

09PM
H1518



Alternate Transcription and Translation of the LIF Gene Produces a Novel Intracellular Protein

A thesis submitted to the University of Adelaide
for the degree of Doctor of Philosophy

by

Bryan Peter Haines, B. Sc. (Hons.)

Department of Biochemistry
University of Adelaide
Adelaide, South Australia

December, 1997

TABLE OF CONTENTS

Table of Contents	i
Summary	viii
Statement	x
Acknowledgements	xi
CHAPTER 1: INTRODUCTION	1
1.1 General Introduction	1
1.2 Growth factors and cytokines	1
1.3 Growth factor function and action	2
1.3.1 Growth factor localisation	2
1.3.2 Receptor mediated growth factor action	4
1.3.2.1 The epidermal growth factor receptor as an example of growth factor signalling.	4
1.3.2.2 From the EGF receptor to the nucleus	5
1.3.2.3 Transcription factor activation	8
1.3.2.4 Complexity of intracellular signalling	9
1.3.2.5 Growth factor receptors lacking intrinsic tyrosine kinase domains	10
1.3.3 Intracellular growth factors	11
1.3.3.1 Evidence for intracellular growth factors	11
1.3.3.2 Biological significance of intracellular growth factor localisation.	12
1.3.3.3 Mechanism of intracellular growth factor action	14
1.4 Cytokines	15
1.4.1 Cytokine action	15
1.4.2 The IL-6 Family of Cytokines	16

1.5 Leukaemia Inhibitory Factor (LIF).....	17
1.5.1 The LIF protein.....	17
1.5.2 LIF mechanism of action.....	18
1.5.2.1 The LIF receptor.....	18
1.5.2.2 LIFR interaction	19
1.5.2.3 LIFR signalling.....	20
1.5.3 Expression of the LIF gene	21
1.5.4 LIF effects	22
1.5.4.1 <i>In vitro</i> LIF effects	22
1.5.4.2 <i>In vivo</i> LIF effects.....	23
1.5.5 The LIF gene	24
1.5.6 Alternative transcription of the LIF gene.....	25
1.6 Aims and Approaches.....	26

CHAPTER 2: IDENTIFICATION AND CHARACTERISATION OF A NOVEL LIF TRANSCRIPT.....28

2.1 Introduction	28
2.2 Identification and cloning of a third LIF transcript.	28
2.2.1 Identification of a novel LIF transcript	28
2.2.2 Cloning of a third LIF transcript	29
2.2.3 Confirmation of the third LIF transcript.....	30
2.3 LIF-T is a conserved mammalian LIF transcript.....	31
2.3.1 Cloning of LIF-T from other species	31
2.3.2 Genomic conservation of the LIF-T transcript.....	32
2.4 LIF-M is a conserved mammalian LIF transcript	33
2.4.1 Cloning of LIF-M transcripts from other species	33
2.4.2 Genomic conservation of the LIF-M transcript.....	33
2.5 Expression of the LIF T transcript.....	34

2.6 Discussion.....	35
2.6.1 The complex conserved structure of the LIF gene includes a novel LIF transcript	35
2.6.2 Alternate LIF transcripts are produced by differential promoter usage	36
CHAPTER 3: TRANSLATION OF THE LIF-T TRANSCRIPT	40
3.1 Introduction	40
3.2 Translation of the third transcript.....	40
3.3 Intracellular localisation of the LIF-T protein	43
3.4 LIF-T is localised to the nucleus and cytoplasm of transfected cells.....	44
3.5 Discussion.....	46
3.5.1 A novel, intracellular LIF protein	46
3.5.2 The significance of an intracellular LIF molecule	48
CHAPTER 4: FUNCTION OF THE LIF-T PROTEIN	51
4.1 Introduction	51
4.2 Apoptosis.....	52
4.2.1 The interleukin-1 β -converting enzyme (ICE) related cysteine protease gene family.....	54
4.2.2 The Bcl-2 gene family.....	55
4.2.3 Death Domain proteins	56
4.2.4 Growth factors/cytokines and apoptosis	57
4.2.5 Alternate apoptotic pathways.....	58
4.3 Results	58
4.3.1 Overexpression of the LIF-T protein causes apoptosis.....	58
4.3.2 LIF-T induced apoptosis is inhibited by CrmA but not Bcl-2	61
4.3.3 Lower level overexpression of LIF-T in Cos-1 cells causes CrmA inhibitable apoptosis	63

4.4 Discussion.....	65
4.4.1 Overexpression of LIF-T causes apoptosis.....	65
4.4.2 LIF-T induced apoptosis occurs via a specific pathway.....	67
4.4.3 Considerations for <i>in vivo</i> LIF-T function.....	68
CHAPTER 5: MECHANISM OF INTRACELLULAR LIF ACTION.....	70
5.1 Introduction.....	70
5.2 Apoptosis results from intracellular localisation of the LIF protein.....	71
5.3 Activity of the intracellular LIF protein is receptor independent.....	73
5.4 LIF-T intracellular activity requires conserved leucines that are not required for extracellular action.....	75
5.4.1 A conserved leucine zipper exists in the LIF amino acid sequence.....	75
5.4.2 Conserved leucines within the zipper are required for intracellular but not extracellular LIF activity.....	77
5.4.3 A conserved non-repeat leucine affects LIF-T intracellular localisation and activity.....	79
5.4.4 Conserved leucine mutations abolishes apoptosis induced by lower level LIF-T expression.....	81
5.5 Discussion.....	82
5.5.1 An alternate domain mediates intracellular LIF function.....	82
5.5.2 The LIF leucine zipper?.....	84
CHAPTER 6: FINAL DISCUSSION.....	86
6.1 Complex genomic organisation of the LIF gene.....	86
6.2 Potential function of the LIF-T protein.....	89
6.3 Mechanism of LIF-T action.....	90
6.4 Future work.....	92
6.4.1 Analysis of LIF protein.....	93
6.4.2 Analysis of LIF gene expression.....	93

6.4.3	Analysis of intracellular LIF protein function.....	94
6.4.4	Analysis of intracellular LIF structure	95
CHAPTER 7: MATERIALS AND METHODS		96
7.1	Abbreviations	96
7.2	Materials	98
7.2.1	Chemicals and reagents.....	98
7.2.2	Radiochemicals.....	98
7.2.3	Buffers	98
7.2.4	Enzymes.....	100
7.2.5	Antibodies.....	101
7.2.6	Kits	101
7.2.7	Cloning and expression vectors	101
7.2.8	Cloned DNA sequences	102
7.2.9	Oligonucleotides.....	102
7.2.10	DNA markers	103
7.2.11	Bacterial strains	104
7.2.12	Bacterial growth media.....	104
7.2.13	Miscellaneous materials.....	104
7.3	Tissue Culture Materials.....	105
7.3.1	Cell lines	105
7.3.2	Solutions	105
7.3.3	Media.....	106
7.3.4	Miscellaneous materials	106
7.4	Molecular Methods	107
7.4.1	General recombinant DNA techniques.....	107
7.4.2	Restriction endonuclease digestion of DNA	107
7.4.3	End-filling DNA fragments	107
7.4.4	Purification of DNA fragments.....	108

7.4.5	DNA ligation reactions	108
7.4.6	Preparation of competent cells.....	108
7.4.7	Rapid small scale preparation of DNA (Mini-prep).....	108
7.4.8	Double stranded sequencing of plasmid DNA.....	109
7.4.9	Large scale plasmid preparation	109
7.4.10	Preparation of genomic DNA	110
7.4.11	Synthesis of radioactive DNA probes.....	110
7.4.12	Bacterial colony lift.....	110
7.4.13	Hybridisation of radioactive probes to DNA immobilised to nitrocellulose filters	111
7.4.14	PCR-RACE	111
7.4.15	Reverse Transcription.....	111
7.4.16	Polymerase Chain Reaction	112
7.4.17	PCR mutagenesis	112
7.4.18	Isolation of cytoplasmic RNA from cultured cells.....	112
7.4.19	Isolation of RNA from tissue samples	113
7.4.20	Ribonuclease protection.....	113
7.4.21	Whole cell protein extracts and Western blotting.....	114
7.4.22	³⁵ S-labelling of cellular proteins and immunoprecipitation with anti- LIF antibodies	114
7.4.23	Transcription factor recognition sequence comparison.....	115
7.4.24	Containment Facilities.....	115
7.4.25	Phosphorimager Analysis and Autoradiograph Scanning	115
7.5	Tissue Culture Methods.....	116
7.5.1	Maintenance of cell cultures.....	116
7.5.2	Electroporation of Cos-1 cells.....	116
7.5.3	Lipofection of Cos-1 and 293T cells.....	117
7.5.4	Immunohistochemical staining of transiently transfected cells.....	117
7.5.5	Staining for β -galactosidase activity.....	118

7.5.6 Stable transfection of Cos-1 cells	118
7.5.7 Maintenance of ES cells	118
7.5.8 ES cell LIF biological activity assay	118
7.5.9 Differentiation of ES cells.....	119

THESIS SUMMARY

Cytokines are important biological molecules involved in a wide range of cellular processes. The classic mode of cytokine action involves interaction of the secreted molecule with a specific cell surface receptor that transduces a signal into the cell. This signal is transmitted into the nucleus, by a series of signalling pathways, producing an alteration in gene transcription and a cellular response. This mechanism of cytokine action may require modification due to the identification of cytokines that are localised within the cell, with intracellular localisation required for the cytokine response in some cases.

Leukemia inhibitory factor (LIF) is a member of the IL-6 family of cytokines and elicits a wide variety of effects on various cell types, *in vitro* and *in vivo*. The mouse LIF gene has been shown to express two proteins arising from alternate transcripts consisting of novel first exons spliced onto common second and third exons. The first exons of both transcripts contain an in-frame initiation codon that mediates translational initiation in exon 1, yielding secreted proteins of identical sequence that are localised to different cellular locations, one being a diffusible protein (LIF-D) and the other localised to the extracellular matrix (LIF-M). The two transcripts are differentially regulated indicating distinct biological functions for each protein.

In this work a third novel LIF transcript (LIF-T) was identified and cloned from murine, human and porcine sources. This transcript, which also contains an alternate first exon spliced onto common third and second exons, was differentially regulated in cultured cell lines and mouse tissues, indicating an important biological function. Genomic sequence comparison indicated a conserved, complex genomic organisation of the mammalian LIF gene in which three alternate transcripts containing novel first exons are expressed by differential promoter usage. Two classes of transcript are produced from the mammalian LIF gene; transcripts containing an in-frame initiation codon in exon 1, which include LIF-D from all species and murine LIF-M, and those that contain no initiation codon in exon 1, including LIF-T from all species and LIF-M from all species except mouse.

The absence of an in-frame ATG in the first exon of the LIF-T transcript was shown to direct initiation of translation to the first in-frame ATG in exon 2. This produced a truncated,

biologically active 17 kDa LIF protein that does not contain a signal sequence and is retained within the cell. Immunohistochemical detection of overexpressed intracellular LIF protein showed nuclear and cytoplasmic protein localisation.

Transfection studies showed that high and low level overexpression of intracellular LIF caused cells to undergo apoptosis, via a specific cellular pathway that was inhibitable by CrmA but not Bcl-2. Therefore, the cellular effect of the LIF-T protein was different to that of the extracellular cytokine, indicating an alternate mechanism of action and novel biological function for intracellular LIF. LIF-T induced apoptosis was shown to be a function of LIF intracellular localisation, and mutational analysis demonstrated that LIF-T action was independent of LIF receptor interaction and signalling. A novel, conserved structural motif was identified, containing a leucine repeat structure similar to a leucine zipper. Mutation of single leucines in this structure abolished nuclear LIF localisation and intracellular activity, without affecting extracellular LIF activity, demonstrating that extracellular and intracellular activity domains are localised in different regions of the protein. It was postulated that the intracellular LIF protein adopts an alternate structure to the extracellular molecule, involving a leucine repeat protein-protein interaction domain. This structure may act in the nucleus by dimerisation with other leucine repeat containing proteins, affecting gene transcription and causing a cellular response.

This study demonstrates a conserved structural organisation of the mouse LIF gene that produces three differentially localised proteins. This provides a mechanism for specific control of the sites of LIF action and mechanisms for mediating the variety of putative actions for the LIF gene. Intracellular localisation of the LIF protein provides another example of intracellular cytokine action, mediated by a novel mechanism and provides a system for separate analysis of intracellular and extracellular cytokines.