos-1 cells transfected with pmLIF-DX and pmLIF-TX. Between 2 and 3 d after transfection the proportion of mLIF-T (iLIF)-overexpressing cells demonstrating nuclear and cytoplasmic LIF staining with a round uniform nucleus in the plane of focus decreased from 46 ± 2 to 25 ± 8%. All other LIF staining cells, 54 ± 2% (day 2) to 75 ± 8% (day 3), stained intensely, were rounded and raised out of the plane of focus of nontransfected cells, and had a nucleus containing compacted or reduced DNA (Figure 2, A and B). This phenotype is indicative of apoptosis,

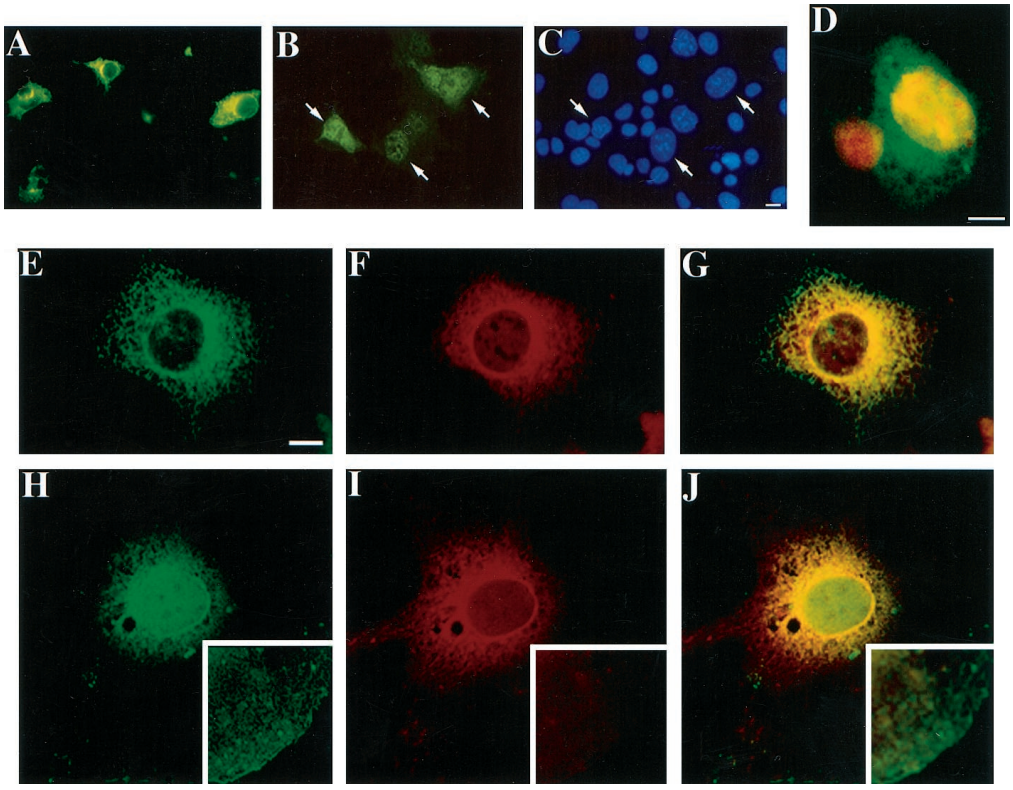

Figure 1. Cellular localization of secreted and iLIF proteins in Cos-1 cells transfected with LIF expression vectors 2 d after transfection. (A) LIF-D (pmLIF-DX)-transfected cells stained with anti-mLIF. (B) LIF-T (pmLIF-TX)-transfected cells stained with anti-mLIF. (C) Cells in B stained with Hoechst DNA stain. Cells were visualized using conventional optics. Arrows indicate selected LIF-staining cells and their nuclei. (D) Confocal laser scanning image of a Cos-1 cell transfected with an expression vector for LIF-T (pmLIF-TX) stained for LIF protein (green) and DNA (red). Colocalization of LIF protein and DNA in the nucleus appears yellow. The red nucleus (lower left) is an untransfected cell. (E–J) Confocal laser scanning images of Cos-1 cells transfected with pmLIF-DX (E–G) and pmLIF-TX (H–J) stained with anti-LIF (E and H) and concanavalin A (F and I). Merged images are presented in G and J. Insets show contrast-enhanced regions of the corresponding cellular cytoplasm. Bars, 10 μm.

which is typically accompanied by cell rounding and shrinkage, membrane blebbing, compaction of chromatin into condensed masses, and degradation of DNA into internucleosomal fragments (Jacobsen *et al.*, 1997). Cells exhibiting this morphology were confirmed as apoptotic by costaining for LIF protein expression and apoptosis-specific internucleosomal DNA fragmentation (Figure 2C). Nontransfected cells with round, healthy nuclei showed no staining for internucleosomal DNA fragmentation, whereas pmLIF-TX-transfected cells with condensed chromosomal DNA or little DNA staining showed weak and strong staining for internucleosomal DNA fragmentation, respectively.

The specificity of apoptosis for iLIF protein was confirmed by analysis of Cos-1 cells transfected with pmLIF-DX. The endoplasmic reticulum staining pattern did not vary at 2 and 3 d after transfection, and the nuclei of these cells were round, uniformly staining and within the plane of focus of nontransfected cells, indicating that overexpression of secreted LIF protein did not induce apoptosis (Figure 2, D and E).

Three days after transfection there were threefold fewer LIF staining cells in transfections with pmLIF-TX compared with pmLIF-DX transfections (Figure 3). This is attributed to shrinkage and rounding of the cells during apoptosis, causing loss of attachment to the culture surface (Kumar *et al.*, 1994; Hsu *et al.*, 1995).

iLIF Activity Is Mediated by Specific Intracellular Pathways

Overexpression of the apoptosis inhibitors Bcl-2 and CrmA in cultured cells specifically inhibits apoptosis elicited by distinct signaling pathways (Hockenbery *et al.*, 1990; Gagliardini *et al.*, 1994). Three days after transfection, Cos-1 cells cotransfected with a 1:3 ratio of pmLIF-DX and the control plasmid pXMT2 showed an endoplasmic reticulum staining pattern (Figure 3A), which was not affected by cotransfection with vectors directing expression of the CrmA (pCXN2-CrmA) or Bcl-2 (pRSV-hBcl-2) proteins (our unpublished results). Cotransfection of the iLIF expression vector pmLIF-T_KX with pXMT2 or pRSV-hBcl-2 resulted in the previously described apoptotic morphology of 75 ± 8% of LIF-staining cells (Figure 3, B and D) after 3 d. Cotransfection of pmLIF-T_KX with pCXN2-CrmA reduced these levels of apoptosis to 21 ± 3% of LIF-staining cells, with the remainder (69 ± 3%) of LIF-staining cells exhibiting nuclear and cytoplasmic

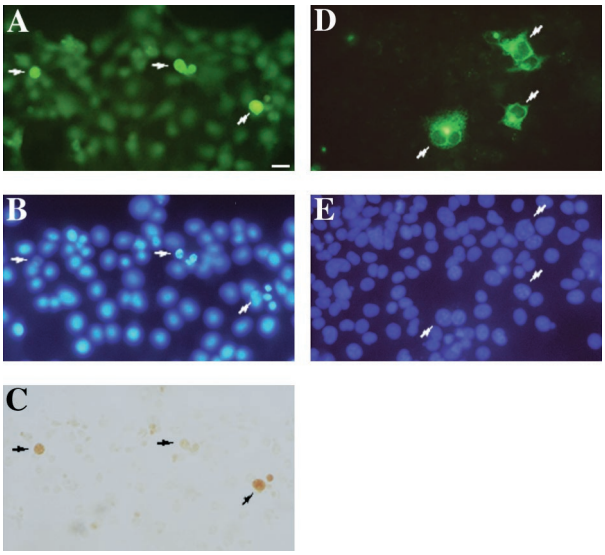

Figure 2. Overexpression of iLIF but not secreted protein induces apoptosis. Cos-1 cells transfected with the LIF-T expression vector pmLIF-TX were stained 3 d after transfection for LIF protein (A), DNA (B), and internucleosomal DNA fragmentation (C). Arrows indicate the nuclei of selected LIF-staining cells. Apoptotic cells with varying degrees of DNA condensation and fragmentation are shown. The plane of focus in B is that of the rounded apoptotic cells, seen most clearly in the central cell. Elevated background staining in A reflects interference from antibodies used in costaining for DNA fragmentation (Apoptag). Cos-1 cells transfected with pmLIF-DX stained with anti-LIF antibody (D) and Hoechst DNA stain (E) 3 d after transfection. The healthy nuclear morphology of pmLIF-DX-transfected cells is indicated by arrows. Bar, 20 μm.

staining and a healthy morphology as assessed by DNA staining (Figure 3C). The differential response to CrmA and Bcl-2 expression indicated that iLIF-induced apoptosis is associated with activation of specific signaling pathways and caspases.

CrmA-inhibitable apoptosis was also induced by overexpression of iLIF protein from pmLIF-TX in the human kidney cell line 293T and by overexpression of cDNAs encoding intracellular hLIF protein (Voyle *et al.*, 1999) in Cos-1 and 293T cells (our unpublished results). The apoptotic action of the iLIF protein is therefore unlikely to be cell line or species specific.

The XMT2-based replicating expression vectors used in this work direct high levels of protein expression, which may not be biologically relevant. For this reason expression from nonreplicating expression vectors was used to analyze the cellular effects of iLIF expression at levels closer to those seen in vivo (Haines *et al.*, 1999). Transient expression from nonreplicating vectors in Cos-1 cells was found to reduce the level of mLIF protein below the levels detectable by immunocytochemical staining using the available anti-mouse LIF antibody. To overcome this problem, mLIF-D and mLIF-T cDNAs were cloned into IRES-containing vectors (Mountford and Smith, 1995) to produce pmLIF-DXIres and pmLIF-TXIres in which LIF protein is translated from a dicistronic mRNA, which also directs β-galactosidase expression. Expression of β-galactosidase could therefore be used as a marker for LIF expression. Cos-1 cells transfected with pmLIF-TXIres exhibited a twofold decrease in the number of blue-staining cells compared with Cos-1 cells transfected with pmLIF-DXIres (Figure 3E). Reduced cell numbers in pmLIF-TXIres-transfected cells could be restored to control levels by cotransfection with pCXN2-CrmA but not pRSV-hBcl-2. These results demonstrated that the reduced numbers of blue-staining cells in pmLIF-TXIres transfections resulted from CrmA-inhibitable apoptosis induced by low levels of iLIF protein.

iLIF Activity Results from Intracellular Localization of the LIF Protein

The apoptotic action of iLIF protein could potentially result from the distinct nuclear and cytoplasmic localization of the protein or from N-terminal truncation by 22 amino acids relative to mature secreted LIF protein. To test these possibilities, LIF expression vectors (Figure 4A) were constructed to secrete the N-terminally truncated, 17-kDa LIF protein outside the cell (LIF-T_{EXTRA}; Haines *et al.*, 1999) and to express the 20-kDa mature LIF protein, containing the first 22 amino acids but lacking the secretion signal peptide, inside the cell (LIF-D_{INTRA}).

pmLIF-D_{INTRA}X, pmLIF-T_{EXTRA}X, pmLIF-TX, and pmLIF-DX were transfected into Cos-1 cells and assayed for iLIF activity. Two and 3 d after transfection, Cos-1 cells transfected with pmLIF-T_{EXTRA}X (Figure 4B) showed endoplasmic reticulum staining identical to cells transfected with pmLIF-DX, demonstrating that sequestration of the N-terminally truncated LIF protein within the endoplasmic reticulum and Golgi apparatus eliminated its apoptotic activity. Immunohistochemical staining of cells transfected with pmLIF-D_{INTRA}X showed a staining pattern equivalent to cells transfected with pmLIF-TX. Two days after transfection, healthy transfected cells showed LIF staining in the nucleus and cytoplasm (Figure 4B), whereas 3 d after transfection, equivalent numbers of apoptotic LIF-staining cells were present in transfections with pmLIF-D_{INTRA}X (79 $\pm$ 1%) and pmLIF-TX (80 $\pm$ 2%).

Conditioned media from cells transfected with pmLIF-D_{INTRA}X and pmLIF-TX showed very low levels of LIF activity by bioassay (Table 1). This was consistent with the immunocytochemistry data and indicated that signal peptide-mediated secretion of the 20-kDa protein translated from pmLIF-D_{INTRA}X had been abolished. The detection of low levels of extracellular LIF bioactivity in cells overexpressing intracellularly localized LIF proteins relative to pXMT2-transfected cells is attributed to loss of membrane integrity in apoptotic cells. High levels of extracellular LIF bioactivity were expressed from pmLIF-T_{EXTRA}X- and pmLIF-DX-transfected cells, confirming that these proteins are secreted from cells and that the presence or absence of the first 22 amino acids of the core LIF peptide, including two residues of helix A (Robinson *et al.*, 1994), does not prevent productive interaction with the LIF receptor (Haines *et al.*, 1999). These data indicate that iLIF-induced apoptosis is a consequence of intracellular localization of the LIF protein and not N-terminal truncation of the protein.

iLIF Activity Is Independent of Receptor Interaction

To investigate the effect of LIF–LIF receptor interaction on iLIF activity, LIF-T and LIF-D expression vectors were con-

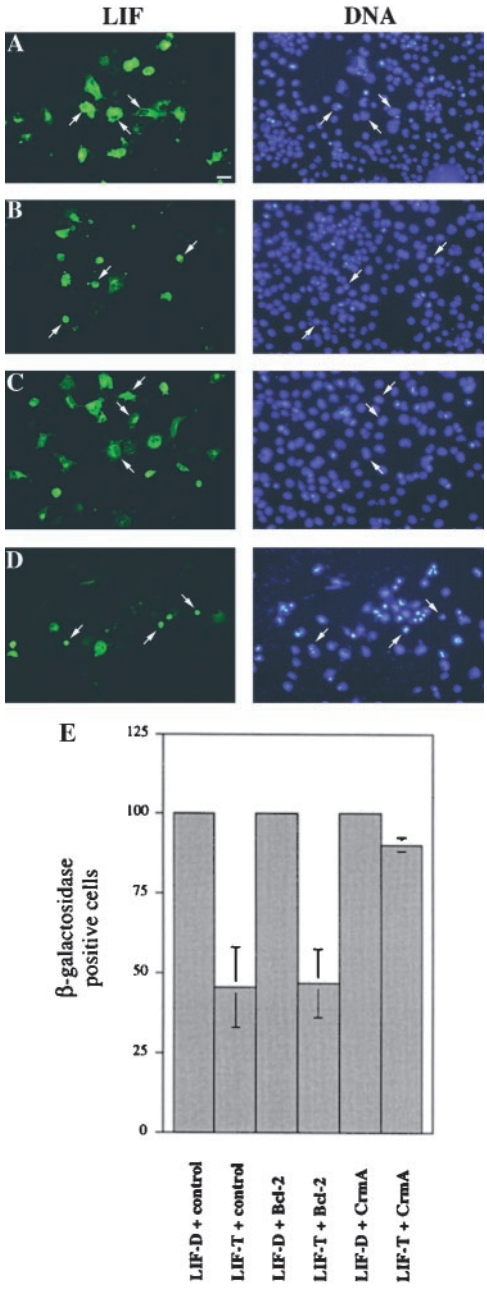

Figure 3. iLIF-induced apoptosis is inhibited by CrmA but not Bcl-2 expression. Cos-1 cells cotransfected with LIF-D or LIF-T expression vectors, at a 1:3 ratio with expression vectors for Bcl-2 (pRSV-hBcl-2), CrmA (pCXN2-CrmA), or the control vector pXMT2, were stained 3 d after transfection for LIF protein (LIF) and DNA. Cells were transfected with pmLIF-DX (LIF-D) and pXMT2 (A), pmLIF-TX (LIF-T) and pXMT2 (B), pmLIF-TX and pCXN2-CrmA (C), and pmLIF-TX and pRSV-hBcl-2 (D). Arrows indicate the nuclei of selected LIF-staining cells. Bar, 20 μm. (E) Graphical representation of relative numbers of β-galactosidase-positive cells 3 d after cotransfection of Cos-1 cells with a 1:3 ratio of pmLIF-DXIres (LIF-D) or pmLIF-TXIres (LIF-T) and pXMT2 (control), pRSV-hBcl-2, or pCXN2-CrmA. The number of staining cells in pmLIF-TX transfections is normalized to the corresponding transfections with pmLIF-DX. Means and SDs were calculated from two experiments.

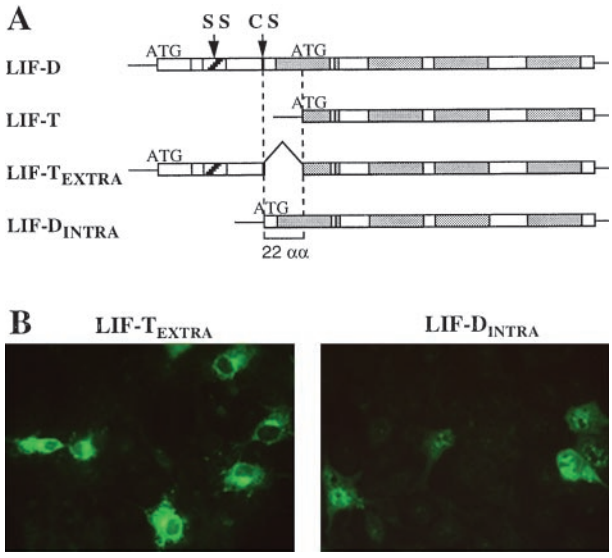

Figure 4. iLIF activity results from intracellular localization of the LIF protein. (A) Schematic diagram of engineered LIF cDNAs and their translation products. LIF-T_{EXTRA} directs secretion of the 17-kDa N-terminally truncated iLIF protein by fusing the LIF-D secretory signal sequence with the iLIF open reading frame. LIF-D_{INTRA} directs intracellular localization of the 20-kDa mature LIF protein by replacement of the secretory signal sequence with an ATG initiation codon. SS, signal sequence; CS, proteolytic cleavage site for mature, secreted LIF protein. ATG initiation codons are indicated. (B) Anti-LIF immunostaining of Cos-1 cells 2 d after transfection with expression vectors containing the LIF-T_{EXTRA} (pmLIF-T_{EXTRA}X) and LIF-D_{INTRA} (pmLIF-D_{INTRA}X) cDNAs. Magnification as in Figure 2.

structed in which the phenylalanine 179 and lysine 182 residues (referenced from the initiation methionine of mLIF-D; Gearing *et al.*, 1987) required for LIF–LIF receptor interaction (Hudson *et al.*, 1996) were mutated to alanine (pmLIF-T FK-AX and pmLIF-D FK-AX; Figure 5A). Cos-1 cells transfected with pmLIF-D FK-AX (Figure 5B) showed staining of the endoplasmic reticulum and Golgi apparatus equivalent to cells transfected with pmLIF-DX. However, no extracellular LIF activity could be detected by bioassay of medium conditioned by these cells, contrasting with the high level of LIF activity in medium conditioned by Cos-1 cells transfected with pmLIF-DX (Table 1). Western blot analysis of extracts from cells transfected with pmLIF-DX and pmLIF-D FK-AX (Figure 5C) confirmed the presence of similar levels and glycosylation variants of LIF-D and LIF-D FK-A proteins. This confirmed that the FK-A mutation abolished formation of a functional LIF receptor complex and activation of the extracellular signaling pathway.

Immunocytochemical staining of Cos-1 cells transfected with pmLIF-T FK-AX revealed a nuclear and cytoplasmic staining pattern equivalent to cells transfected with pmLIF-TX 2 d after transfection (Figure 5B). A similar number of apoptotic LIF-staining cells were present in transfections with pmLIF-T FK-AX (82 $\pm$ 3%) and pmLIF-TX (80 $\pm$ 2%) 3 d after transfection. This indicated that the intracellular apoptotic action of LIF-T was not affected by the FK-A mutation and therefore occurred independently of signaling through the receptor complex.

Table 1. Assays for extracellular LIF bioactivity of media conditioned by Cos-1 cells transfected with LIF expression vectors

	Media dilutions					
	1:10	1:100	1:500	1:1000	1:10000	1:20000
LIF-D	+	+	+	+	+	+/−
LIF-T	+	+/−	−	−	−	−
LIF-D_{INTRA}	+	+/−	−	−	−	−
LIF-T_{EXTRA}	+	+	+	+	+/−	−
LIF-D_{FK-A}	−	−	−	−	−	−
LIF-T_{FK-A}	−	−	−	−	−	−
XMT2	−	−	−	−	−	−

The assay is maintenance of undifferentiated ES cell colonies, which requires activation of the LIF receptor complex. Media dilutions are indicated. Media were obtained from cells overexpressing LIF-D (pmLIF-DX), LIF-T (pmLIF-TX), LIF-D_{INTRA} (pmLIF-D_{INTRA}X), LIF-T_{EXTRA} (pmLIF-T_{EXTRA}X), LIF-D FK-A (pmLIF-D FK-AX), LIF-T FK-A (pmLIF-T FK-AX), and pXMT2 (parental vector). +, LIF activity (maintenance of undifferentiated ES cell colonies); −, no LIF activity (differentiated cells only); +/−, some LIF activity (undifferentiated and differentiated colonies).

iLIF Activity Requires Conserved Leucine Residues That Are Not Required for Signaling through Cell Surface Receptors

A heptad repeat of leucine and isoleucine residues (Leu^{106}, Leu^{113}, Ile^{120}, and Leu^{127} and Leu^{134}; referenced from the initiation methionine of iLIF; Gearing *et al.*, 1987; Haines *et al.*, 1999), similar to the leucine zipper protein dimerization domain found in many intracellular transcription factors (Kerppola and Curran, 1991), was conserved among the LIF proteins of six mammals (Figure 6A), including the marsupial *Sminthopsis crassicaudata* (Cui, 1998). This region is potentially able to form an α helical structure (Figure 6B) in which the two most highly conserved positions are the leucine/isoleucine heptad repeat at position 1 and hydrophobic residues at position 5, an arrangement similar to leucine zippers of the *jun* family (Kerppola and Curran, 1991). This motif is located outside the regions required for interaction with LIF receptor subunits (Owczarek *et al.*, 1993; Robinson *et al.*, 1994; Hudson *et al.*, 1996) and the leucine residues are not all surface exposed in the extracellular LIF structure (Robinson *et al.*, 1994).

The role of specific residues within the potential zipper structure and outside the heptad repeat was tested by mutation to alanine (Figure 7A). This region has been identified by measurement of amide exchange (S. Yao, D.K. Smith, M.G. Hinds, J.-G. Zhang, N.A. Nicola, and R.S. Norton, unpublished data) as a relatively plastic region of the generally rigid LIF structure. Given the suggestion that the LIF bundle might "unzip" from the BC loop, conserved heptad leucines within and adjacent to the BC loop were mutated. The role of conserved, nonheptad leucine residues was tested by mutation of Leu^{117}, and the role of nonconserved residues in this region was tested by mutation of Val^{126}. An L2I3 double mutant was constructed to eliminate formation of a potential leucine zipper. LIF-T and LIF-D expression vectors were constructed to analyze the effect of these mutations on intracellular and extracellular LIF activity.

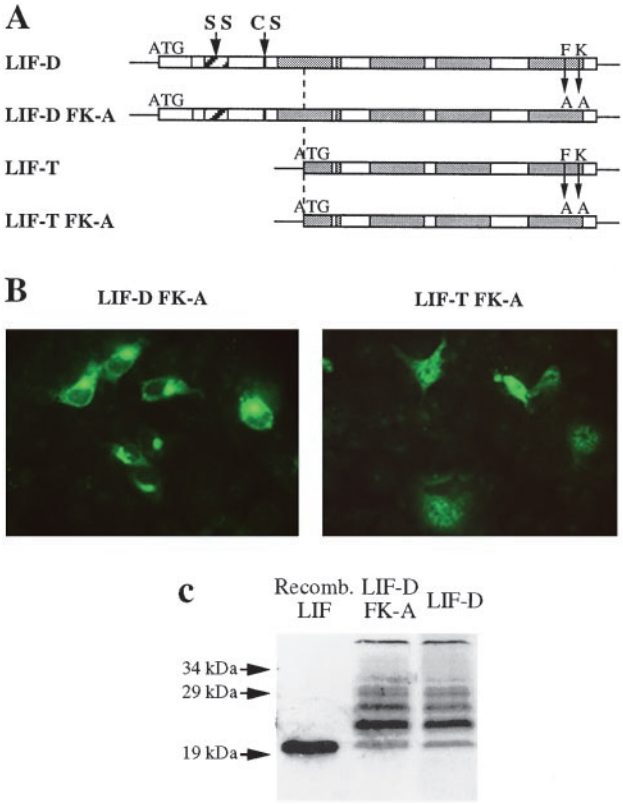

Figure 5. iLIF activity does not require productive receptor interaction. (A) Schematic diagram showing mutant LIF-D and LIF-T cDNAs carrying the double mutation Phe^{179}-Ala and Lys^{182}-Ala (LIF-D FK-A and LIF-T FK-A, respectively), which abrogates LIF-LIF receptor complex assembly and activation (Hudson *et al.*, 1996). (B) Anti-LIF immunostaining of Cos-1 cells 2 d after transfection with expression vectors containing the LIF-D FK-A (pmLIF-D FK-AX) and LIF-T FK-A (pmLIF-T FK-AX) cDNAs. Magnification as in Figure 2. (C) Western blot analysis of LIF proteins in extracts from Cos-1 cells 2 d after transfection with pmLIF-D FK-AX and pmLIF-DX. LIF is 50 ng of recombinant, unglycosylated (20 kDa) mLIF (ESGRO; AMRAD, Richmond, Victoria, Australia).

Cos-1 cells expressing the mutated iLIF proteins translated from pmLIF-T L2I3-AX, pmLIF-T L2-AX, pmLIF-T L4-AX, and pmLIF-T L5-AX all showed an iLIF staining pattern that was markedly different from that of cells transfected with pmLIF-TX or pmLIF-DX. Nuclear localization was lost, and cytoplasmic staining was restricted to vesicle-like structures or aggregates, concentrated around the nucleus (Figure 7B). This alternate localization of the mutant iLIF proteins correlated with reduced apoptotic activity (Figure 7C). Although 80 ± 2% of cells transfected with pmLIF-TX showed an apoptotic morphology 3 d after transfection, this was reduced to 32 ± 2 and 35 ± 2% for cells transfected with pmLIF-T L2I3-AX and pmLIF-T L5-AX, respectively. Cells transfected with pmLIF-T L4-AX and pmLIF-T L2-AX exhibited an intermediate level of apoptosis, 53 ± 5 and 52 ± 3%, respectively. Immunofluorescence (Figure 7B) and Western blot analysis (our unpublished data) indicated that similar LIF protein levels were expressed from each plasmid. Interestingly, cells transfected

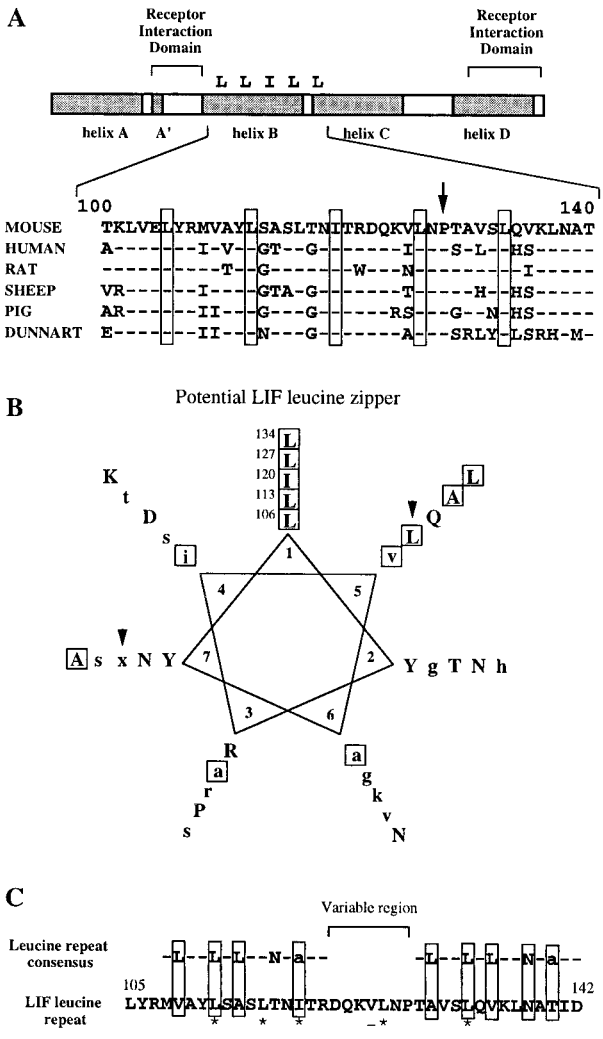

Figure 6. Conserved protein motifs in the LIF sequence. (A) Schematic diagram of the LIF protein showing the position of the conserved leucine zipper-like structure relative to the receptor interaction domains in five eutherian mammals and one marsupial. Dashes indicate residues conserved with the mLIF gene. Boxes indicate conserved leucine residues. A conserved proline residue is marked by the arrow. Helices in the LIF secondary structure (Robinson *et al.*, 1994) are shaded. Numbers indicate corresponding residues in the mLIF sequence described by Gearing *et al.* (1987). (B) Potential LIF leucine zipper represented as a wheel diagram. Residues conserved in at least five of the six LIF sequences are capitalized; lowercase residues are conserved in at least three of the six LIF sequences; x indicates a nonconserved residue; and hydrophobic residues are boxed. Arrowheads indicate nonheptad residues that were mutated in this analysis. (C) Alignment of the mLIF leucine motif with the consensus sequence for a leucine-rich repeat. Asterisks indicate residues required for iLIF activity as determined by mutation. The valine residue that is nonessential for iLIF activity is underlined.

with pmLIF-T L117-AX, in which a nonheptad leucine was mutated to alanine, showed the vesicle-like LIF protein localization characteristic of heptad leucine mutants and a slight reduction in apoptotic number (68 ± 2%; Figure 7C). This indicates that iLIF function can be modified by residues outside the heptad leucine repeat. Cos-1 cells transfected with pmLIF-T V126-AX showed nuclear and cytoplasmic localization and apoptosis at levels similar to cells transfected with pmLIF-TX.

The identity of the vesicle-like structures associated with mutated, inactive iLIF protein was investigated by costaining with Lysotracker, a marker of lysosomes and acidic organelles (Figure 7B, iv–vi). This could not be achieved using the existing LIF expression plasmids because of incompatibility between the fixing procedures required for the alternate staining methods. Accordingly, pmLIF L2I3-A GFP, which directs expression of a GFP/LIF-T L2I3-A fusion protein, was constructed. Simultaneous visualization by confocal laser scanning revealed that LIF-T L2I3-A fused to GFP also localized to nuclear-adjacent vesicle-like structures or more condensed aggregates in the cytoplasm. These structures did not colocalise with lysosomal or acidic vesicles and may represent aggresomes, sites of proteasome-associated protein degradation (Johnston *et al.*, 1998).

Biological assay of conditioned media from Cos-1 cells expressing the mutated extracellular LIF proteins translated from pmLIF-D L2I3-AX, pmLIF-D L2-AX, pmLIF-D L4-AX, pmLIF-D L5-AX, pmLIF-D L117-AX, and pmLIF-D V126-AX demonstrated that the mutant LIF proteins exhibited high-level extracellular biological activity identical to wild-type secreted LIF protein (our unpublished data). Immunocytochemical staining and Western blot analysis showed that levels and localization of mutant LIF-D proteins were equivalent to cells transfected with pmLIF-DX (our unpublished data), and that expression of these proteins did not result in cell apoptosis (Figure 7C). These results demonstrate that the conserved leucines required for iLIF protein activity are not required for secretion or activity of the extracellular LIF protein.

The role of these residues in induction of apoptosis was supported by low-level expression of the mutant LIF proteins from IRES-based expression vectors containing mutant LIF-T cDNAs. Transfection of Cos-1 cells with pmLIF-TXIres showed the previously described twofold decrease in blue-staining cells (Figure 7D), shown to be a result of CrmA-inhibitable apoptosis. Transfection of pmLIF-T L2I3-AXIres, pmLIF-T L2-AXIres, pmLIF-T L4-AXIres, and pmLIF-T L5-AXIres resulted in β-galactosidase-positive cell numbers equivalent to those obtained in control transfections with pmLIF-DXIres and pXIres.

DISCUSSION

iLIF Protein Localizes to the Nucleus and Cytoplasm

Intracellular growth factor and cytokine localization can broadly be achieved by two mechanisms, synthesis of proteins lacking a secretory signal sequence and internalization after receptor interaction. In the latter case, evidence points to a role for the internalized ligand in the receptor-mediated cellular response. For example, internalization and nuclear localization of extracellular FGF-2 and Schwannoma-derived growth factor have been shown to be an essential component of the receptor-mediated mitogenic signal (Imamura *et al.*, 1990; Kimura *et al.*, 1990; Wiedlocha *et al.*, 1996). The biochemical demonstration that other growth factors colocalize with soluble receptors within the cell and

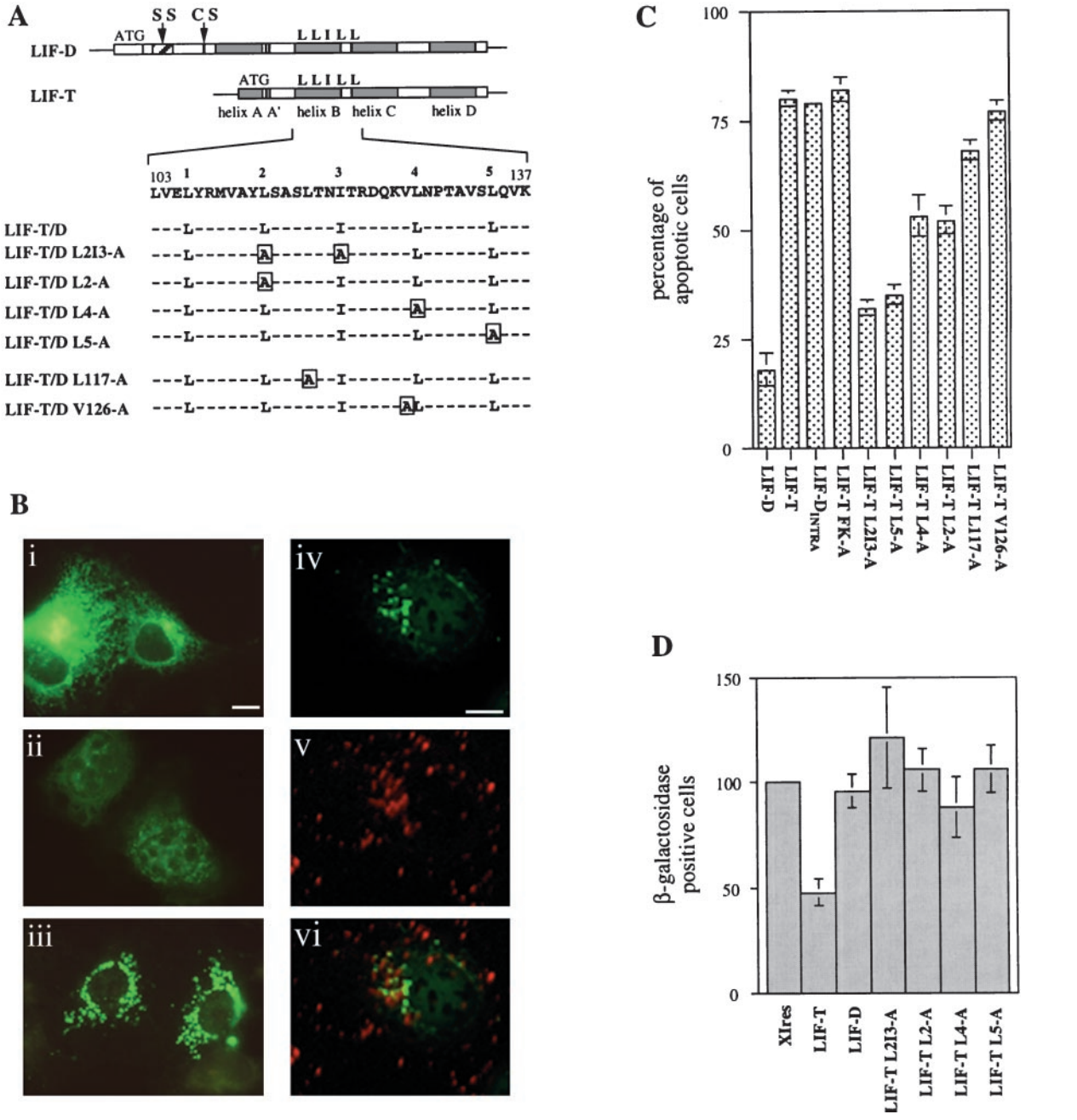

Figure 7. Conserved leucine residues outside the receptor interaction regions are required for iLIF activity. (A) Schematic representation of LIF-D and LIF-T cDNAs and the mutations introduced into these cDNAs. (B, i–iii) Anti-LIF immunostaining of Cos-1 cells 2 d after transfection with expression vectors for LIF-D (pmLIF-DX) (i), LIF-T (pmLIF-TX) (ii), and LIF-T L2I3-A (pmLIF-T L2I3-AX) (iii). All leucine mutants showed similar LIF staining to B, iii. (B, iv–vi) Confocal laser scanning images of a Cos-1 cell transfected with pmLIF L2I3-A GFP stained with Lysotracker lysosome and acidic vesicle stain. (iv) GFP; (v) Lysotracker; (vi) merged images. Bar, 10 μm. (C) Graphical representation of the percentage of LIF-staining Cos-1 cells exhibiting an apoptotic morphology 3 d after transfection with expression vectors directing expression of LIF-D, LIF-T, and mutated LIF-T cDNAs. (D) Graphical representation of β-galactosidase-positive cells 3 d after transfection with low-level expression vectors coupling expression of β-geo to no cDNA (pXIres), LIF-T (pmLIF-TIres), LIF-D (pmLIF-DXIres), LIF-T L2I3-A (pmLIF-T L2I3-AXIres), LIF-T L2-A (pmLIF-T L2-AXIres), LIF-T L4-A (pmLIF-T L4-AXIres), and LIF-T L5-A (pmLIF-T L5-AXIres). The number of staining cells is normalized to control transfections with the parental vector pXIres. Means and SDs were derived from three experiments.

nucleus (Jans and Hassan, 1998) and can associate with chromatin (Curtis *et al.*, 1990; Lobie *et al.*, 1994), DNA, and nuclear proteins (Amalric *et al.*, 1994; Kolpakova *et al.*, 1998) suggests that direct involvement of cytokines in intracellular signaling may be widespread.

There are now a number of cases in which translation of a cytokine lacking a secretory signal sequence results in its retention within the cell (Jans and Hassan, 1998). Production of alternative transcripts encoding proteins that lack a signal sequence, as exemplified by the LIF (Haines *et al.*, 1999), IL-1 receptor antagonist (Haskill *et al.*, 1991), and IL-15 (Tagaya *et al.*, 1997) genes, provides a mechanism for controlled localization of a cytokine in different cellular compartments.

Subcellular localization of iLIF protein was investigated by overexpression of the mLIF-T cDNA, which encodes iLIF protein exclusively (Haines *et al.*, 1999). iLIF protein was found to be distributed throughout the nucleus and cytoplasm, providing a possible mechanism for iLIF action via interaction with cytosolic or nuclear proteins. This distribution was clearly distinct from that of secreted LIF translated from the LIF-D transcript, which localized to subcellular components of the secretory pathway. Localization of iLIF protein parallels localization of the intracellular IL-15 protein, which is also translated from an alternative transcript and appears to be nuclear and cytoplasmic (Tagaya *et al.*, 1997).

Distinct Cellular Activities of Intracellular and Extracellular LIF Proteins

Extensive use of Cos-1 cells for overexpression of LIF-D transcripts (Rathjen *et al.*, 1990a) indicates that biologically active extracellular LIF protein has no effect on these cells. Immunohistochemistry indicated that Cos-1 cells expressing and cultured in extracellular LIF protein were morphologically indistinguishable from untransfected cells. By contrast, high- and low-level overexpression of iLIF protein induced cell apoptosis. The specificity of this effect is indicated by the fact that overexpression of other proteins in Cos-1 cells, including secreted LIF, α1–4-galactosidase (R.B. Voyle, unpublished observations), and β-galactosidase (our unpublished data; Kumar *et al.*, 1994), does not induce an apoptotic response. The induction of apoptosis was induced by mouse and human iLIF proteins in both Cos-1 and 293T cells, suggesting that iLIF activity is unlikely to be species or cell line dependent.

We have shown that intracellular activity of iLIF does not require assembly of a functional LIF receptor complex and is mediated by internalization of the LIF protein. Furthermore, iLIF activity was deduced to result from interaction of the protein with known intracellular signaling pathways. In particular, the inhibition of iLIF activity by the serine protease inhibitor CrmA but not Bcl-2 indicates that iLIF protein interacts with caspase-activating pathways (Komiyama *et al.*, 1994; Tewari and Dixit, 1995), which are distinct from Bcl-2-inhibitable pathways mediating γ-irradiation- and etoposide-induced apoptosis. It remains to be determined whether the involvement of iLIF in apoptosis is direct or reflects perturbation of other essential cellular pathways.

The importance of intracellular cytokines for transduction of some extracellular signals (Imamura *et al.*, 1990), and as a pool of presynthesized ligand awaiting extracellular release in response to environmental cues, has been recognized. We have extended knowledge of intracellular cytokine biology with the first demonstration of an intracellular cytokine that is distinct from its extracellular counterpart in terms of its means of production, activity, and mechanism of action.

Although apoptosis could be a normal cellular function of iLIF protein, the effects of iLIF expression may well be context dependent. For example, many cytokines, including LIF, exhibit pleiotropic activities in vitro that are dependent on the molecular constitution of the responsive cell, whereas intracellular regulatory molecules such as myc proteins have been implicated in a wide range of cellular events that are dependent on the identity of the target cell and its environment (Vastrik *et al.*, 1994). Many of the biological effects of targeted LIF and LIF receptor gene disruption in mice (Stewart *et al.*, 1992; Escary *et al.*, 1993; Rao *et al.*, 1993; Sendtner *et al.*, 1996; Kurek *et al.*, 1997; Chesnokova *et al.*, 1998) have not been elaborated at the molecular and cellular levels and could reflect iLIF activity in vivo. Defects apparent in mice lacking LIF but not LIF receptor genes, such as the reduction in numbers of hematopoietic stem and progenitor cells seen in LIF knockout mice (Escary *et al.*, 1993; Ware *et al.*, 1995), could be explained by the absence of iLIF activity in LIF knockout animals. Resolution of these issues will require mapping of cellular sites of expression for specific LIF isoforms in vivo and correlation with cellular defects or the creation of knockout mice deficient for individual LIF transcripts and proteins.

Distinct Structural Domains Mediate Alternate Intracellular and Extracellular LIF Activities

Mutational analysis indicated that residues required for interaction between extracellular LIF protein and the receptor complex were not involved in iLIF nuclear localization or induction of apoptosis. Furthermore, mutation of a leucine-rich motif located outside the region of the LIF protein essential for interaction with receptor subunits inhibited intracellular activity but had no effect on extracellular receptor activation. This motif has been maintained within the LIF genes of eutherian mammals and marsupials, which are separated evolutionarily by >150 million years (O'Brien and Graves, 1990). The alternate intracellular and extracellular LIF activities therefore require spatially distinct regions of the LIF protein, which presumably underlie interaction with distinct molecular pathways.

Several intracellular protein–protein interaction domains contain conserved leucine residues that are of functional importance. The heptad leucine repeat in the LIF sequence, shown by mutation to be required for intracellular localization and activity, is similar to the leucine zipper motif, which is important for a variety of intracellular protein–protein interactions (Kerppola and Curran, 1991). Localization of iLIF protein within the nucleus suggests that it might interact with leucine zipper-containing transcription factors known to be involved in apoptosis. For example, formation of a leucine zipper within the c-Myc protein (Vastrik *et al.*, 1994; Kohlhuber *et al.*, 1995) is required for tumor necrosis factor-induced apoptosis that is inhibited by CrmA but not Bcl-2 (Janicke *et al.*, 1996). A variety of leucine zipper proteins have been implicated in apoptosis (Inaba *et al.*, 1996; Johnstone *et al.*, 1996; Matsumoto *et al.*, 1996; Metzstein *et al.*, 1996; Wang *et al.*, 1996) and are candidate binding partners for iLIF protein.

Mutation of critical leucine residues inhibited both iLIF activity and nuclear entry, suggesting that nuclear localization might be important for iLIF activity. Nuclear localization of the LIF protein could be mediated by passive diffusion or by active transport, making it available to interact with other nuclear proteins. A conserved sequence (RKK in mouse and rat, KKK in other sequenced LIF genes) with homology to p53 and Max nuclear localization sequences (Boulikas, 1993) is located at the C terminus of the LIF protein, within a region required for receptor interaction. Alternatively, iLIF could be transported to the nucleus by interaction with another nuclear-targeted protein. iLIF proteins containing mutations within the conserved leucine motif showed exclusive cytoplasmic localization to granular structures. Because these structures did not colocalize with acidic vesicles, it is possible that mutant iLIF proteins may be targeted for degradation via proteasome-associated aggresomes (Johnston *et al.*, 1998) in the absence of protein–protein interactions leading to apoptosis in Cos-1 cells.

Sequences within the conserved region required for iLIF activity may also be consistent with formation of a leucine-rich repeat, a structure that is also involved in protein–protein interaction (Figure 6C; Kobe and Deisenhofer, 1994). Formation of this structure may explain why mutation of the conserved Leu^{117} reduced iLIF-induced apoptosis even though mutations outside the heptad repeat are normally tolerated within a zipper structure. Furthermore, a conserved proline residue at position 128, between the fourth and fifth leucines of the putative zipper would disrupt the α-helix required for zipper formation but not a leucine rich repeat. Although formation of zipper motifs containing four leucine residues has been reported (Ransone *et al.*, 1990), the fifth leucine of the LIF heptad repeat was shown by mutation to be required for iLIF activity.

The leucine repeat motif is partially hidden within the four-helical bundle structure of the mature extracellular LIF protein (Robinson *et al.*, 1994). The alternate intracellular and extracellular actions of the LIF protein may therefore be consequences of differential protein folding in alternate cellular compartments: formation of the extracellular structure by folding in the oxidizing environment of the endoplasmic reticulum and the intracellular structure, containing a leucine zipper or leucine-rich repeat, within the reducing environment of the cytoplasm. Differential localization of chaperones within these cellular compartments has been described (Frydman and Hohfeld, 1997) and could conceivably have a role in the generation of alternative LIF protein structures. It is interesting that the leucine-rich domain required for iLIF intracellular function corresponds to a region of the LIF protein determined to be relatively plastic compared with the otherwise rigid LIF structure (S. Yao, D.K. Smith, M.G. Hinds, J.-G. Zhang, N.A. Nicola, and R.S. Norton, unpublished data). This structural plasticity may provide the opportunity for formation of alternative structures in this region.

There is increasing recognition that individual proteins can have multiple distinct activities, which confound existing classification systems (Prochiantz and Theodore, 1995). In several cases individual proteins have been shown to be multifunctional in terms of both their cellular activities and interactions with signaling pathways (He and Furmanski, 1995; Jeffery, 1999). Furthermore, alternate activities and interactions can be mediated by distinct regions of a protein. We have extended this observation to cytokines and show that different protein motifs underlie the alternate biological activities resulting from differential compartmentalization of the LIF protein. It will be interesting to elucidate the biochemical nature of the iLIF intracellular signaling pathway and to determine the biological function of this protein in vivo.

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