UNIVERSITY OF CALGARY

Identification of the Soluble Granulocyte-Macrophage Colony Stimulating Factor Receptor Protein in Vivo and Development of a Soluble Model of the High Affinity Cell Surface Receptor for Granulocyte-Macrophage Colony Stimulating Factor

by

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DEDICATION

To my brothers, Lee and Dean, for providing support and encouragement throughout my life. To an incredible friend, Tara Gibson, for her love, encouragement and generosity. To Hanna and Niska, my favorite canine companions. Finally, to my wonderful parents Lorne and Bernie, who have provided loving support throughout my life and shown me the true meaning of honesty, compassion and selflessness.

TABLE OF CONTENTS

LIST OF FIGURES

NOMENCLATURE

βc	the common signal-transducing, affinity-converting β subunit that is shared by the GM-CSF, IL-3 and IL-5 receptor complexes
μM	micro molar
1C1	anti-βc monoclonal antibody
8G6	anti-GMRα monoclonal antibody
BHK-21	baby hamster kidney fibroblast cell line
Bm-5	ovarian cell line of the silk moth *Bombyx mori*
BSA	bovine serum albumin
CNTF	ciliary neurotrophic factor
ConA	concanavalin A
DNA	deoxyribonucleic acid
ECDα	an engineered, soluble isoform of GMRα containing the extracellular domain of GMRα
ECDβc	an engineered, soluble isoform of βc containing the extracellular domain of βc
EPO	erythropoietin
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
G-CSF	granulocyte-colony stimulating factor
GH	growth hormone
GM-CSF	granulocyte-macrophage colony stimulating factor
GMRα	the cell surface, ligand binding α subunit of the GM-CSF receptor
gp130	the common signal-transducing, affinity-converting subunit that is shared by the IL-6, CNTF, OSM and LIF receptor complexes
HPC	hematopoietic progenitor cell
HRP	horse radish peroxidase
IEF	isoelectric focusing
IEX	ion exchange chromatography

IgG	immunoglobulin class G
IL-3	interleukin-3
IL-5	interleukin-5
IL-6	interleukin-6
LIF	leukemia inhibitory factor
mRNA	messenger RNA
NHS	N-hydroxy succinimide
nM	nano molar
OSM	oncostatin M
PCR	polymerase chain reaction
pI	isoelectric point
pM	pico molar
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RPC	reverse phase chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
sGMRα	the naturally occuring, soluble isoform of the GMRα subunit
WGA	wheat germ agglutinin

CHAPTER 1
INTRODUCTION

1.1 Hematopoiesis and cytokines. Blood cells of the hematopoietic system are critical mediators of gas exchange, host defense, clotting and wound repair. However, all blood cells have a limited life span and must therefore be continually replaced by new functional cells. Maintenance of the different populations of circulating blood is the responsibility of a small population of hematopoietic progenitor cells (HPC) located in the bone marrow of the adult. The HPCs are uniquely capable of perpetual self-renewal and therefore act as a lifetime reservoir of potential blood cells. However, while the self-renewal capacity of HPCs is a critical feature of hematopoiesis, it is the HPCs extraordinary ability to progress through multiple stages of development and maturation to eventually give rise to a diverse group of blood cells that makes them such an important part of homeostasis.

Hematopoiesis is a tightly regulated event that is mediated in part by a large, heterogeneous family of glycoprotein growth factors called cytokines. Cytokines are responsible for recruiting HPCs into the cell cycle and stimulating their cell division. Cytokines are also responsible for driving the daughter cells of the proliferating HPCs through the various stages of maturation and differentiation. However, while individual cytokines have specific roles in hematopoiesis, there is also substantial overlap in their hematopoietic activities. Surprisingly, despite the apparent redundancy that is built in to the control of hematopoiesis, hematopoietic diseases such as anemia and leukemia often arise through aberrant cytokine expression which can lead to loss of control of the process of hematopoiesis.

Cytokines are responsible for initiating HPC cell division. When a HPC undergoes cell division, one of the daughter cells remains in the same undifferentiated, pluripotent state as its parent HPC, and therefore remains capable of perpetual self-renewal (Figure 1.1). The other daughter cell will, upon stimulation with a specific set of cytokines, begin a long and complex journey through the various stages of differentiation, proliferation and maturation that eventually lead to the formation of a fully functional,

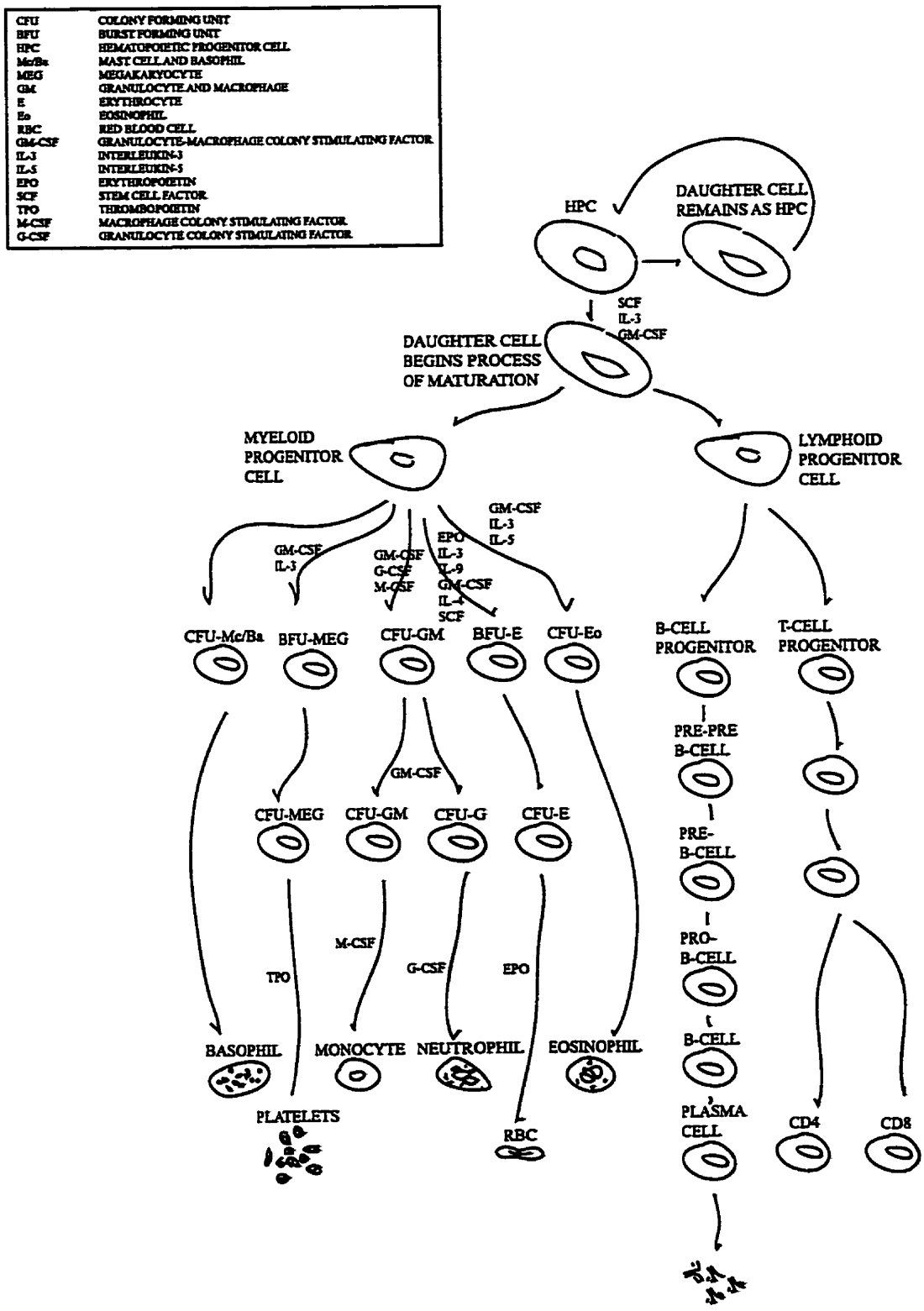


Figure 1.1 Cytokines and hematopoiesis. A simplified schematic showing the heirarchial organization of the hematopoietic system and the role of selected cytokines in myelopoiesis.

terminally differentiated blood cell. Importantly, the mature blood cell, whether of myeloid (erythrocyte, platelet, monocyte/macrophage or granulocyte) or lymphiod (B or T cell) origin, remain highly dependent upon specific cytokines for intiation of their respective biological activites. Therefore, while cytokines are critical mediators of hematopoietic cell division and differentiation, they are also important regulators of mature blood cell function.

1.2 Identification, cloning and expression of GM-CSF. In 1974 Golde and Cline[1] demonstrated that a factor expressed by phytohemaglutinin-stimulated human lymphocytes could drive hematopoietic progenitor cells to proliferate and differentiate *in vitro*. The isolated growth factor was referred to as granulocyte-macrophage colony stimulating factor (GM-CSF) by virtue of its ability to drive the hematopoietic progenitor cells to mature into granulocytes and macrophages. A murine growth factor with GM-CSF-like activity was later purified from mouse lung conditioned media[2]. Using the N-terminal amino acid sequence a cDNA encoding murine GM-CSF was cloned[3]. Recombinant murine GM-CSF encoded a 141 amino acid polypeptide with a predicted molecular weight of 13,138 Daltons that migrated by SDS-PAGE as a broad 22,000 Dalton smear suggesting substantial post-translational modification.

Human GM-CSF was expression cloned in 1984 from an HTLV-II transformed human T-cell line[4]. The following year Cantrell et al.[5] cloned the same cDNA from a human lymphocyte library using the murine GM-CSF cDNA as a probe. The isolated cDNA had a single 423 base pair open reading frame encoding a 144 amino acid polypeptide showing 54% homology to murine GM-CSF. The first 17 amino acids of the translated GM-CSF sequence corresponded to a signal peptide that targeted the mature protein for secretion resulting in a mature protein of 127 amino acids with a predicted molecular weight of 13,976 Daltons.

The naturally occuring human GM-CSF protein expressed by the HTLV-II transformed T-cell line was purified and characterized by SDS-PAGE[4]. Despite a predicted molecular weight of 13,976 Daltons purified GM-CSF migrated as a 22, 000 to 25,000 Dalton protein. However, enzymatic deglycosylation of the purified protein resulted in the predicted migration pattern suggesting that the size discrepancy was due to

substantial glycosylation of the mature protein. Surprisingly, the carbohydrate moieties of human GM-CSF were shown not to be required for full biological activity *in vitro*[6-8] or *in vivo*[9-11].

Comparison of the crystal structure of GM-CSF[12,13] with that of other hematopoietic ligands such as growth hormone (GH)[14], granulocyte-colony stimulating factor (G-CSF)[15,16], interleukin-2 (IL-2)[17,18] and leukemia inhibitory factor (LIF)[19] showed that GM-CSF had the same overall tertiary structure as other members of the cytokine superfamily characterized by four anti-parallel α-helices arranged in a barrel structure.

1.3 Biological activities of GM-CSF. GM-CSF was initially characterized by its ability to stimulate the proliferation of hematopoietic progenitor cells and drive the progeny to differentiate into mature granulocytes and macrophages[20-26]. The myelostimulatory activity of GM-CSF has been demonstrated clinically: GM-CSF is used to reduce the myelosuppressive effects of chemotherapy and radiation therapy[27-33], to accelerate stem cell engraftment following bone marrow transplantation[34] and to mobilize hematopoietic progenitor cells into the peripheral blood for harvest[35,36]. However, like other hematopoietic cytokines, GM-CSF has a diverse range of biological activities outside the setting of hematopoiesis. GM-CSF can stimulate the antimicrobial[37-40], antifungal[41-47] and antitumor[48-63] activities of phagocytes. This can occur through direct cell stimulation or, more commonly, by enhancing the ability of both the phagocytic cells and other antigen presenting cells to respond to a secondary stimuli. For example, GM-CSF can prime immune cell function by increasing the expression of the cell surface fMLP receptor on neutrophils and by increasing MHC-II receptor expression on macrophages and dendritic cells. Interestingly, locally administered GM-CSF has also been shown to prevent emmigration of phagocytic cells from sites of infection and injury thereby enhancing both the local immune response and the healing process[64-69].

Overexpression of GM-CSF in mice leads to a non-neoplastic myeloproliferative syndome that resembles chronic myelogenous leukemia in humans[70,71]. Overexpression of GM-CSF in mice also leads to pulmonary hypersensitivity[72] and hyperplasia[72-74] and infiltration of activated macrophages into the lungs and the striated muscle[75] of the mice resulting in severe tissue damage and death. Surprisingly the GM-CSF/GM-CSF receptor

knockout mouse shows no major hematological abnormalities[76-79] except for a reduced capacity to manage parasitic[78] and pulmonary bacterial infections[80] suggesting that GM-CSF is not required for normal hematopoiesis. However loss of functional GM-CSF activity leads to the accumulation of surfactant in the lungs resulting in a pulmonary alveolar proteinosis-like disease[77-79]. The accumulation of surfactant is believed to result from a decreased capacity of the pulmonary alveolar macrophages to clear the surfactant from the alveolus. Loss of the signalling subunit of the GM-CSF receptor (βc, see below) in children suffering from acute myelogenous leukemia (AML) was shown to correlate with an increased incidence of pulmonary alveolar proteinosis[81,82] suggesting that GM-CSF activity may also be critical for pulmonary homeostasis in humans.

1.4 GM-CSF expression. Basal expression of GM-CSF during hematopoiesis is accomplished in part by bone marrow stromal cells[83-87]. During inflammation GM-CSF is expressed primarily by T-cells[88-92], macrophages[93-95], fibroblasts[96-100], endothelial cells[101-103] and mast cells[104,105], although other non-hematological cell types[106,107] and tissues[72,108-114] are capable of GM-CSF expression. Interestingly a number of non-hematopoietic tumor cell types can also constitutively express GM-CSF[115].

A role for aberrant GM-CSF expression in leukemogenesis has been hypothesized. However the constitutive expression of GM-CSF by certain leukemic cells lines[116-121] is not sufficient for tumorigenesis. On the other hand GM-CSF does contribute to the progression of a fatal pediatric leukemia, juvenile myelomonocytic leukemia (JMML). JMML arises in part through disruption of the ras signalling pathways[122-124] but it is a hypersensitivity to GM-CSF that leads to uncontrolled cell proliferation[125-128], demonstrated by the ability of antagonists of GM-CSF to halt disease progression[129-131].

1.5 Characterization of the cell surface receptor for GM-CSF. Preliminary characterization of the receptor for human GM-CSF was performed by ^{125}I-GM-CSF binding assay using GM-CSF-responsive leukemic cell lines. ^{125}I-GM-CSF binding to the myelomonocytic cell line KG-1 was shown to be rapid at 37°C reaching equilibrium within 30 minutes[132]. Scatchard analysis of ^{125}I-GM-CSF saturation binding revealed 2 potential classes of GM-CSF receptors; a predominant high affinity receptor with a dissociation constant of 18 pM and a lower affinity receptor that could not be saturated

with the available ligand preparations[132]. The presence of two potential GM-CSF receptor subtypes was also reflected by a biphasic dissociation curve showing both rapid ($t_{1/2}$=2 minutes) and slow ($t_{1/2}$=290 minutes) dissociations of ^{125}I-GM-CSF from the cell surface[133].

The high affinity GM-CSF receptor was present on KG-1 cells in low numbers (50/cell)[132,134] but was absent on the GM-CSF unresponsive KG-1 subtype (KG-1a)[132]. The same high affinity receptor (Kd=14 pM) was present on the human promyelocytic leukemia cell line (HL-60) in similarily small numbers (50/cell)[132-134]. Of interest, differentiation of HL-60 cells towards a granulocytic phenotype using dimethylsulfoxide (DMSO) resulted in a 5 fold increase in the number of high affinity GM-CSF receptors[132]. The high affinity receptor was also present on a number of other myeloid cell lines including the histiocytic lymphoma cell line U-937[134], the myelomonocytic cell line THP-1[134], but not on the Jurkat T-cell line[133] or the erythroleukemia cell lines K-562[133] and HEL 92.1.7[134]. Importantly the high affinity GM-CSF receptor was also present on freshly isolated human neutrophils[132,133], mononuclear cells[133,134] and bone marrow cells[134] but not on human lymphocytes[133] suggesting that GM-CSF receptor expression is restricted to cells of the myeloid lineage. Murine cell lines expressing the murine GM-CSF receptor did not bind human ^{125}I-GM-CSF[133] demonstrating a lack of cross reactivity between the human and murine ligands.

1.6 Cloning of the GMRα and βc subunit of the GM-CSF receptor. Cloning of two independent subunits for the GM-CSF receptor in 1989 and 1990 provided physical evidence for the existence of two classes of GM-CSF receptors. The ligand binding subunit of the GM-CSF receptor (GMRα) was expression cloned in 1989 from a human placental cDNA library[135]. Simian COS-7 and murine factor-dependent FDC-P1 cells transfected with the GMRα cDNA showed low affinity (Kd=3.4 nM) GM-CSF binding but did not show high affinity binding suggesting that another cell surface component might be required for high affinity GM-CSF binding. Surprisingly, despite the absence of high affinity binding, the GMRα-transfected FDC-P1 cell line could proliferate in response to GM-CSF, suggesting that the GMRα subunit was sufficient for signal

transduction[136]. However proliferation of the FDC-P1/GMRα cell line required a 1000 fold higher concentration of GM-CSF than cells expressing the high affinity GM-CSF receptor. The reason for this became clear with the cloning of a second subunit of the GM-CSF receptor.

The ability of interleukin-3 (IL-3) to compete with GM-CSF for high but not low affinity binding[137-140] suggested that the two receptors might share a common affinity converting subunit. In 1990 a human homologue of the murine IL-3 receptor AIC2A was cloned from an erythroleukemia cell line cDNA library using the murine cDNA as a probe[141]. The isolated cDNA showed 56% homology to AIC2A and 55% homology to AIC2B (a seperate murine gene with 97% homology to AIC2A that encodes a non-ligand binding cell surface receptor) but showed no binding activity for human GM-CSF, IL-2, IL-3, interleukin-4 (IL-4), interleukin-5 (IL-5), erythropoietin (EPO) or murine IL-3. However transfection of the cloned cDNA into a GMRα expressing cell line (COS-7/GMRα) reconstituted high affinity (Kd=120 pM) GM-CSF binding. Further, the 120 kDa protein encoded by the isolated cDNA could be cross linked with ^{125}I-GM-CSF when co-expressed with GMRα suggesting that it was part of the GMR complex. Finally, co-expression of the human AIC2A/AIC2B homologue (referred to from here in as βc) with the ligand binding subunit of the IL-3 or IL-5 receptors resulted in high affinity binding of their cognate ligands suggesting that βc was a common affinity converting subunit for all three receptors.

Reconstitution experiments using the GMRα and βc subunits showed that when co-transfected into the murine factor dependent cell lines BAF-B03 and CTLL-2 the cells would bind GM-CSF with high affinity[142]. However the BAF-B03/GMRα cell line, like the previously described FDC-P1/GMRα line, could proliferate in response to high concentrations of GM-CSF while the CTLL-2/GMRα cell line could not. The reason for this apparent paradox was that the BAF-B03/GMRα and FDC-P1/GMRα cell lines were responding to GM-CSF by recruiting the murine homologue of human βc, AIC2B, into a signal transducing complex, wherease the CTLL-2/GMRα cell line did not express AIC2B and therefore remained unresponsive. Thus, the functional GM-CSF receptor

was believed to consist of both a ligand binding GMRα subunit and an affinity converting βc subunit that was shared with both the IL-3 and IL-5 receptor systems.

1.7 Structure of GMRα and βc. The GMRα cDNA consists of a single 1,200 base pair open reading frame that encodes a 400 amino acid protein[135]. The first 22 residues of GMRα are a signal peptide that directs expression of the receptor to the cell surface. The mature 378 amino acid subunit has a 298 residue extracellular domain and a stretch of 26 uncharged amino acids that encode a single membrane spanning domain. The cytoplasmic domain of GMRα consists of 54 residues. The predicted molecular weight of GMRα is 43,728 Daltons however it migrates as an 80,000 Dalton protein by denaturing SDS-PAGE reflecting substantial carbohydrate modification of its 11 potential N-glycosylation sites.

The βc cDNA consists of a single open reading frame of 2,691 base pairs encoding an 897 amino acid polypeptide[141]. The first 16 amino acids are also a signal peptide that is cleaved prior to cell surface expression. The mature βc protein consists of 881 residues divided into a 421 residue extracellular domain, a 27 amino acid hydrophobic membrane spanning domain and a 433 amino acid cytoplasmic domain. βc migrates as 120,000 Dalton protein by SDS-PAGE.

1.8 The cytokine receptor superfamily. The cytokine receptor superfamily includes a number of individual receptor families for the interleukins, the colony stimulating factors and related hematopoietins, the interferons, the TNF family of cytokines, the chemokines and the growth factors. GMRα and βc belong to the type I family of cytokine receptors by virtue of shared structural and functional motifs[143]. Type I cytokine receptors have a single membrane spanning region where the N-terminus of the receptor exists outside of the cell and the C-terminus within the cell. None of the type I cytokine receptors have intrinsic kinase activity but instead associate constitutively and/or inducibly with cytoplasmic signaling molecules. The type I family contains four identifiable subfamilies that are defined by the sharing of a single receptor subunit between more than one individual cytokine. For example, the members of the IL-6 subfamily share the gp130 receptor subunit while members of the GM-CSF subfamily

share the βc receptor subunit. The extracellular domain of the type I cytokine receptors consist of at least one cytokine receptor homology (CRH) region that is defined by a pair of fibronectin-type III (FNIII) bundles connected at 90 degrees to each other by a short helical hinge region. The GMRα subunit has a single CRH domain while βc has two. Each FNIII bundle is made up of 7 β strands arranged into two pleated sheets. Most members of the family also have additional structural features such as unique immunoglobulin-like domains or unique N-terminal sequences. Unique amongst the family is the CNTF receptor which does not have a cytoplasmic or transmembrane domain but is instead anchored to the cell surface via a glycophosphatidylinositol (GPI) linkage. The type I cytokine receptor family and the cytokine receptor superfamily in general is also characterized by the existence of soluble variants of nearly all ligand binding subunits as well as soluble variants of certain signal transducing subunits (see below).

Closer inspection of the amino acid sequence of members of the cytokine receptor superfamily shows a number of conserved motifs. The N-terminal domain contains four conserved cysteine residues that mediate two intrachain disulphide bonds, while the membrane proximal region of the extracellular domain has a highly conserved tryptophan-serine-X-tryptophan-serine (WSxWS) motif of unknown function. Within the membrane proximal region of the cytoplasmic domain are two proline rich motifs (box 1 and box 2) that are involved in signal transduction.

1.9 The soluble GM-CSF receptor. A common feature of the cytokine receptor superfamily is the existence of soluble variants of the ligand binding subunits and of certain signal transducing subunits[144-147]. Soluble receptors arise through proteolytic cleavage or alternative splicing. Loss of the membrane spanning and cytoplasmic domains results in expression of the extracellular domain of the cognate cell surface receptor into the pericellular milieu. Soluble receptors can act as agonists, antagonists or carriers of their respective ligands. Surprisingly the activities described are not mutually exclusive as certain receptors have been shown to act as both agonists and antagonists.

Using the available sequence data for GMRα a soluble variant of GMRα (sGMRα) was initially cloned from a human placental cDNA library[148]. The cloned cDNA shared a

nucleotide sequence encoding 317 of the 320 residues of the extracellular domain of GMRα but was missing a 97 bp sequence that encoded the 27 residue transmembrane domain. Further, the excision of an odd number of nucleotides from the gene sequence generated a frameshift in the coding sequence that replaced the 54 residue cytoplasmic domain of GMRα with a unique set of 16 amino acids. The presence of alternative splice consensus sequences flanking the excised transmembrane exon suggested that sGMRα was a naturally occuring splice variant of GMRα and not an artifact of the cloning procedure.

sGMRα binds GM-CSF in solution with the same affinity as GMRα[149,150] and can antagonize GM-CSF-mediated proliferation[151] and bone marrow colony formation[149]. Exogenous sGMRα can not associate with βc even in the presence of GM-CSF[149,150,152]. Paradoxically, co-expression of sGMRα with βc results in their cell surface interaction[152] and the formation of a high affinity GM-CSF binding complex[150]. It is not known why co-expression of sGMRα with βc induces the tethering of sGMRα to βc on the cell surface to form a heteromeric complex that can bind GM-CSF while an independent source of sGMRα, although able to bind GM-CSF in solution, can not associate with βc. Further, it is not clear whether the sGMRα/βc cell surface complex can induce a biological response either in the presence of absence of GM-CSF.

1.10 Activation of the GM-CSF receptor complex. The mechanism through which GM-CSF binding to the GM-CSF receptor stimulates a biological response is poorly understood. The low affinity interaction of GM-CSF with GMRα[135] is necessary for interaction with βc since in the absence of GMRα, βc shows no intrinsic affinity for GM-CSF[141]. However, in the presence of GMRα, βc is believed to directly interact with GM-CSF to form a high affinity complex. A recent set of experiments has demonstrated that high affinity binding of GM-CSF is necessary but not sufficient for signal transduction[153]. GMRα and βc were shown to form a disulphide bonded heterodimer upon GM-CSF binding that is mediated through free sulhydryl groups in the N-terminus of βc and GMRα. Importantly, mutation of these cysteine residues to alanine does not affect high

affinity GM-CSF binding but instead completely abrogates signal transduction suggesting that ligand binding and receptor activation are dissociable events.

While high affinity ligand binding results in the formation of a GMRα-βc heterodimer that is involved in signal transduction[153] formation of this complex is not absolutely necessary for biological activity. Homodimerization of βc using receptor-chimeras[154,155] or through mutation[156-158] results in constitutive activation and signal transduction. On the other hand, the wild type βc-subunit has been demonstrated to exist on the cell surface at least in part as a disulphide bonded homodimer that is inactive in the absence of GMRα and GM-CSF[159] suggesting that facile homodimerization of βc is in itself not sufficient for signal transduction.

GM-CSF signal transduction is initiated by transphosphorylation of non-receptor tyrosine kinase molecules of the Janus kinase family (JAK2) that are associated with the cytoplasmic domain of βc[160]. Assembly of the GM-CSF receptor complex is believed to bring two or more JAK2 subunits into close enough proximity to induce autotransphosphorylation resulting in activation of the tyrosine kinase activity of the JAK2 molecules. Phosphorylation of βc by the JAK2 molecules generates docking sites for src-homology 2 domain (SH2) containing signaling and adapter molecules such as signal transducers and activators of transcription (STATs), members of the src-family of non-receptor tyrosine kinsases and adaptor molecules such as SHC resulting in initiation of the signal transduction cascade[161-185].

HYPOTHESES AND THESIS OBJECTIVES

1.11 The sGMRα transcript is present in all cells that express cell surface GMRα. However, the sGMRα protein has yet to be identified in media conditioned by cells that contain the sGMRα transcript. Since transfection of the sGMRα cDNA into hematopoietic cells results in secretion of ample sGMRα protein into the media, I would hyothesize that the sGMRα protein would also be secreted from non-transfected cells that contain the sGMRα transcript. Therefore, one objective of this thesis was to determine if

the naturally occuring sGMRα protein was secreted by leukemic cell lines and human neutrophils and determine if it was present in normal human plasma and in human plasma isolated during peripheral blood stem cell harvest.

1.12 The mechanism of assembly of the cell surface GMR complex is poorly understood. It is believed that the GMRα and βc subunits have no intrinsic affinity for one another in the absence of GM-CSF and, since βc has no intrinsic affinity for GM-CSF, it is the association of GMRα with GM-CSF that drives the interaction with βc. Furthermore, since the cytoplasmic and transmembrane domains of GMRα are not necessary for binding GM-CSF and the cytoplasmic domain of βc is similarly not required for the interaction of βc with the GMRα/GM-CSF complex, it is believed that the interactions that mediate the stepwise formation of the GMR complex are mediated through the extracellular domains of GMRα and βc. I would hypothesize that the extracellular domains of GMRα and βc are fully capable of reconstituting the stepwise assembly of the high-affinity GMR complex *in vitro*. Therefore, a second objective of this thesis was to engineer, express and characterize soluble isoforms of the GMRα and βc subunits that contain only the extracellular domains of their cognate cell surface receptors in order to determine if the extracellular domains of GMRα and βc mediate the assembly of the high-affinity GM-CSF receptor complex.

CHAPTER 2
GENERAL MATERIALS AND METHODS

Recombinant DNA procedures

2.1 PCR amplification. The polymerase chain reaction was employed to amplify DNA fragments. Each 50 µL reaction contained 5 µL of *Pfu* polymerase buffer, 1 unit of *Pfu* DNA polymerase, 3 mM $MgCl_2$, 0.4 mM each of the four dinucleotide triphosphates, 20 pmol of each primer, and 20 ng of plasmid DNA. The amplification conditions were 30 cycles of (1) denaturation at 94°C for 1 minute, (2) annealing at 50°C for 1 minute and (3) extension at 72°C for 3 minutes.

2.2 Purification of DNA from agarose gels. DNA inserts were routinely purified from melted agarose gels using a silica matrix (Geneclean II, Bio 101 Inc., La Jolla, CA). Briefly 4.5 volumes of a 6M NaI solution and 0.5 volumes of TBE buffer (90 mM Tris-borate, 2 mM EDTA) were added to the DNA/agarose mix. The solution was incubated in a 55°C water bath until the agarose was melted. 5-10 µL of a silica matrix was added to adsorb the DNA. The reaction was incubated for 15 minutes and the silica/DNA was precipitated by centrifugation. The pellet was washed with a NaCl/EDTA buffer and the DNA was eluted from the silica by incubating the pellet with 12 µL of ddH_20 for 3 minutes at 55°C. The elution procedure was repeated twice.

2.3 Agarose gel electrophoresis of DNA. DNA was size fractionated by electrophoresis through a 0.8% agarose/TBE gel containing ethidium bromide (0.5 µg/mL) for 30 minutes at 90 mV. The gel was visualized on a flatbed UV light box at 212 nm.

2.4 DNA ligation. Ligations of DNA fragments into plasmid vectors were routinely performed by incubating 100 ng of linearized plasmid DNA with a 5 fold molar excess of DNA insert in a 20 µL volume containing 1 unit of T4 DNA ligase (Gibco BRL Life Technologies Inc., Mississauga, ON) and 4 µL of ligase buffer. The reaction was allowed to take place at 16°C for 18 hours.

2.5 Transformation of bacteria. 40 μL of competent *E. coli* cells were incubated for one hour on ice with 1 ug of plasmid DNA. The bacteria were induced to take up the DNA by heat shock for 45 seconds at 42°C. The tubes were chilled on ice for 2 minutes and 450 μL of SOC media (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added. The culture was incubated for 1 hour at 37°C before plating on LB-ampicillin/agarose plates (10 g bacto-tryptone, 5 g bacto-yeast, 10 g NaCl, 1 L ddH_20, pH 7.0, 15 g agarose100 ug/mL ampicillin) (the ampicillin resistance gene was rountinely incorporated in both cloning and expression vectors and was therefore used to select for transformed bacteria). For vectors containing the β-galactosidase gene the transformed bacteria were plated in the presence of X-gal (40 μL of a 20 mg/mL solution in dimethylformamide) and IPTG (4 μL of a 200 mg/mL solution) (Sigma) to allow for blue/white selection of bacterial colonies containing the recombinant vector.

2.6 Small scale preparation of plasmid DNA: "minipreps". 1.5 mL of an overnight bacterial culture was transferred to an eppendorf tube and centrifuged at 12,000 x g for 1 minute. The supernatant was discarded, the pellet was vortexed with 100 μL of ice cold solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCL pH 8, 4 mg/mL lysozyme) and incubated at room temperature for 5 minutes. The bacteria were lysed and the nucleic acids denatured by gently mixing 200 μL of freshly prepared solution II (0.2M NaOH, 1% SDS) with the reaction mixture. Following a 5 minute incubation on ice the DNA was renatured and the protein-nucleic acid complexes precipitated by vortexing the mixture with 150 μL of ice cold solution III (90 μL of 3 M potassium acetate, 17.25 μL of glacial acetic acid, 47.25 μL of ddH_20). The tubes were incubated for 5 minutes on ice and centrifuged for 5 minutes at maximum speed to remove the cell debris. The supernatant containing plasmid DNA and bacterial RNA was transferred to a fresh tube containing 1 mL of absolute ethanol and mixed. The tube was centrifuged for 10 minutes at maximum speed and the ethanol was removed. The pellet was washed with 700 μL of 70% ethanol and pelleted by centrifugation for 2 minutes. The ethanol was

removed and the pellet was dried. The pellet was resuspended in 40 µL of 20 µg/mL DNase-free RNase A in ddH_20.

2.7 Restriction digests. Restriction enzyme digests of DNA were routinely performed for 1 hour at 37°C in a 20 µL volume containing 2-15 µL of a plasmid DNA solution, 2 µL of 10 x enzyme buffer and 10 units of restriction enzyme (Gibco BRL Life Technologies Inc).

2.8 PCR-based DNA sequencing. DNA sequencing was done by PCR using fluorescent dideoxynucleotides. A 20 µL solution containing 9.5 µL of sequencing mix (PE Biosystems, Mississauga, ON), 1 µg of plasmid template and 3.2 pmol of primer was subjected to 30 cycles of PCR consisting of (1) denaturation at 96°C for 30 seconds, (2) annealing at 50°C for 15 seconds and (3) extension at 60°C for 4 minutes. The amplified product was separated from the oil and precipitated from solution by incubating the mixture with 2 µL of a 3M sodium acetate solution and 50 µL of 95% ethanol at -80°C for 10 minutes. The DNA was pelleted by centrifugation at 12,000 x g for 10 minutes and washed once with 250 µL of 70% ethanol. The pellet was dried and transferred to the University of Calgary DNA Sequencing Facility for analysis. The resultant chromatographs were analyzed using the SeqEd DNA sequencing program.

2.9 Large scale preparation of plasmid DNA from bacteria: "maxipreps". A 2 mL overnight bacterial culture was used to innoculate 500 mL of LB broth containing 500 µg of ampicillin. The culture was incubated overnight at 37°C. The culture was divided in half and transferred to 250 mL centrifuge tubes. The bacteria was precipitated by centrifugation for 5 minutes at 7,000 rpm and the supernatant was discarded. The individual pellets were re-suspended in 6.5 mL of solution I and transferred to 50 mL Oakridge tubes. The bacteria were lysed during a 10 minute incubation on ice with 13 mL of freshly prepared solution II. 6.5 mL of ice cold solution III was added to each tube and the mixture was incubated on ice for 20 minutes after which the debris was pelleted by centrifugation at 12,000 rpm for 10 minutes. The supernatant was transferred to fresh 50 mL Oakridge tubes and mixed with 0.6 volumes of isopropanol. The tubes were centrifuged at 8,000 rpm for 5 minutes and the supernatant was discarded. The pellets

were resuspended together in 2.4 mL of ddH_20. 2.64 g of cesium chloride and 300 µL of ethidium bromide (10 mg/mL) were added to the resuspended DNA and the mix was transferred to a 5 mL ultracentrifugation tube (Beckman). The tube was centrifuged for 4 hours at 95,000 rpm. The band containing the plasmid DNA was removed using a syringe and 21 gauge needle. The ethidium bromide was removed by extraction with butanol and the plasmid DNA was dialyzed overnight. The DNA was precipitated by incubating the solution with 0.1 volumes of 3M sodium acetate and 2.5 volumes of 95% ethanol for 30 minutes at -70°C followed by centrifugation for 10 minutes. The supernatant was discarded, the pellet was dried and resuspended in 100 µL of ddH_20. The DNA concentration was determined by UV spectrometry at 280 nm.

Tissue culture and cell transfections

2.10 Propagation of mammalian cell lines. BHK-21 and HL-60 cell lines were grown to confluence in static cell culture at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12), 10% fetal calf serum and 1% antibiotic-antimycotic solution (Life Technologies, Inc., Mississauga, ON). U-937, THP-1 and K-562 cells were grown to confluence in static cell culture at 37°C and 5% CO_2 in RPMI-1640, 10% fetal calf serum and 1% antibiotic-antimycotic solution. BHK-21 cells were subcultured at confluence (approximately every 48 hours) by removing the culture media, washing the cell monolayer twice with 5 mL of Puck's media (5.4 mM KCl, 140 mM NaCl, 4.2 mM $NaHCO_3$, 5 mM dextrose, 10 mM HEPES, 1 mM EDTA) and incubating the plates at 37°C for 5 minutes. The monolayer was disrupted by tapping the plates and the cells were rendered into suspension by gentle pipetting with 1 mL of tissue culture media. 20% of the cells were replated into 10 mL of fresh culture media. Non-adherent hematopoietic cells (HL-60, U-937, THP-1 and K-562) were subcultured by removing approximately 80% of the culture media and cells and replacing this with fresh culture media.

2.11 Propagation of insect cell lines. Bm-5 cell lines were grown in IPL-41 insect media (Life Technologies) supplemented with 10% fetal bovine serum (JRH Biociences, Lenexa, KA) and 0.2 g/L L-glutamine, 1.0 g/L D-glucose. Cells were grown in 25 cm^2

T-flasks in ambient air at 28°C. Bm-5 cells were subcultured weekly by removing one fifth of the cells and culture media and replacing it with fresh media. Bm-5 cells were rountinely cultured in 125 mL spinner flasks on stirrer plates in 100 mL of media containing 10^5 viable cells/ mL.

2.12 Transfection of recombinant plasmid DNA into mammalian cell lines by calcium phosphate precipitation. 10 µg of plasmid DNA was resuspended in sterile ddH_20 to a final volume of 440 µL. 62.5 µL of a 2M $CaCl_2$ solution was added to the DNA and mixed. 500 µL of a 2x HEPES buffered saline solution (HBS, 50 mM HEPES buffer, 280 mM NaCl, pH 6.95) and 10 µL of a 0.07M Na_2PO_4 solution were mixed together in a 5 mL glass tube. The DNA/$CaCl_2$ solution was slowly pipetted into the bottom of the glass tube and mixed by gently bubbling the solution. This was allowed to sit for 30 minutes at room temperature prior to pipetting over a monolayer of adherent BHK-21 cells (30% confluent) in 10 mL of tissue culture media. The cells were incubated overnight in the transfection media before adding fresh media to the plates.

2.13 Transfection of recombinant plasmid DNA into insect cell lines by lipid uptake. Bm-5 cells were resuspended in IPL-41 media at a density of $5x10^5$ cells/mL. 2 mL of the suspension was plated into each well of a 6-well plate (35 mm diameter, Falcon) and allowed to adhere during overnight incubation. Prior to transfection, the cells were washed twice with 1 mL IPL-41 media. Plasmid DNA and Lipofectin (Life Technologies) were made up separately in IPL-41 media, then re-combined to make a transfection solution containing 30 µg/mL Lipofectin and 6 µg/mL DNA. The solution was incubated on ice for 15 minutes and 550 µL was added to each well. The transfection was allowed to proceed for 5 hours at 28°C at which time the cells were washed once with 2 mL IPL-41 and 2 mL complete medium was added to each well.

2.14 Propagation of cell lines in the absence of fetal calf serum. BHK-21 cells were first subcultured into DMEM/F12 basal media supplemented with 10% fetal calf serum for four hours to allow the cells to adhere to the plates prior to being transferred to a complete serum free media (OPTI-MEM, Gibco BRL Life Technologies Inc.). BHK-

21 cells could be cultured for 24-48 hours in the absence of FCS without significant loss of viability.

Bm-5 cell lines were adapted to long term growth in serum free media (EC401, JRH Bioscience) by sequential dilution out of serum containing media into serum free media. The cells were subcultured twice in the previous dilution of tissue culture media before the proportion of IPL-41/10%FCS was reduced again by half. The adaptation process took approximately two months.

Protein assays

2.15 Quantitation of GM-CSF. Clinical grade GM-CSF of known concentration was used for all experiments (Sargramostim Leukine, a gift if Immunex Inc., Seattle, WA). The concentration of ^{125}I-GM-CSF (Dupont NEN, Mississauga, ON, Canada) was confirmed independently of the manufactureres claims using a GM-CSF specific sandwich ELISA. All incubation steps in the following ELISA protocol were followed by three washes in wash buffer and unless otherwise indicated all solutions were made up in blocking buffer (phosphate-buffered saline, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, containing 2% BSA, 0.05% Tween-20, BDH Inc. Toronto, ON) and were used at 100 µL/well. A 96 well plate was incubated with a 1/2000 dilution (dilution buffer 0.1M Na_2CO_3, pH 9.6) of an anti-GM-CSF monoclonal antibody for 2 hours at 37°C and blocked for 2 hours. The plate was incubated with unknown antigen or GM-CSF standard for one hour at 37°C. The plate was incubated with a 1/2000 dilution of rabbit anti-GM-CSF anti-sera for one hour at 37°C followed by one hour with a 1/1000 dilution of streptavidin conjugated goat-anti-rabbit antibody. The plate was incubated with a 1/1000 dilution of biotinylated-horse radish peroxidase (HRP) for one hour followed by OPD solution (4 mg o-phenylenediamine substrate, 1 µL H_2O_2, 10 mL 1M NaCitrate, pH 5.0). The colorimetric reaction was stopped by incubating the plate with 50 µL/well of 1M H_2SO_4 and the results were visualized on a multiwell UV absorbance reader at 492 nm. The effective range of the ELISA was between 8 pg/mL and 1 ng/mL.

2.16 Quantitation of sGMRα and ECDα. sGMRα and ECDα were quantitated using a commercially available ELISA (rGM-CSF, Immunotech, Marseille, France).

Briefly, 50 μL/well of sample or affinity purified and quantified sGMRα protein (20 pM to 1280 pM) was added to a 96 well plate that was pre-coated with an anti-sGMRα monoclonal antibody. Following washing, 50 μL/well of a biotinylated-rabbit anti-sGMRα antibody and 100 μL of streptavidin-conjugated-HRP goat anti-rabbit antibody was added to each well. The presence of receptor was detected using a chromogenic peroxidase substrate that was visualized at 450 nm on a microplate reader. The effective range of the assay was 20 pM to 1280 pM.

2.17 SDS-PAGE. Proteins were boiled in 2X SDS-PAGE loading buffer (62.5 mM Tris-HCL pH 6.8, 20% glycerol, 4% SDS, 0.025% bromophenol blue) in the presence or absence of 1.5M of the reducing agent β-mercaptoethanol (βME, Sigma). The solubilized and denatured proteins were electrophoresed on 8-10.5% SDS polyacrylamide minigels for 2 hours at 100 volts using a mini-gel apparatus (Mini-Protean II, Bio-Rad Laboratories Ltd., Mississauga, ON).

2.18 Western blotting. Fractionated proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P PVDF, Millipore) according to the manufacturers instructions using a mini trans-blot apparatus. The blots were air dried, re-wet in methanol and blocked for 12 hours in 2-5% BSA in Tris-buffered saline (TBS, 20 mM Tris pH 7.4, 137 mM NaCl). The blots were probed with either anti-GMRα mAb 8G6 or anti-βc mAb 1C1 diluted 1/5000 in TBS-T (TBS containing 0.2% Tween-20) for 2 hours. The blots were washed once with TBS-T and incubated for 1 hour with a horse radish peroxidase-conjugated rabbit-anti-mouse IgG secondary antibody (Bio/Can Scientific, Mississauga, ON) diluted 1/22,000 in TBS-T. The blots were washed extensively and visualized by enhanced chemiluminesence (ECL, Amersham Life Sciences, Oakville, ON) and exposure to x-ray film.

2.19 Dot blotting. Dot blotting was performed using a vacuum filtration apparatus (Gibco BRL Life Technologies Inc.) and PVDF membrane. The PVDF membrane was wet in methanol, equilibrated in phosphate-buffered saline (PBS, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl) and assembled into the filtration device. Cell conditioned media or chromatography fractions (100-500 μL) were pipetted into

individual wells and adsorbed to the membrane by vacuum filtration. 200 µL of PBS was then washed through each well to ensure that the entire sample reached the membrane. The apparatus was dismantled and the membrane was dried. Western blotting was performed as described in 2.18.

2.20 Silver staining of SDS-PAGE gels. The gel was fixed in 50% methanol/10% acetic acid for 10 minutes then 5% methanol/7% acetic acid for 10 minutes. The gel was soaked in freshly made 1% gluteraldehyde for 15 minutes and washed extensively in water for one hour. The gel was soaked in 5 µg/mL solution of dithiotreital for 10 minutes then in 0.1% silver nitrate for 10 minutes. The gel was rinsed twice in ddH_20 then once in developer (50 µL 37% formaldehyde in 100 µL of 3% Na_2CO_3) before developing to the desired exposure. The reaction was stopped with 5 mL of glacial acetic acid.

2.21 Coomassie blue staining of SDS-PAGE gels. SDS-PAGE gels were fixed in 50% methanol/10% acetic acid for 30 minutes and stained with 0.1% Coomassie Blue R250 in 10% acetic acid for 30 minutes. The gel was destained in 10% acetic acid until the background was clear.

2.22 Coomassie blue staining of PVDF membranes. PVDF membranes were rinsed in water for 5 minutes then stained with 0.1% Coomasie Brilliant Blue R250 in 50% methanol for 2 minutes. The membrane was destained for 5-10 minutes in 45% methanol then rinsed in ddH_20 and dried.

2.23 Preparation of the GM-CSF-Sepharose 4B affinity column. 1 mL of N-hydroxysuccinimide (NHS)-activated agarose beads (NHS-Sepharose 4B Fast Flow, Pharmacia Biotech, Baie d'Urfe, PQ) was washed three times with 10 mL of an ice cold 1 mM HCl solution (pH 3.0) . The beads were incubated overnight at 4°C with 1 mL of a 1mg/mL solution of GM-CSF. Unconjugated NHS active sites were blocked by incubating the slurry with Tris-buffered saline (TBS, 20mM Tris pH 7.4, 137 mM NaCl) for 4 hours at 4°C. The slurry was transferred to a 10 mL plastic column and washed extensively with phosphate-buffered saline (PBS, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl). The column was stored at 4°C in PBS containing 0.02% sodium azide.

2.24 Affinity purification of sGMRα and ECDα. sGMRα and ECDα were purified from cell conditioned media using the GM-CSF affinity column. 200 mL of BHK-21/sGMRα conditioned media or 20 mL of Bm-5/ECDα conditioned media were allowed to flow through the column by gravity. The column was washed with 70 mL of phosphate-buffered saline (PBS, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl) and the adsorbed proteins were eluted with a 0.1M glycine buffer (pH 2.5). The column was eluted in 1 mL fractions which were immediately neutralized with 20 µL of 1M Tris buffer (pH 9.0).

2.25 Preparation of GM-CSF, sGMRα and ECDα protein-bead conjugates. Clinical grade GM-CSF and affinity purified sGMRα/ECDα were conjugated to NHS-activated Sepharose 4B as described in 2.23.

2.26 Protein affinity adsorption assays. A typical GM-CSF affinity adsorption assay was performed as follows. 100 µL of a 10% slurry of GM-CSF-beads were incubated at room temperature for 2 hours with 12 mL of BHK-21/sGMRα+ECDβc conditioned media or BHK-21/sGMRα conditioned media or 1 mL of Bm-5/ECDα conditioned media. The protein complexes bound to the beads were pelleted by centrifugation at 12,000 x g and washed in phosphate buffered saline containing Tween-20 (PBS-T, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 0.02% Tween-20). The protein-bead aggregates were then incubated for 12 hours at 4°C with either 12 mL of BHK-21/ECDβc conditioned media or 1 mL of Bm-5/ECDβc conditioned media. The beads were again pelleted and washed and the protein complexes were eluted from the beads by boiling in 2X-SDS-PAGE gel loading buffer containing 1.5M β-mercaptoethanol.

2.27 Immunoprecipitations. 1 mL of cell conditioned media was incubated with 10 µL of a 50% slurry of protein-A sepharose (Protein-A Sepharose Fast Flow, Pharmacia) in phosphate-buffered saline (PBS, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl) for 30 minutes at 4°C to reduce non-specific interactions. Receptor subunits were precipitated from conditioned media by incubation for 2 hours with 1 µg of either an anti-GMRα or anti-βc mAb (gifts of A. Lopez), respectively. The

immune complexes were immobilized on 30 μL of a 50% slurry of protein-A sepharose/PBS by rocking at 4°C for 12 hours. The immobilized proteins were pelleted from solution by centrifugation for 10 minutes at 12,000 x g and washed three times with 500 μL of PBS-T (PBS with 0.02% Tween-20). The proteins were eluted from the sepharose beads by boiling for 10 minutes in 2X SDS-PAGE loading buffer containing 1.5M β-mercaptoethanol.

2.28 ^{125}I-GM-CSF cell-associated receptor binding assays. Adherent cells were harvested, counted and washed as described. A ligand solution containing 5 pM to 10 nM of ^{125}I-GM-CSF alone or in combination with a 100 fold excess of unlabelled GM-CSF was prepared in receptor binding buffer (RPMI 1640, 1% BSA, 25mM HEPES, pH 7.4). 10^6 cells were incubated with 20 μL of either ligand solution for 1 hour at room temperature at which time 80 μL of ice cold binding buffer was added to each tube. Following a 15 minute incubation on ice, the samples were layered on a 100 μL cushion of phthalate oil (60:40 dibutyl phthalate:dinonyl phthalate) and centrifuged at 12,000 rpm for 6 minutes to separate free ligand from cell-associated ligand. The radioactivity of the cell pellet and supernatant was measured using a γ-counter.

2.29 ^{125}I-GM-CSF soluble receptor binding assays. Samples were incubated in receptor binding buffer with varying concentrations of ^{125}I-GM-CSF in the presence or absence of a 100 fold molar excess of unlabelled GM-CSF to a final volume of 50 μL. After one hour at room temperature the reaction was stopped with the addition of 50 μL of ice cold receptor binding buffer containing 20 mg/mL each of BSA and bovine IgG followed by 100 μL of ice-cold binding buffer containing 500 mg/mL polyethylene glycol 6000. The reaction was incubated for 15 minutes on ice, layered on a 100 μL cushion of phthalate oil (90% dibutyl phthalate:10% dinonyl phthalate) and centrifuged for 6 minutes at 12,000 rpm at 4°C. The receptor bearing pellets were separated from the supernatant by cutting off the bottom of the tube. The radioactivity of the pellet and the supernatant was measured on a γ-counter.

2.30 Cold saturation binding assays and Dixon analysis. Dissociation constants for receptor subunits were determined by Dixon analysis[236-238] of ^{125}I-GM-CSF soluble

receptor binding assay data performed using fixed concentration of radioligand and saturating concentrations of unlabelled GM-CSF. Briefly, soluble receptor binding assays were performed in the presence of two fixed concentrations of ^{125}I-GM-CSF (determined empirically as the concentration that provided a strong radioactive signal but remained low enough to allow complete competition using unlabelled GM-CSF) and 5 pM to 800 nM unlabelled GM-CSF. Non-specific binding of ^{125}I-GM-CSF (calculated as the number of bound counts remaining when the assay was performed in the presence of 800 nM of unlabelled GM-CSF) was subtracted from all results. The reciprocal of the number of specifically bound counts per minute was plotted against the concentration of unlabelled GM-CSF added to each tube (nM) according to the method of Dixon. The results of the experimental sets performed at different concentrations of ^{125}I-GM-CSF were plotted on the same graph. Regression analysis was performed on the linear portion of the lines and the point of intersection of the two lines was extrapolated to the x-axis. The absolute value of the extrapolated point of intersection was an estimate of the dissociation constant.

2.31 Fluorescent activated cell sorting (FACS) analysis. Adherent cells were harvested according to the method described. 10^6 cells were washed 3 times with 1 mL of phosphate buffered saline (PBS, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl). The cells were incubated for one hour at room temperature with 1 mL of a 1/2000 dilution of primary antibody (anti-GMRα mAb 8G6 or anti-βc mAb 1C1, gifts of A. Lopez)) or PBS alone. The cells were washed 3 times with PBS and incubated for one hour at room temperature with 4 μL of a fluoroisothiocyanate (FITC) conjugated goat-anti-mouse secondary antibody (Becton Dickinson, Mississauga, ON) in minimal volume. The cells were washed 3 times with PBS, resuspended in 1 mL PBS and the relative fluorescence was analyzed using a flow cytometer (Becton Dickinson).

CHAPTER 3
IDENTIFICATION OF THE NATURALLY OCCURING SOLUBLE GM-CSF RECEPTOR PROTEIN

3.1 Introduction

The ligand binding subunit of the GM-CSF receptor (GMRα) was cloned in 1989 from a human placental cDNA library[135]. GMRα is a 400 amino acid membrane spanning protein consisting of a 320 amino acid extracellular domain, a single membrane spanning domain and a short cytoplasmic domain. Using the available sequence data for GMRα a soluble variant of GMRα (sGMRα) was cloned from a human placental cDNA library[148]. The cloned cDNA shared a nucleotide sequence encoding 317 of the 320 residues of the extracellular domain of GMRα but was missing a 97 bp sequence that encoded the 26 residue transmembrane domain. Further, the excision of an odd number of nucleotides from the gene sequence generated a frameshift in the coding sequence that replaced the 54 residue cytoplasmic domain of GMRα with a unique set of 16 amino acids. Following cleavage of the 22 amino acid N-terminal signal sequence the putative soluble GMRα subunit was predicted to consist of 311 amino acids. The presence of alternative splice consensus sequences flanking the excised transmembrane exon suggested that sGMRα was a naturally occuring splice variant of GMRα.

The sGMRα transcript was later cloned from a human megakaryocytic leukemia cell line (MO-7e)[186], a human choriocarcinoma cell line (BeWo)[187], a myelomonocytic cell line (AM-193)[151] and from a human placental cDNA library[149]. RNase protection assays using MO-7e cells, BeWo cells, COS-7 simian kidney cells, JAR human choriocarcinoma cells, human bone marrow and placenta confirmed that the sGMRα transcript was naturally occurring and not a product of non-specific PCR amplification. Subcloning and expression of the sGMRα cDNA resulted in the presence of GM-CSF binding activity in the cell conditioned media demonstrating that the cloned cDNA likely encoded a soluble variant of GMRα. The difficulty in identifying GM-CSF binding activity in concentrated JAR conditioned media[187] suggested that the sGMRα protein, like GMRα, was normally

expressed at low levels. However, the expression of the sGMRα protein in another human choriocarcinoma cell line (JEG3) was confirmed indirectly the following year by chemical cross-linking of a soluble protein to ^{125}I-GM-CSF[188] that migrated as a 79 kDa protein complex by SDS-PAGE. Taking the molecular weight of yeast expressed GM-CSF to be 17 kDa the putative sGMRα protein would have a molecular weight of 62 kDa. Interestingly, the group was unable to demonstrate sGMRα expression from the human histiocytic lymphoma cell line U937 despite high level expression of GMRα on the cell surface[134] and the presence of the sGMRα transcripts[189]. It was therefore suggested that sGMRα may not normally be expressed in human hematopoietic cells[188].

Functional characterization of sGMRα began in 1994 with the observation that recombinant sGMRα (rsGMRα) can inhibit GM-CSF-mediated proliferation of human leukemic cell lines[151]. Further study revealed that rsGMRα could also antagonize binding of GM-CSF to human neutrophils and inhibit GM-CSF-induced bone marrow colony formation in vitro[149]. The presence of varying levels of sGMRα transcript in a number of cell types and human tissues[151,189] suggested that transcription of sGMRα was a regulated event. This hypothesis was strengthened following the observation that sGMRα transcription is regulated independently of GMRα transcription during HL60 cell differentiation[189]. However, in spite of the initial success in characterising the activities of rsGMRα in vitro the protein had yet to be identified directly as a normal expression product of hematopoietic cells. Therefore the purpose of this study was to determine if sGMRα was expressed by hematopoietic cells and if it was present in human peripheral blood.

3.2 Materials and Methods

3.2.1 Cell lines. Cell lines were obtained from the American Type Culture Catalogue (ATCC, Rockville, MY). The human promyelocytic leukemia cell line HL-60 was maintained in DMEM/F12 supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic (Gibco BRL, Life Technologies, Inc., Mississauga, ON). For some experiments, the cells were transferred to serum free media (OPTI-MEM, Gibco BRL,

Life Technologies, Inc.) 48 hours prior to experimentation in order to reduce the presence of contaminating proteins in the conditioned media. The human histiocytic lymphoma cell line U-937, the human monocytic cell line THP-1 and the human erythroleukemic cell line K-562 were maintained in RPMI/1640 supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic (Gibco BRL, Life Technologies, Inc.), 50 mM glutamine, 1mM sodium pyruvate, 1% non-essential amino acids and 50 μM β-mercaptoethanol. All cells were maintained in static culture in a humidified incubator at 37°C and 5% CO_2. Conditioned media was routinely harvested during the exponential phase of cell growth. Cell debris was removed by centrifugation at 6,000 rpm for 10 minutes. The clarified media was stored at -20°C.

3.2.2 Isolation and culturing of human neutrophils. Neutrophils from healthy donors were purified by dextran sedimentation, hypotonic lysis and Histopaque centrifugation as previously described[190]. Except for the dextran sedimentation step, which was performed at room temperature, the cells were kept at 4°C throughout the isolation procedure. Cell preparations contained greater than 95% neutrophils with greater than 99% viability using trypan blue dye exclusion. After isolation neutrophils were resuspended at a final concentration of 10^6 cells/mL in phosphate buffered saline (PBS, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl) and cultured at 37°C for 24 hours.

3.2.3 Normal human plasma. One unit of blood was drawn from two healthy volunteers and collected into heparanized tubes. Cells were removed by centrifugation at 1,000 rpm for 10 minutes. The plasma was further clarified prior to affinity purification by centrifugation at 16,000 rpm for 30 minutes.

3.2.4 Peripheral blood stem cell product (PBSCP). Plasma conditioned by human hematopoietic progenitor cells was obtained from peripheral blood stem cell (PBSC) products collected as previously described[191,192] from stem cell donors undergoing leukapheresis for autologous or allogeneic stem cell transplant. Briefly, normal donors for allogeneic transplants were administered recombinant granulocyte-colony stimulating factor (G-CSF, Filgrastim Neupogen, Amgen Inc., Thousand Oaks, CA), 5-10 μg/kg subcutaneously for 4 days prior to harvest. The donors for autologous transplant also

received G-CSF, 5-10 μg/kg subcutaneously starting on day 7 after the administration of systemic chemotherapy. In the autologous patients the G-CSF was continued for 4-14 days according to peripheral blood counts. The administration of G-CSF resulted in the proliferation of myeloid elements and the mobilization of large numbers of progenitor cells from the marrow into the peripheral blood. The PBSC product was collected by a 3-8 hour leukapheresis procedure during which the cells accumulated in a small volume of autologous plasma. The stem cell product was centrifuged at low speed and the cells cryopreserved for later transplantation. Our lab received the remaining supernatant, consisting of 50-200 mL of conditioned plasma which was stored at -80°C. Prior to use the plasma was centrifuged at 16,000 rpm for 30 minutes to remove insoluble matter. The clarified plasma was then applied to the GM-CSF-affinity column for purification.

3.2.5 Affinity adsorption using GM-CSF immobilized on agarose beads. GM-CSF was immobilized on agarose beads as in 3.23. 100 μL of a 10% slurry of GM-CSF-beads or mock-conjugated beads (in PBS) were incubated with 15-75 mL of cell conditioned media. The samples were incubated at 4°C and rocked for 12 hours. The beads and associated proteins were pelleted by centrifugation at 2,400 rpm for 10 minutes. The supernatant was discarded and the pellet was washed 3 times in 15 mL of PBS containing 1% Tween-20. After the final wash the beads were resuspended in 50 μL of SDS-PAGE loading buffer containing β-mercaptoethanol. The suspension was boiled for 5 minutes, centrifuged to pellet the beads and the supernatant was assayed by SDS-PAGE and Western blotting as described in 2.17 and 2.18. All Western blots were probed with the anti-GMRα monoclonal antibody 8G6 (a gift of A. Lopez).

3.2.6 GM-CSF affinity chromatography. Construction of the GM-CSF affinity column is described in detail in 2.24. Purification of conditioned media and plasma is described individually for each sample in their respective Results sections. The purified products were analyzed by soluble ^{125}I-GM-CSF binding assay as described in 2.29 and by SDS-PAGE and Western blotting with the anti-GMRα mAb 8G6 and a horse radish peroxidase conjugated rabbit anti-mouse secondary antibody as described in 2.17 and 2.18.

3.3 Results

3.3.1 HL-60 cells secrete a soluble GM-CSF binding protein. The sGMRα transcript has been extensively characterized in the human promyelocytic leukemia cell line HL-60[189] and it was therefore considered a possible source of sGMRα expression. 15 mL of serum free HL-60 conditioned media was incubated at with GM-CSF-agarose beads or mock-conjugated beads for 12 hours at 4°C. The beads were pelleted by centrifugation, washed extensively with PBS containing 1% Tween-20 (PBS-T) and boiled in SDS-PAGE loading buffer in the presence of the reducing agent β-ME. The eluent was analyzed by SDS-PAGE and Western blotting with the anti-GMRα mAb 8G6. A 55 kDa protein band corresponding in size to rsGMRα (Figure 3.1, lane 1) was adsorbed with the GM-CSF-beads (lane 3) but not with mock-conjugated beads (lane 4). Lane 2 was loaded with SDS-PAGE loading buffer only.

A GM-CSF-affinity column was used to purify a larger volume of HL-60 conditioned media. 200 mL of HL-60 conditioned media was applied to the affinity column and allowed to run through by gravity. The column was washed extensively with PBS and was eluted with 0.1M glycine buffer (pH 2.5) as 7 individual fractions of 1 mL each. 30 μL of each eluted fraction was analyzed by SDS-PAGE in the presence of β-ME and Western blotting with the anti-GMRα mAb 8G6. Two GM-CSF binding proteins were purified from the HL-60 conditioned media - a 48 kDa protein band that was clearly resolved in fractions 5-7 and a poorly resolved 55 kDa protein smear in fractions 3-6 (Figure 3.2). A constituent of fraction 5 showed specific ^{125}I-GM-CSF binding activity (data not shown) suggesting that a soluble GM-CSF binding proteins was secreted by the HL-60 cells. Of interest, when a larger aliquot of fractions 4-7 was pooled, volume reduced and re-analyzed by SDS-PAGE and Western blotting both the 55 kDa and the 48 kDa protein bands were clearly visible (Figure 3.3, lane 3). The larger protein band (p55) corresponded in size to mammalian expressed rsGMRα (Figure 3.3, lane 2, partially obscured by air bubble) while the smaller protein band (p48) corresponded in size to insect expressed rsGMRα (Figure 3.3, lane 1). The significance of this was not explored further. Taken together these results suggest that HL-60 cells can secrete a soluble GM-

CSF binding protein that is similar in size to rsGMRα and is specifically recognized by an anti-GMRα mAb.

3.3.2 THP-1 and U-937 cells but not K-562 cells secrete a soluble GM-CSF binding protein. To determine if sGMRα was also expressed by other leukemic cell lines the GM-CSF-adsorption assay was repeated using media conditioned by THP-1, U-937 and K-562 cell lines. 75 mL of conditioned media from each of the cell lines was incubated with GM-CSF-agarose beads or mock-conjugated beads for 12 hours at 4°C. The beads were pelleted by centrifugation and washed extensively with PBS containing 1% Tween-20. The adsorbed proteins were eluted from the beads by boiling in SDS-PAGE loading buffer containing 1.5M β-ME. The eluted protein were fractionated by SDS-PAGE in the presence of β-ME and analyzed by Western blotting with the anti-GMRα mAb 8G6. A 60 kDa protein band was isolated from the THP-1 conditioned media using the GM-CSF-beads but not the mock-conjugated beads (Figure 3.4, lanes 2 and 3 respectively). A faint 55 kDa protein band was isolated from the U-937 conditioned media with the GM-CSF-beads but not the mock-beads (Figure 3.4, lanes 4 and 5, respectively). No unique protein bands corresponding in size to rsGMRα were isolated from the K-562 conditioned media (Figure 3.4, lanes 6 and 7). A constituent of the THP-1 and U-937 but not the K-562 conditioned media could also specifically bind ^{125}I-GM-CSF in solution (data not shown). It is not clear why the GM-CSF binding protein isolated from the THP-1 conditioned media migrated as a 60 kDa protein band rather than the predicted 55 kDa protein. However, heterogeneous glycosylation of recombinant sGMRα and other soluble cytokine receptors has been observed and may account for the size discrepency.

The faintness of the putative sGMRα protein band isolated from the U-937 conditioned media did not correspond with the strength of the ^{125}I-GM-CSF soluble receptor binding assay signal (data not shown). To further characterize the potential of U-937 cells to express sGMRα, 1L of U-937 conditioned media was purified over the GM-CSF-affinity column. The column was washed extensively with PBS and the adsorbed proteins were eluted with 8 mL of 0.1M glycine buffer (pH 2.5). The eluent

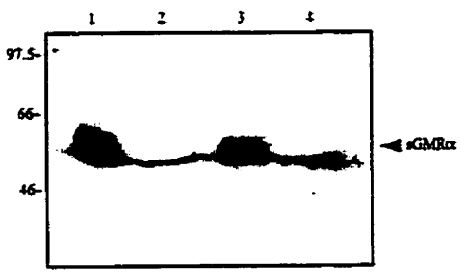
was collected into 8 fractions of 1 mL each and 30 μL of each fraction was analyzed by SDS-PAGE in the presence of β-ME and Western blotting with the anti-GMRα mAb 8G6. 25 μL aliqouts of each fraction were also analyzed for GM-CSF binding by ^{125}I-GM-CSF binding assay. A 55 kDa protein band corresponding in size to rsGMRα was purified from the U-937 conditioned media (Figure 3.5B, lanes 4-7). The elution of this protein band corresponded with the presence of ^{125}I-GM-CSF binding acitivity (Figure 3.5A, lanes 4-7). Taken together these results demonstrate that a soluble GM-CSF binding protein that is similar in size to rsGMRα is also secreted by THP-1 and U-937 leukemic cell lines but not by the K-562 erythroleukemic cell line.

3.3.3 Secretion of a soluble GM-CSF binding protein by cultured human neutrophils. Freshly isolated human neutrophils (200 x 10^6) were cultured in 200 mL of PBS at 37°C for 24 hours. The cell conditioned media was harvested and applied to the GM-CSF affinity column. The column was washed extensively with PBS and eluted with 10 mL of 0.1M glycine buffer (pH 2.5). 30 μL aliqouts of each fraction were analyzed for the presence of sGMRα by SDS-PAGE and Western blotting with the anti-GMRα mAb 8G6 and by ^{125}I-GM-CSF binding assay. No soluble GM-CSF binding protein could be detected in any of the fractions. To increase the sensitivity of the assay the remaining eluent from the GM-CSF affinity column was pooled and incubated with GM-CSF-agarose beads for 12 hours at 4°C. The beads were pelleted by centrifugation, washed extensively with PBS containing 1% Tween-20 and the adsorbed proteins were eluted from the beads by boiling them in SDS-PAGE loading buffer containing β-ME. The eluent was fractionated by SDS-PAGE and analyzed by Western blotting with the anti-GMRα mAb 8G6. A 55 kDa protein band was present in the neutrophil conditioned PBS (Figure 3.6, lane 2) that corresponded in size to rsGMRα (Figure 3.6, lane 1). There was not sufficient sample remaining to determine if the purified protein could also bind ^{125}I-GM-CSF in solution. These results suggest that cultured human neutrophils can secrete a soluble GM-CSF binding protein that is similar in size to rsGMRα and is recognized by the anti-GMRα mAb.

3.3.4 Identification of a soluble GM-CSF binding protein in human plasma. 440 mL of human plasma isolated from two healthy volunteers was applied to the GM-CSF-affinity column. The column was washed extensively with PBS and eluted with 6 mL of 0.1M glycine buffer (pH 2.5) into 6 individual fractions of 1 mL. The fractions were volume reduced to approximately 150 µL. 25 µL aliqouts of each fraction were analyzed by ^{125}I-GM-CSF binding assay (Figure 3.7A) and by reducing SDS-PAGE and Western blotting with mAb 8G6 (Figure 3.7B). A constituent of fractions 2-6 showed specific ^{125}I-GM-CSF binding activity with a peak of activity in fraction 4 (Figure 3.7A). A 60 kDa protein band corresponding in size to rsGMRα was present also present in fractions 2-6 but the pattern of immunological activity did not correspond with the pattern of ^{125}I-GM-CSF binding activity (Figure 3.7B). There was also a smeared 48 kDa protein band present in fractions 2-6 that may correlate with the pattern of GM-CSF binding seen in 3.7 A. These results suggest that a GM-CSF binding protein is present in normal human plasma and that it may correspond in size to rsGMRα.

3.3.5 Identification of a soluble GM-CSF binding protein in peripheral blood stem cell product (PBSCP) isolated from a leukemic individual. 200 mL of PBSCP that was isolated from a leukemic patient and was conditioned by hematopoietic progenitor cells was applied to the GM-CSF affinity column. The column was washed extensively with PBS and eluted with 9 mL of 0.1M glycine buffer (pH 2.5) into 9 individual fractions of 1 mL each. The eluted fractions were volume reduced and 25 µL aliqouts were analyzed by reducing SDS-PAGE and Western blotting with the anti-GMRα mAb 8G6. A distinct 60 kDa protein band corresponding in size to rsGMRα was present in fractions 3 and 4 (Figure 3.8) that corresponded with ^{125}I-GM-CSF binding activity (data not shown) suggesting that a GM-CSF binding protein that corresponds in size to rsGMRα is secreted by hematopoietic progenitor cells and is present peripheral blood stem cell products.

Figure 3.1. Secretion of a soluble GM-CSF binding protein by HL-60 cells. 15 mL of HL-60 conditioned media was incubated for 12 hours at 4C with GM-CSF-agarose beads or mock-conjugated beads. The beads were pelleted by centrifugation and washed extensively in PBS containing 1% Tween-20. The adsorbed proteins were eluted off the beads by boiling in SDS-PAGE loading buffer in the presence of 1.5M 2-mercaptoethanol. The eluent was analyzed by SDS-PAGE and Western blotting with the anti-GMRα mAb 8G6. 1: recombinant sGMRα; 2: SDS-PAGE loading dye; 3: Eluent from the GM-CSF-beads; 4: Eluent from the mock-conjugated beads.



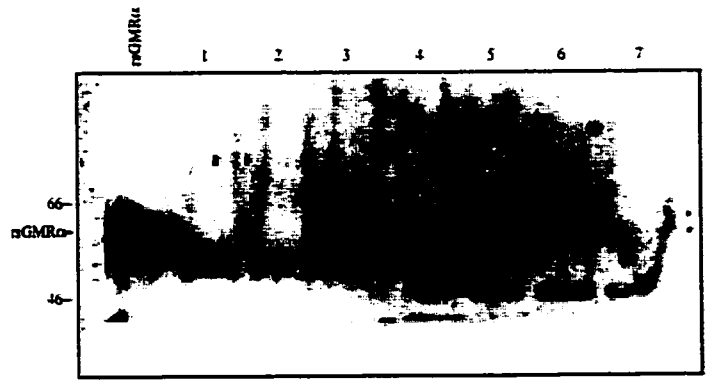


Figure 3.2. SDS-PAGE and Western blotting of proteins that were purified from HL-60 cell conditioned media using a GM-CSF affinity column. 200 mL of HL-60 conditioned media was applied to a GM-CSF-affinity column. The column was washed extensively with PBS and eluted with 7 mL of 0.1M glycine buffer (pH 2.5). The eluent was collected in 7 individual fractions of 1 mL each. 30 uL of each fraction (1-7) were fractionated by SDS-PAGE in the presence of 2-mercaptoethanol and analyzed for the presence of sGMRα by Western blotting with the anti-GMRα mAb 8G6.

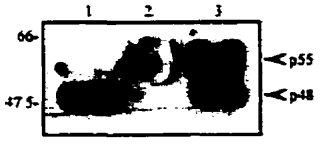


Figure 3.3. SDS-PAGE and Western blotting of a larger aliqout of fractions 4-7 from figure 4.2. A 30 uL aliqout of each of fractions 4-7 from figure 4.2 were pooled and volume reduced. The pooled eluent was fractionated by SDS-PAGE in the presence of 2-mercaptoethanol and analyzed by Western blotting with the anti-GMRα mAb 8G6. Insect expressed rsGMRα (lane 1) and mammalian expressed rsGMRα (lane 2, partially obscured by an air bubble) were analyzed along side the pooled eluent (lane 3) as size standards.

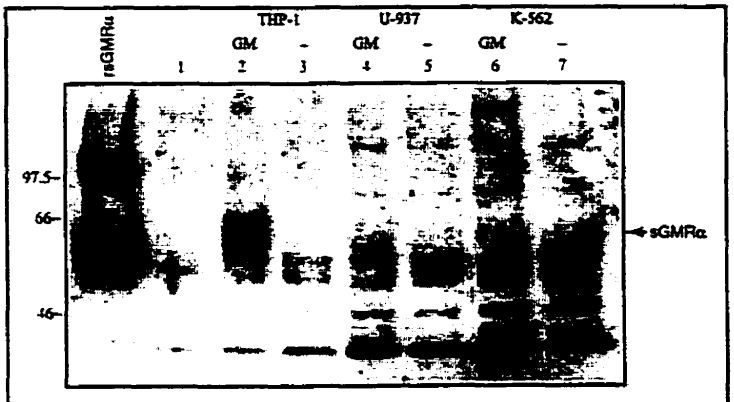


Figure 3.4. Secretion of a soluble GM-CSF binding protein by THP-1 and U-937 but not K-562 leukemic cell lines. 75 mL of THP-1, U-937 and K-562 cell conditioned media was incubated with either GM-CSF-agarose beads (2,4,6) or mock conjugated beads (3,5,7). The beads were pelleted by centrifugation and washed extensively with PBS containing 1% Tween-20. The adsorbed proteins were eluted from the beads by boiling in SDS-PAGE loading buffer containing 1.5M 2-mercaptoethanol. The eluted protein were analyzed by SDS-PAGE and Western blotting with the anti-GMRα mAb 8G6. 30 uL of recombinant sGMRα (rsGMRα) was analyzed along side the leukemic samples as a standard. 1: SDS-PAGE loading buffer. 2,3: THP-1; 4,5: U937; 6,7: K-562.

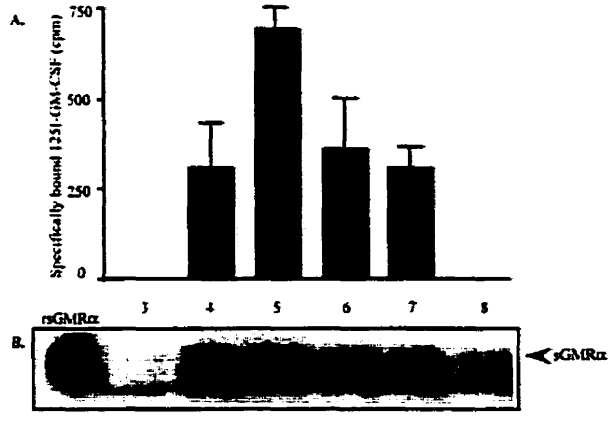


Figure 3.5. U-937 cells secrete a soluble GM-CSF binding protein. 1L of U-937 conditioned media was applied to the GM-CSF affinity column. The column was washed extensively with PBS and the adsorbed protein were eluted with 8 mL of 0.1M glycine buffer (pH 2.5). The eluent was collected into 8 individual 1 mL fractions. 30 uL of each of fractions 3-8 were analyzed by ^{125}I-GM-CSF binding assay (A) for the ability to bind GM-CSF in solution, and by reducing SDS-PAGE and Western blotting with anti-GMRα mAb 8G6 (B).

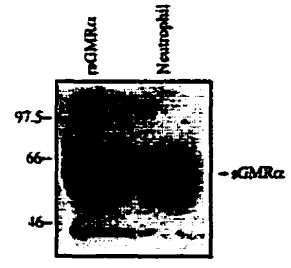


Figure 3.6. Cultured human neutrophils secrete a GM-CSF binding protein. Freshly isolated human neutrophils were cultured in PBS at 37C for 24 hours. The culture supernatant was applied to the GM-CSF affinity column. The column was washed extensively with PBS and eluted with 10 mL 0.1M glycine buffer (pH 2.5). The 10 mL eluent was then incubated with GM-CSF-agarose beads for 12 hours at 4C. The beads were pelleted by centrifugation and washed with PBS containing 1% Tween-20. The adsorbed proteins were eluted off the beads by boiling them in SDS-PAGE loading buffer containing 2-mercaptoethanol. The eluent was fractionated by SDS-PAGE and analyzed by Western blot with the anti-GMRα mAb 8G6.

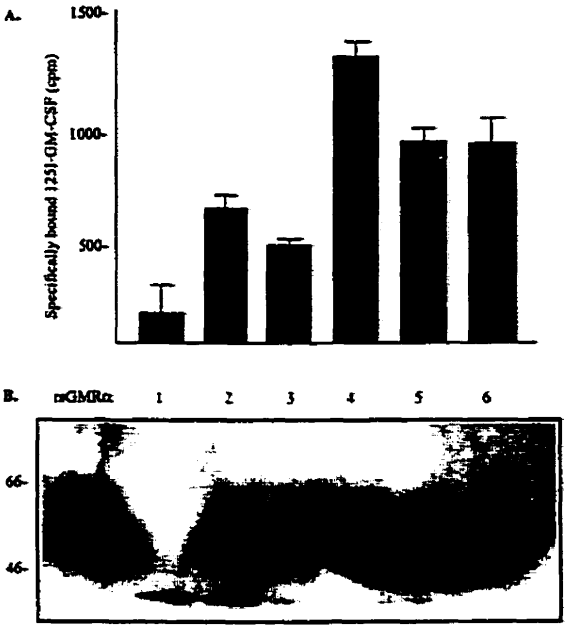


Figure 3.7. Identification of a GM-CSF binding protein in normal human plasma. 440 mL of human plasma was applied to the GM-CSF affinity column. The column was washed extensively with PBS and eluted with 6 mL of 0.1M glycine buffer (pH 2.5) into 6 individual fractions of 1 mL each. 25 uL of of each fraction was analyzed by 125I-GM-CSF binding assay (A) and by reducing SDS-PAGE and Western blotting with the anti-GMRα mAb 8G6 (B).

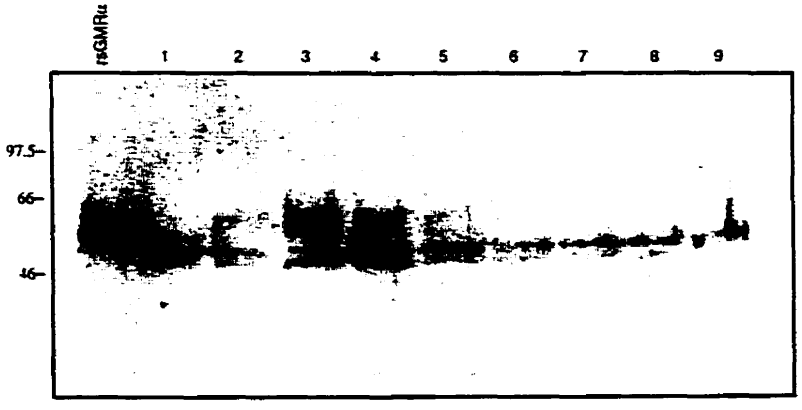


Figure 3.8. Identification of a soluble GM-CSF binding protein in the peripheral blood stem cell product from a leukemic patient. 200 mL of PBSCP that was conditioned by hematopoietic progenitor cells during leukapheresis was applied to the GM-CSF affinity column. The column was washed extensively with PBS and eluted with 9 mL of 0.1M glycine buffer (pH 2.5) as 9 individual 1 mL fractions (1-9). 25 uL of each fraction was analyzed by reducing SDS-PAGE and Western blotting with the anti-GMRα mAb 8G6.

3.4 Discussion

This chapter describes the identification of a naturally occuring soluble GM-CSF binding protein in the conditioned media of leukemic cell lines, human neutrophils, normal human plasma and the peripheral blood stem cell product of a leukemic individual. The isolated proteins were potentially identified as the soluble GMRα receptor (sGMRα) by their ability to adsorb to immobilized GM-CSF, by the ability of a highly specific anti-GMRα monoclonal antibody to recognize them by Western blot, and by their ability to specifically precipitate ^{125}I-GM-CSF from solution in a radioligand binding assay.

The precipitation of a 55 kDa protein band from HL-60 conditioned media using immobilized GM-CSF (Figure 3.1) suggested that a soluble GM-CSF binding protein similar to sGMRα was secreted by hematopoietic cells. The two different sized protein bands that were purified from HL-60 conditioned media (Figure 3.2.3) suggest that the soluble GM-CSF binding protein may also normally undergo differential glycosylation. Interestingly, the 48 kDa protein band corresponded in size to insect expressed rsGMRα which is believed to undergo only simple carbohydrate modification (see 4.3.2). The existence of naturally occurring carbohydrate variants of soluble cytokine receptors has been observed for both the soluble gp130 receptor and the soluble LIF receptor[193] and has also been observed for rsGMRα[149,194]. As both the larger and the smaller sized putative-sGMRα protein bands were purified by GM-CSF-affinity chromatography it is unlikely that any potential difference in glycosylation affects their ability to bind GM-CSF. However the possibility that the 48 kDa protein band is not a naturally occuring glycosylation variant but is instead a degradation product of the 55 kDa band that that has retained the ability to bind GM-CSF cannot be ruled out.

Two other human leukemic cell lines that express GMRα on their cell surface (the myelomonocytic leukemia cell line THP-1 and the histiocytic lymphoma cell line U-937)[134] and one that is believed not to express GMRα (the erythroleukemia cell line K-

562)[133,189] were analyzed for the secretion of sGMRα. A 60 kDa GM-CSF binding protein band was isolated from THP-1 conditioned media (Figure 3.4, lanes 2 and 3) that showed specific ^{125}I-GM-CSF binding activity (data not shown). The significance of the size difference between the GM-CSF binding protein secreted by the THP-1 cells and rsGMRα are unknown but could again be due to heterogeneous glycosylation. The faint 55 kDa band isolated from U-937 cell conditioned media (Figure 3.4, lanes 4 and 5) along with the ability of the isolated protein to bind ^{125}I-GM-CSF (data not shown) suggested that this cell line, previously believed not to secrete sGMRα[188], could secrete a soluble GM-CSF binding protein similar to rsGMRα. This was confirmed by the elution of a 55 kDa protein from the GM-CSF-affinity column (Figure 3.5A) that correponded in size to rsGMRα. Further, the eluted fractions showed a pattern of ^{125}I-GM-CSF binding activity (Figure 3.5B) that corresponded with the elution profile of the 55 kDa protein. In retrospect the U-937 cell line showed the highest level of secretion of the putatuve sGMRα protein of the cell lines tested (data not shown) which is consistent with the presence of a large number of sGMRα mRNA transcripts in these cells[189]. The absence of secretion of a GM-CSF binding protein by the K-562 cells (Figures 3.4, lanes 6 and 7) is consistent with the lack GM-CSF binding activity on their cell surface[133] and with the absence of either GMRα or sGMRα mRNA transcript in these cells[189].

Freshly isolated human neutrophils secreted a small amount of a GM-CSF binding protein that was similar in size to rsGMRα (Figure 3.6). This is consistent with the expression of GMRα on their cell surface[132,133] and their responsiveness to GM-CSF stimulation. Unfortunately there was not sufficient protein available to determine if the isolated protein could also bind ^{125}I-GM-CSF.

A soluble GM-CSF binding protein was purified from normal human plasma (Figures 3.7). SDS-PAGE and Western blotting of the purified protein with the anti-GMRα mAb 8G6 demonstrated that at least two sGMRα-like proteins were present in human plasma. The reason for the lack of correlation between the ^{125}I-GM-CSF binding activity (Figure 3.7A) and the immunological data (Figure 3.7B) is unknown. Perhaps the 60 kDa protein band visualized in Figure 3.7B is not sGMRα but is simply the result of non-specific

interaction with the anti-GMRα antibody. In this case the constituent of fractions 2-6 that shows specific ^{125}I-GM-CSF binding activity could be the 48 kDa protein band that was also present on the blot.

A soluble GM-CSF binding protein was purified from the peripheral blood stem cell product (PBSCP) of a leukemic individual that was undergoing leukaphersis for autologous stem cell transplantation (Figure 3.8). During the leukaphersis procedure the PBSCP was conditioned by hematopoietic progenitor cells suggesting that these cells can secrete a soluble GM-CSF binding protein that is similar in size to rsGMRα. However since a proportion of the cells that are harvested during the leukaphersis procedure are not progenitor cells but mature myeloid and lymphoid cells, the GM-CSF binding protein present in the PBSCP is not necessarily a product of the progenitor cells.

This report demonstrates that a soluble GM-CSF binding protein is secreted by a number of different cell types and is present in normal human plasma. While the soluble GM-CSF binding protein identified here is of similar size as sGMRα and is recognized by a highly specific monoclonal antibody raised against the shared extracellular domain of sGMRα and GMRα, the possibility remains that the proteins identified here are not the same a sGMRα but are instead different splice variants of GMRα or a unique GMRα cleavage product that is shed into the cell conditioned media. Monoclonal antibodies directed towards the unique 16 amino acid C-terminal domain of sGMRα are currently being raised in order to determine if the naturally occuring GM-CSF binding proteins identified in this study are the same as sGMRα.

The physiological role of sGMRα is currently unknown. *In vitro* data suggest that sGMRα acts as an antagonist of GM-CSF activity by binding to it and preventing it from activating cell surface GM-CSF receptors. This evidence comes from the observation that an exogenous source of sGMRα inhibits GM-CSF-mediated cell proliferation[151] and bone marrow colony formation[149]. However the data presented in this manuscript would suggest that, considering the 100 fold lower affinity of sGMRα for GM-CSF when compared with the high affinity cell surface receptor, that sGMRα is expressed at too low a concentration to be an effective antagonist *in vivo*. On the other hand the concentration

of sGMRα could be substantially higher than what was observed here if it were expressed in a localized environment. Indeed the majority of cytokines and soluble cytokine receptors are expressed at very low levels and remain highly functional suggesting they are present in substantially higher concentrations in the localized environment where they exert their function. Further, the observation that sGMRα can be retained on the cell surface in association with βc and can form a high affinity GM-CSF receptor complex[150,152] suggests that sGMRα may exert antagonist activity by remaining localized to the membrane surface of the cell it was expressed from. However despite the apparent requirement of the cytoplasmic domain of GMRα for signal transduction[195,196], the potential for a sGMRα/βc complex to be a functional GMR complex that acts as a GM-CSF agonist rather than antagonist can not be ruled out.

Another potential mechanism that sGMRα may "use" to increase its ability to antagonize GM-CSF activity is by associating with other soluble molecules that can increase its affinity for GM-CSF. The extracellular domain of βc can associate with a soluble variant of the GMRα receptor in solution to form a GM-CSF binding complex that has a 5-20 fold higher affinity for GM-CSF than sGMRα alone (see Chapter 5). Thus a soluble variant of βc could act as a potentiator of sGMRα anatagonist activity. A recent report has isolated soluble variants of βc mRNA from human monocytes[234] suggesting that a soluble variant of βc may exist in vivo. Indeed the ability of a soluble variant of a non-ligand binding, signal transducing subunit to potentiate the activities of a soluble cytokine receptor are well characterized in the IL-6/gp130 system where a natuarally occuring soluble variant of gp130 can increase the affinity of soluble IL-6 receptor for IL-6[193] and prevent it from activating cell surface gp130 receptors[197].

A third possibility is that the sGMRα expression levels observed in this investigation do not represent the protein levels that are present during the period when sGMRα would be expected to function. That is sGMRα expression may normally be upregulated in response to specific stimuli that allow it to function as an effective GM-CSF anatagonist both locally and perhaps systemically. Our group has recently observed increased expression of sGMRα in the plasma of acute myelomonocytic leukemia patients (300-

500 pM) compared with healthy individuals (30-50 pM) demonstrating the potential for a substantial increase in sGMRα. On the other hand GM-CSF itself does not appear to upregulate sGMRα expression in a megakaryocytic cell line (MO7e)[151] suggesting that upregulation of sGMRα expression may not be a mechanism of moderating GM-CSF levels in the pericellular milieu. Similarly terminal differentiation of HL-60 cells is not associated with an increase in sGMRα mRNA levels in spite of a 20-50 fold increase in GMRα receptor expression[189]. Thus upregulation of sGMRα expression does not appear to play a role in maturation of myeloid precursors.

Finally sGMRα may not have to compete with a higher affinity cell surface receptor for GM-CSF binding. In chapter 5, I demonstrate that the GM-CSF receptor complex assembles in a step-wise manner where GM-CSF must first associate with GMRα before it can bind βc to form the higher affinity complex. Since sGMRα and GMRα have the same affinity for GM-CSF, sGMRα could conceivably compete equally well with GMRα for GM-CSF binding thereby acting as an effective antagonist. Therefore, whether by localized expression, potentiation by another molecule or upregulation the potential for sGMRα to act as an effective antagonist of GM-CSF activity exists. Whether any or all of these mechanisms are involved in normal sGMRα activity needs to be investigated.

CHAPTER 4
ENGINEERING, EXPRESSION AND PRELIMINARY CHARACTERIZATION OF SOLUBLE ISOFORMS OF THE GMRα AND βc RECEPTOR SUBUNITS

4.1 Introduction

Assembly and activation of the GM-CSF receptor complex (GMR) is a multistep process. In the absence of GM-CSF, the GMR is believed to exist as a co-localized aggregate of free and/or weakly-associated[198-200] GMRα and βc subunits. The presence of ligand is believed to drive the sequential association of GM-CSF with GMRα and βc resulting in a tightly-associated heteromeric complex of GMRα, GM-CSF and βc[143,201]. While formation of this complex is required for signal transduction, the mechanism by which it assembles and the domains of each subunit that are required for complex formation are unclear.

Experiments designed to deal with these issues have focused on analysis of the GMR complex on the cell surface[153,155-159,195,198-200,202-206]. The advantages of this approach are obvious as this situation best represents a physiological environment. However analysis of ligand/receptor interactions on the cell surface often requires cell lysis and solubilization of the plasma membrane to gain access to the individual subunits. Detergent solubilization can disrupt molecular interactions that may normally be present between proteins on the cell surface. For this reason chemical cross-linking of cell surface proteins has become a favored method of stabilizing protein-protein interactions prior to solubilization. Unfortunately the use of chemical cross-linking reagents can overestimate the importance of an individual interaction by stabilizing interactions that may normally be weak and/or transient in nature. Thus while characterization of protein-protein interactions using cell surface expression systems is the experimental model of choice other strategies are also needed to identify other interactions that can potentially mediate receptor complex assembly.

One such strategy involves expressing the extracellular domains of cell surface proteins in solution and analyzing their interactions in solution phase assays. The rationale for this approach is that for many cell surface proteins the interactions that

mediate complex formation are driven almost exclusively by the extracellular domains of the protein. That is, all the regions necessary to recapitulate normal ligand/receptor interactions are often contained within the extracellular domain. Solution phase modelling of cytokine receptors has been used successfully for studying structure/function relationships for other members of the cytokine receptor superfamily such as the IL-6 receptor[197,207-214], the IL-5 receptor[215-217] and the EPO receptor[218-224]. Solution phase assays can be performed under physiological buffer conditions and the resultant protein complexes can be assayed without need for detergent solubilization. This method preserves the intergrity of subtle interactions that can be sensitive to detergent lysis conditions. In addition, solution phase assays are amenable to quantitative analysis as the concentration of individual constituents can be manipulated. Finally powerful biophysical techniques for elucidation of structure/function relationships such as x-ray crystalography, analytical ultracentrifugation and biosensor analysis all require soluble reagents. Therefore the use of a solution based strategy for analyzing protein interactions in addition to conventional cell surface analysis can provide complementing information to better understand the nature of receptor/ligand interactions.

This chapter describes the engineering, expression and preliminary characterization of soluble isoforms of GMRα and βc that consist solely of the extracellular domains of their cognate cell surface counterparts. The reagents developed in this study provide the foundation for experiments in the following chapter that deal with unresolved questions concerning protein-protein interactions involving the GMR complex and the sGMRα subunit, and demonstrate the order of assembly of the GMR complex. Together these two studies demonstrate the applicability of this experimental approach for studying questions that can not be easily addressed through examination of cell surface interactions.

4.2 Materials and Methods

4.2.1 Engineering soluble variants of GMRα. The recombinant DNA procedures used for the engineering of the soluble constructs are described in detail in Chapter 2 and are referenced in parentheses in the following sections. Four soluble isoforms of GMRα

were engineered by PCR mutagenesis (2.1) using λgt-11.sGMRα[149] as a template. Each mutant consisted of a portion of the extracellular domain of GMRα (ECDα), where each mutant was truncated at a position within 4 amino acids of the putative transmembrane domain of GMRα[135]. ECDα^{317}, which was truncated following serine^{317}, the last residue encoded by the sGMRα cDNA prior to the unique 16 amino acid C-terminal domain[148,151,187], was amplified using *Pfu* polymerase and the following synthetic oligonucleotides: (1) 5'-AT ACA GTC AAG CTT AGC ACC ATG CTT CTC CTG GTG AC-3' that starts 20 nucleotides 5' of the ATG start site and contains an engineered HindIII restriction site (AAG CTT); (2) 5'-CTA TCA GGA ACC AAA TTC AAT GGC TTC ACT CCA-3' that contains two inframe termination codons immedietly 3' of serine^{317}. ECDα^{318}, which was truncated following aspartate^{318} , was constructed using *Pfu* polymerase and the following unique synthetic oligonucleotide: 5'-CTA TCA GTC GGA ACC AAA TTC AAT GGC TTC ACT-3' which generated a unique aspartate residue and two stop codons immedietly 3' of the serine^{317} codon. ECDα^{319} was truncated following aspartate^{319} of the GMRα sequence by PCR amplification using *Taq* polymerase and the following synthetic oligonucleotide: 5'-CTA TCA GTC GTC GGA ACC AAA TTC AAT GGC TTC-3' which generated two aspartates and two stop codons following the last residue encoded by the sGMRα open reading frame. Finally, ECDα^{320} was truncated following glycine^{320}, believed to be the last residue within the extracellular domain of GMRα before the transmembrane domain[135], by PCR mutagenesis using *Taq* polymerase and the following synthetic oligonucleotide: 5'-CTA TCA CCC GTC GTC GGA ACC AAA TTC AAT GGC-3' which encoded two aspartates and one glycine residue and two stop codons immedietly 3' of the last residue encoded by the sGMRα open reading frame.

ECDα^{317} (971 bp) and ECDα^{318} (974 bp) were purified (2.2,3) and ligated (2.4) into the SrfI site of the pCR-Script Amp SK(+) cloning vector (Stratagene, La Jolla, CA) using T4 DNA ligase (Gibco BRL, Life Technologies, Inc., Mississauga, ON). ECDα^{319} (977 bp) and ECDα^{320} (980 bp) were purified and ligated into the pCR-II cloning vector (TA cloning kit, Invitrogen, Carlsbad, CA) using T4 DNA ligase. DH5α cells

(Invitrogen) were transformed (2.5) with the recombinant plasmids and plated. Positive colonies were used to innoculate overnight cultures from which plasmid DNA was later purified (2.6). The presence of the cDNA insert in the plasmid was confirmed by restriction digest (2.7) with HindIII and NotI (ECD$\alpha^{317/318}$) or EcoRI (ECD$\alpha^{319/320}$) followed by agarose electrophoresis. The insertion of the ECDα^{317}, ECDα^{318}, ECDα^{319} and ECDα^{320} fragments into the pCR-Script and pCR-II vectors in the correct orientation was confirmed by restriction digest. The sequence of pCR-Script.ECDα^{317}, pCR-Script.ECDα^{318}, pCR-II.ECDα^{319} and pCR-II.ECDα^{320} was confirmed by DNA sequencing (2.8).

ECDα^{317} and ECDα^{318} were excised from pCR-Script with HindIII and NotI and subcloned into the HindIII/NotI site of the pRC/CMV mammalian expression vector (Stratagene). ECDα^{319} and ECDα^{320} were excised from pCR-II with BstXI and subcloned into the BstXI site on pRC/CMV. DH5α cell were transformed with the recombinant expression plasmids and plated. Overnight cultures were grown from individual colonies and the presence of the proper insert was confirmed by digestion of purified plasmid DNA with HindII/NotI (ECD$\alpha^{317/318}$) or BstXI (ECD$\alpha^{319/320}$). Maxipreps (2.9) were performed for each positive clone to isolate a large quantity of recombinant plamid. The integrity of plasmids were analyzed once more by digestion and electrophoresis and were quantified and frozen.

ECDα317 was also subcloned into the pIE/153A insect expression vector[225] to generate pIE/153A.ECDα. Briefly, the ECDα317 insert was excised from pRC/CMV.ECDα with BamHI/NotI and purified by agarose gel electophoresis. ECDα was ligated into the BamHI/NotI site of pIE/153A to produce pIE/153A.ECDβc. DH5α cells were transformed and plated. Individual colonies were expanded in overnight cultures. Plasmid DNA was purified and the presence of the insert was confirmed by digestion with BamHI/NotI. pIE/153A.ECDα suitable in quantity and purity for cell transfection was prepared.

4.2.2 Engineering a soluble variant of βc. A soluble receptor consisiting of the extracellular domain of βc (ECDβc) was engineered by PCR mutagenesis using

pREP4.βc$^{1-54}$ as a template and the following synthetic oligonucleotides: (1) 5'-ATG GTG CTG GCC CAG GGG CTG CTC-3' which begins at the start site of the open reading frame of βc$^{1-41}$, and (2) 5'-CTA TCA CGA CTC GGT GTC CCA GGA GC-3' which introduces 2 stop codons immedietly following the last codon before the transmembrane domain of βc. The amplified DNA fragment was purified and ligated into the SrfI site of pCR-Script Amp SK(+). DH5α cell were transformed with pCR-Script.ECDβc and plated. Overnight cultures were grown using positive colonies as the innoculant. The presence of the ECDβc cDNA insert was confirmed by digestion of the purified plasmid with NotI an EcoRI followed by agarose eletrophoresis. The correct cDNA sequence for ECDβc was confirmed by DNA sequencing.

ECDβc was excised from pCR-Script with HindIII and NotI and was ligated into the HindIII/NotI site of pRC/CMV. DH5α cells were transformed and plated. Individual colonies were expanded in overnight cultures. The presence of the ECDβc insert was confirmed by digestion with HindIII/NotI. Insertion of ECDβc into pRC/CMV in the correct orientation was confirmed by digestion with AccI yielding three individual fragments of unique length. pRC/CMV.ECDβc suitable in quantity and purity for cell transfection was prepared.

ECDβc was also subcloned into the pIE/153A insect expression vector to generate pIE/153A.ECDβc. Briefly, the ECDβc insert was excised from pRC/CMV.ECDβc with BamHI/NotI and purified by agarose gel electophoresis. ECDβc was ligated into the BamHI/NotI site of pIE/153A to produce pIE/153A.ECDβc. DH5α cells were transformed and plated. Individual colonies were expanded in overnight cultures. Plasmid DNA was purified and the presence of the insert was confirmed by digestion with BamHI/NotI. pIE/153A.ECDβc suitable in quantify and purity for cell transfection was prepared.

4.2.3 Transfection and selection of stable cell lines. The procedure for calcium phosphate and lipid mediated transfection of mammalian and insect cell lines is detailed in 2.12 and 2.13, respectively.

BHK-21 cells (ATCC, American Type Culture Catalogue, Rockville, MY) were transfected with pRC/CMV.ECDα or pRC/CMV using the calcium phosphate precipitation method. The cells were cultured in the presence of the neomycin analog G418 (Sigma) to select for those cells that incorporated the plasmid DNA. Surviving cell colonies were individually subcultured and expanded in the presence of G418. Expression of ECDα was assessed by ^{125}I-GM-CSF soluble receptor binding assay of cell conditioned media and a stable clonal population was established.

Bm-5 cells (ATCC) were transfected with pIE/153.ECDα or pIE/153A using Lipofectin (Gibco BRL Life Technologies Inc.). The cells were cultured in the presence of hygromycin (Sigma) to select for those cells that incorporated the plasmid DNA. Expression of ECDα was assessed by ^{125}I-GM-CSF soluble receptor binding assay of cell conditioned media and a stable cell line was selected by limiting dilution of the transfected cell population.

BHK-21 cells were transfected with pRC/CMV.ECDβc or pRC/CMV using the calcium phosphate method. Cells were cultured in the presence of G418 to select for those cells that incorporated the plasmid DNA. Expression of ECDβc was confirmed by SDS-PAGE and Western blot of cell conditioned media. The BHK-21/ECDβc cells were maintained as a polyclonal population.

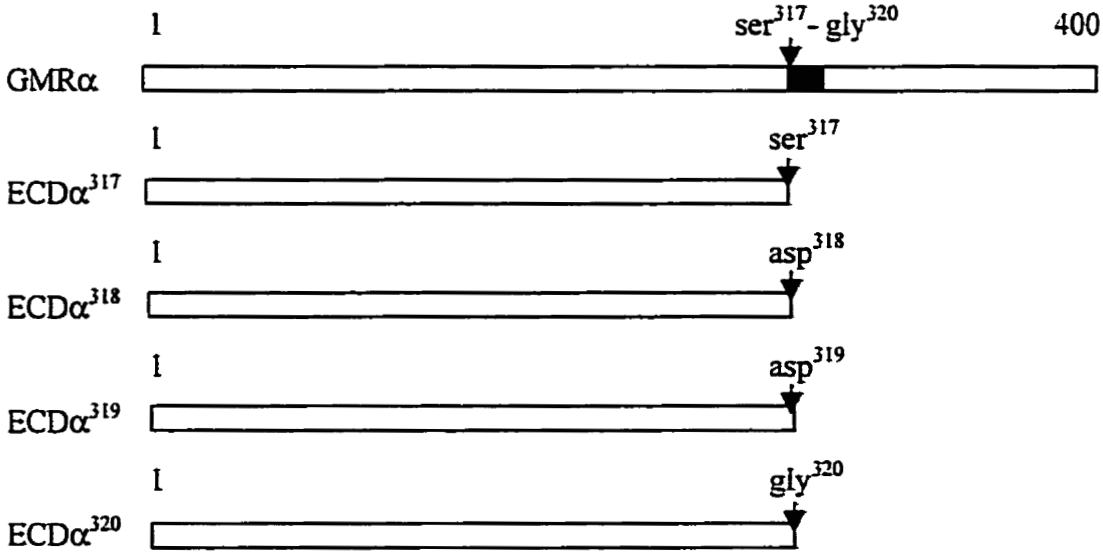
Bm-5 cells were transfected with pIE/153A.ECDβc or pIE/153A using Lipofectin. Transfected cells were selected for resistance to hygromycin. A clonal population of ECDβc expressing cells was established by limiting dilution and the highest ECDβc expressing populations were identified by SDS-PAGE and Western blotting of cell conditioned media.

4.2.4 Characterization of the soluble receptor isoforms. Concanavalin A-sepharose and wheat germ agglutinnin-sepharose adsorption assays were performed as described in 2.26 using ConA-Sepharose and Wheat Germ Agglutinin-Sepharose (Pharmacia Biotech, Baie d'Urfe, PQ) as the adsorption agents. Immunopreciptations, SDS-PAGE and Western blotting were described in detail in sections 2.27, 2.17 and 2.18 respectively. Western blotting was performed with the anti-GMRα mAb 8G6 and with

the anti-βc mAb 1C1 (gifts of A. Lopez) and a rabbit anti-mouse horse radish peroxidase conjugated secondary antibody. Soluble ^{125}I-GM-CSF binding assays and Dixon analysis of cold saturation binding data were described in 2.29 and 2.30, respectively. FACS analysis was described in detail in 2.31; the anti-βc mAb 1C1 was used for FACS analysis of cell surface retention of the ECDβc subunit.

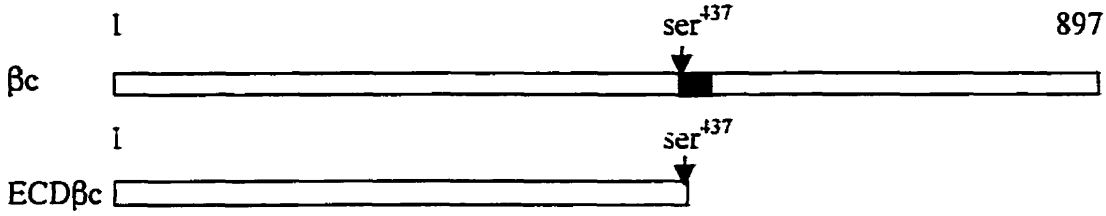
4.3 Results

4.3.1 Construct design. Soluble isoforms of GMRα and βc were constructed by PCR mutagenesis. Four different soluble isoforms of GMRα, truncated following either serine^{317}, aspartate^{318}, aspartate^{319} or glycine^{320}, were amplified by PCR using λgt-11.sGMRα$^{1\text{-}49}$ as a template. ECDα$^{317/318}$ were ligated into the pCR-Script Amp SK(+) cloning vector and ECDα$^{319/320}$ were ligated into the pCR-II cloning vector where the sequence was confirmed by DNA sequencing. All four ECDα fragments were subcloned into the pRC/CMV mammalian expression vector for transfection of BHK-21 cells. ECDα^{317} was also subcloned into the pIE/153A insect expression vector for the transfection of Bm-5 cells.



The extracellular domain of βc was amplified by PCR using pREP4.βc$^{1\text{-}54}$ as a template. The amplified fragment was ligated into the pCR-Script Amp SK(+) cloning

vector where the sequence was confirmed by DNA sequencing. The fragment was subcloned into the pRC/CMV mammalian expression vector and the pIE/153A insect expression vector for transfection of BHK-21 and Bm-5 cells, respectively.



4.3.2 Expression and characterization of ECDα. BHK-21/sGMRα, BHK-21/ECDα and mock-transfected BHK-21 cell conditioned media was immunoprecipitated with the anti-GMRα mAb 8G6 and analyzed by SDS-PAGE and Western blotting with 8G6. The high level of protein expression by the Bm-5/ECDα cell line allowed for direct characterization of the conditioned media by SDS-PAGE and Western blotting with 8G6. A 55 kDa protein band was isolated from the conditioned media of the BHK-21/ECDα cell line but not the mock transfected BHK-21 cell line (Figure 4.1, lanes 2 and 3, respectively). The 55 kDa band corresponded in size to sGMRα (Figure 4.1, lane 1). A 48 kDa protein band was also present in the conditioned media of the Bm-5/ECDα cell line (Figure 4.1, lane 4). A 55 kDa protein band could also be isolated from BHK-21/ECDα conditioned media with both ConA-sepharose and WGA-sepharose; the 48 kDa protein expressed by the Bm-5/ECDα cell line could only be adsorbed with ConA-sepharose (data not shown).

Dixon analysis of saturation binding data derived from ^{125}I-GM-CSF soluble receptor binding assays showed that a constituent of the BHK-21/ECDα conditioned media could bind ^{125}I-GM-CSF with the same affinity as sGMRα and GMRα (Figure 4.2A, Kd=3.3 nM). A constituent of the Bm-5/ECDα conditioned media bound ^{125}I-GM-CSF with a similar affinity (Figure 4.2B, Kd=2.8 nM).

4.3.3 Expression and characterization of ECDβc. BHK-21/ECDβc and mock-transfected BHK-21 cell conditioned media and whole cell lysates from a cell line expressing the full length cell surface βc subunit (BHK-21/βc)^{150} were

immunoprecipitated with the anti-βc mAb 1C1. The precipitate was fractionated by SDS-PAGE and analyzed by Western blotting with 1C1. A 55 kDa protein band was present in the conditioned media of the BHK-21/ECDβc cell line but not the mock-transfected BHK-21 cell line (Figure 4.3, lanes 1 and 2, respectively). A 135 kDa protein band was present in the immunoprecipitate of the BHK-21/βc whole cell lysate corresponding to the full length cell surface βc subunit (Figure 4.3, lane 3). ECDβc was expressed entirely in solution as it could not be detected on the cell surface of BHK-21/ECDβc cells by flow cytometry using the anti-βc mAb 1C1 (data not shown).

Bm-5/ECDβc conditioned media was analyzed by SDS-PAGE and Western blotting with 1C1 in the absence (Figure 4.4, lanes 1-4) or presence (lanes 5-8) of β-mercaptoethanol. A 48 kDa protein band was present in the conditioned media of the Bm-5/ECDβc cell line (lane 1) but not the Bm-5/ECDα cell line (lane 2), mock-transfected Bm-5 cells (lane 3) or in the IPl-41 cell culture media (lane 4). In the absence of β-mercaptoethanol a faint 135 kDa band was also present along with the 48 kDa protein band in the conditioned media of the Bm-5/ECDβc cell line (lane 5), but not the Bm-5/ECDα conditioned media, mock-Bm-5 cell line conditioned media or IPl-41 cell culture media (lanes 6-8, respectively).

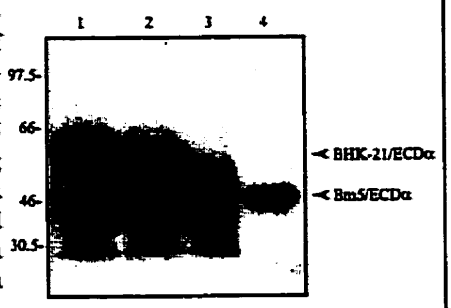


Figure 4.1. Expression of ECDα by BHK-21/ECDα and Bm5/ECDα cell lines. 1 mL of BHK-21/sGMRα (1), BHK-21/ECDα (2) or sham-transfected BHK-21 cell (3) conditioned media was immunoprecipitated with anti-GMRα mAb 8G6 and analyzed by SDS-PAGE and Western blotting with 8G6. 10 uL of Bm5/ECDα conditioned media (4) was analyzed directly by SDS-PAGE and Western blotting. The IgG heavy chain is present in lanes 1-3 at the same postion as Bm-5/ECDα in lane 4.

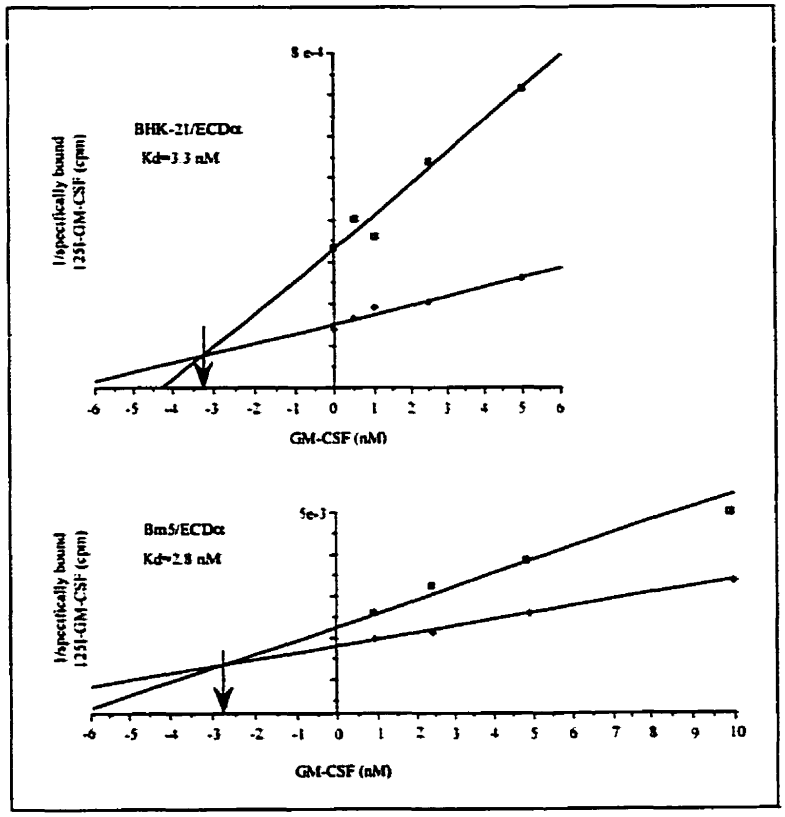


Figure 4.2. Constituents of BHK-21/ECDα and Bm-5/ECDα conditioned media bind GM-CSF with similar affinities. BHK-21/ECDα (A) and Bm-5/ECDα (B) conditioned media was subject to GM-CSF saturation binding assays using two different concentrations of 125I-GM-CSF as a label. The concentration of unlabelled GM-CSF added to the reaction mixture was plotted against the inverse of the concentration of specifically bound 125I-GM-CSF (counts per minute) according to the method of Dixon. The dissociation constant was estimated by extrapolation of the intersection of the lines generated by linear regression to the x-axis (nM).

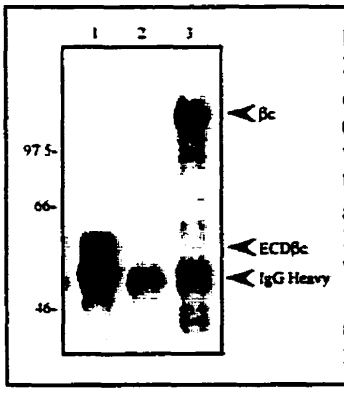


Figure 4.3. Expression of ECDβc by BHK-21/ECDβc cells. Cell conditioned media (1,2) or whole cell lysates (3) were immunoprecipitated with the anti-βc mAb 1C1 and analyzed by reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1. 1:BHK-21/ECDβc;2:mock-transfected BHK-21; 3:BHK-21/βc.

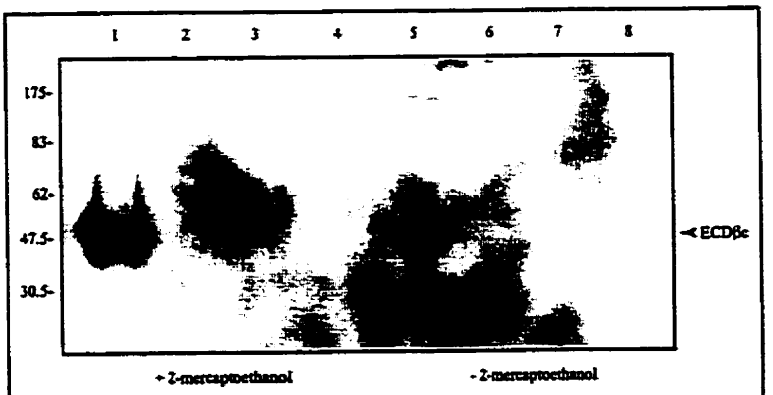


Figure 4.4. Expression of ECDβc by Bm-5/ECDβc cells. 10 uL of Bm-5/ECDβc conditioned media (1,5), Bm-5/ECDα conditioned media (2,6), mock-transfected Bm-5 conditioned media (3,7) or IPL-41 cell culture media (4,8) was analyzed by SDS-PAGE and Western blotting with the anti-βc mAb 1C1 in the presence and absence of 2-mercaptoethanol.

4.4 Discussion

4.4.1 Rational for construct design. The purpose of this study was to generate soluble isoforms of the GMRα and βc subunits for the GM-CSF receptor so that the interactions that mediate receptor complex assembly could be studied in solution. The interactions that mediate ligand binding and formation of the high affinity functional GMR complex are believed to be mediated through the extracellular domains of GMRα and βc. For example the low affinity interaction of GMRα and GM-CSF is believed to be mediated though the hinge region of GMRα located within the extracellular domain of GMRα[143,202]. Indeed sGMRα, which lacks both the transmembrane and cytoplasmic domains of GMRα but is otherwise identical in sequence, can bind GM-CSF with the same affinity as GMRα[149,150,152]. Similarly, loss of the cytoplasmic domain of GMRα has no effect on low affinity GM-CSF binding[154,195]. The cytoplasmic domain of βc is also not required for high affinity interaction with GMRα and GM-CSF presumably because βc interacts with GM-CSF through the hinge region of its extracelluluar domain[226,227] and with GMRα through reactive sulfhydryl residues located within the membrane distal region of its extracellular domain[153]. Thus the extracellular domains of GMRα and βc appear to contain all the necessary domains for assembly of a high affinity receptor complex.

The location of the putative transmembrane domains of GMRα and βc, and therefore the location chosen to truncate the mutant subunits, are reasonably well defined. Exon 11 of GMRα encodes a stretch of 32 amino acids that are believed to consist of the transmembrane domain of GMRα[135] since removal of this exon during processing of the GMRα pre-mRNA results in loss of the transmembrane domain and secretion of the soluble form of GMRα (sGMRα)[148,149,151,186,187]. sGMRα, identical in sequence to the extracellular domain of GMRα up to and including serine[317], appears to include the entire extracellular domain of GMRα. However, hydrophobicity plots[135] of the amino acid sequence of GMRα predict the transmembrane domain to begin following glycine[320] rather than serine[317]. For this reason, distinct GMRα mutants were constructed that were

truncated following one of the four residues preceding the putative transmembrane domain (serine^{317}, aspartate^{318}, aspartate^{319}, glycine^{320}). ECDα^{317} was selected for expression and preliminary characterization as it is identical in sequence to sGMRα except that it does not encode the unique 16 amino acid C-terminal domain thereby allowing identification of molecular interactions that are mediated by the common ligand binding domain of GMRα and sGMRα.

Hydrophobicity plots of the amino acid sequence of βc[141] predict the transmembrane domain to begin following serine^{437}. As there was no evidence to suggest that the transmembrane domain was at a residue other than this one ECDβc was constructed by amplifying only the region encoding these first 437 residues to give a mature peptide of 421 amino acids.

4.4.2 Protein expression systems. Glycosylation of the extracellular domain of GMRα is critical for its activity[228,229] necessitating the use of higher eukaryotic expression systems that are capable of carbohydrate modification of the translated peptide. While the importance of carbohydrate modification for the function of βc is unknown, for simplicity sake the same expresion systems were employed for production of ECDβc.

The pRC/CMV mammalian expression vector uses the cytomegalovirus promotor/enhancer system for driving protein expression and the neomycin resistance gene for selection of transfectants. Coupled with the BHK-21 cell line, an adherent rodent fibroblast line capable of high level protein expression and complex carbohydrate modification, this expression system proved highly effective for expression of both ECDα and ECDβc. Unfortunately, the expression levels of this system were not sufficiently high to allow for direct analysis of the recombinant proteins in cell conditioned media. This required concentration of the recombinant protein in the cell conditioned media by either immunoprecipitation, ultrafiltration or affinity purification prior to use. This additional step prompted us to examine the usefullness of a novel insect expression system[225] that purportedly expressed 10-100 fold more protein per unit volume than most mammalian expression systems. Importantly, unlike the baculovirus expression systems which, due to the lytic cycle of the virus, results in only transient

protein expression the pIE/153A expression vector could be used to generate stable cell lines for long term production of recombinant protein. Protein expression in the pIE/153A vector is driven by the silkmoth actin promotor and selection is accomplished using hygromycin. This vector has been coupled with a lepidopteran ovarian cell line generated from the *Bombyx mori* silk moth (Bm-5) and is capable of long term culture at high cell densities. Importantly the Bm-5 cell line is capable of carbohydrate modification of recombinant proteins.

4.4.3 Characterization of expression products. ECDα was expressed in the conditioned media of the BHK-21/ECDα cell line as a 55 kDa protein (Figure 4.1). ECDα migrated primarily as a monomer but could form higher molecular weight aggregates at higher protein concentrations (data not shown). This result is consistent with the ability of sGMRα to form homodimers and homotrimers in solution. However, only the monomeric species is able to bind GM-CSF[194]. ECDα was also expressed in solution by the Bm-5/ECDα cell line (Figure 4.1). The 48 kDa protein band was substantially smaller than that of mammalian expressed ECDα suggesting a lack of post-translational modification by the insect cell line. However, both the mammalian and insect expressed ECDα could bind concanavalin A in solution suggesting that terminal mannose residues were present on the insect expressed ECDα as well as the mammalian expressed subunit (data not shown). The ability of BHK-21/ECDα but not Bm-5/ECDα to associate with wheat germ agglutinin is consistent with the inability of insect cells to add terminal N-acetylglucosamine residues to expressed proteins [230,231] (data not shown).

Removal of the 16 amino acid C-terminal domain of sGMRα to generate ECDα had no affect on its ability to bind GM-CSF in solution (Figure 4.2) demonstrating that the ligand binding activity of GMRα and sGMRα is present in the 295 residues that constitute the shared extracellular domain of these two proteins. Importantly, the simple glycosylation pattern of insect expressed ECDα had no effect on its ability to bind GM-CSF (Figure 4.2). In contrast blocking glycosylation of GMRα using tunicamycin has been shown to abrogate GM-CSF binding activity[228]. Similarly GMRα expressed in yeast, capable of only very simple carbohydrate modification, is also unable to bind GM-

CSF[229]. Taken together these results demonstrate that carbohydrate modification of GMRα at a level that can be achieved by lepidopteran cells is sufficient for GM-CSF binding activity.

ECDβc was expressed in solution by the BHK-21/ECDβc cell line as a 55 kDa protein (Figure 4.3). The lower apparent molecular weight of ECDβc compared with ECDα was likely due to the extensive glycosylation of ECDα. Indeed, there are only 3 potential N-linked glycosylation sites on the extracellular domain of ECDβc[141] compared with 11 for ECDα[135].

The Bm-5/ECDβc cell line expressed ECDβc as a 48 kDa protein (4.4). Again the lower molecular weight of insect expressed ECDβc compared with mammalian expressed ECDβc was likely due to a difference in glycosylation. Similar to insect expressed ECDα, insect expressed ECDβc could be precipitated from solution with concanavalin A but not wheat germ agglutinin demonstrating the ability of the lepidopteran cell line to modify expressed proteins with simple mannose residues only. Interestingly, analysis of ECDβc under non-reducing conditions revealed a higher molecular weight protein band that may have corresponded to a disulphide bonded homodimer or homotrimer of ECDβc (Figure 4.4). The importance of this was not explored further.

This study demonstrates the successful engineering and high level protein expression of soluble isoforms of GMRα and βc. The ECDα subunit could bind GM-CSF with the same affinity as GMRα demonstrating the validity of using this subunit to model cell surface interactions in solution.

CHAPTER 5
DETERMINANTS OF THE FUNCTIONAL INTERACTION BETWEEN THE SOLUBLE GM-CSF RECEPTOR AND βc

5.1 Introduction

Previous work by our lab has demonstrated that, whether in the absence or presence of GM-CSF, an exogenous source of sGMRα is unable to interact with cells expressing βc on their surface[149]. Yet paradoxically, co-expression of sGMRα and βc results in the anchoring of a small amount of sGMRα on the cell surface[152] through a direct interaction with βc[150]. The resultant sGMRα/βc complex, though only a very small proportion of the total cell surface complexes, is fully capable of binding GM-CSF with the same high affinity as GMRα and βc[150]. The tethering of sGMRα to βc requires the cysteine at position 6 of the unique 16 amino acid C-terminal domain of sGMRα[150]. However the domains of βc involved in this interaction are not known.

The purpose of this study was to determine if the ligand-independent association between sGMRα and βc was mediated through the extracellular domain of βc and to deal with the puzzling observation that endogenous sources of sGMRα can form a complex with βc on the cell surface[150] while exogenous sources cannot[149]. A solution phase assay was developed to answer these questions and its use revealed the importance of the extracellular domains to the functional interactions of the GM-CSF receptor subunits.

5.2 Materials and Methods

5.2.1 Cell lines and protein expression. The construction and preliminary characterization of the BHK-21/ECDα, Bm-5/ECDα, BHK-21/ECDβc and Bm-5/ECDβc cell lines was described in Chapter 4. A cell line that co-expressed sGMRα and ECDβc (BHK-21/sGMRα+ECDβc) was established by calcium phosphate transfection (section 2.12) of pRC/CMV.ECDβc into the BHK-21/sGMRα cell line[149] to generate BHK-21/sGMRα+ECDβc. Cells that contained both plasmids, pZEM229.sGMRα[149] (conferring resistance to methotrexate) and pRC/CMV.ECDβc, were selected for

resistance to methotrexate (David Bull Laboratories Pty. Ltd., Mulgrave, Victoria, Australia) and G418. Surviving cell colonies were subcultured individually and screened for co-expression of sGMRα and ECDβc by ^{125}I-GM-CSF soluble receptor binding assay (section 2.29) and SDS-PAGE and Western blotting with the anti-βc mAb 1C1 (sections 2.17, 2.18), respectively. Unless otherwise indicated the source of the recombinant proteins for the following experiments was crude cell conditioned media.

5.2.2 Interaction of ECDα and GM-CSF with cell surface expressed βc: Flow cytometry. Cell surface expression of βc and GMRα receptor subunits on BHK-21 cells (BHK-21/βc^{150} and BHK-21/GMRα^{149}, respectively) was confirmed by flow cytometry using anti-βc mAbs 1C1 and the anti-GMRα mAb 8G6 as described in section 2.31. Detection of ECDα/GM-CSF complexes on the surface of βc expressing cells was performed as follows. A 50 nM ligand solution containing ECDα alone or in combination with an equimolar amount of GM-CSF was prepared in PBS. 10^6 βc-expressing cells (BHK-21/βc) or controls (BHK-21) were incubated at room temperature with 20 µL of either ligand solution for one hour. The tubes were maintained on ice to limit receptor internalization and were washed three times in ice cold PBS. The presence of ECDα on the surface of the cells was detected by incubation with a 1/2000 dilution of anti-GMRα mAb 8G6 for 1 hour. Cells were washed 3 times with ice cold PBS and were incubated with 1µg of FITC-labelled goat-anti mouse IgG secondary antibody for one hour. Cells were washed in PBS and analyzed on a flow cytometer. Non-specific interaction of the secondary antibody was controled for by performing all experiments with secondary antibody alone. The ability of the anti-GMRα mAb 8G6 to bind to ECDα in the presence and absence of GM-CSF was confirmed by analyzing the ability of 8G6 to identify BHK-21/GMRα cells that had been incubated with or without 50 nM of GM-CSF.

5.2.3 Interaction of ECDα and GM-CSF with cell surface expressed βc: ^{125}I-GM-CSF cell-associated receptor binding assays. Cell-associated GM-CSF receptor binding assays were performed as described in section 2.29 with the following modification: 10^6 βc-expressing cells (BHK-21/βc) or control cells (BHK-21) were

incubated with 20 μL of a ligand solution containing either 10 nM ^{125}I-GM-CSF alone or in combination with 10 nM affinity purified ECDα. Non-specific interaction of ^{125}I-GM-CSF was determined by performing the assays in the presence of 100 fold excess of unlabeled GM-CSF. The functionality of the assay was confirmed by ^{125}I-GM-CSF soluble receptor binding assay as described in section 2.29. All assays were performed in duplicate.

5.3 Results

5.3.1 The extracellular domain of βc is sufficient for the formation of a ligand-independent receptor complex between sGMRα and βc. sGMRα and βc can associate in the absence of GM-CSF when co-expressed in the same cell line[152]. While this unique association is dependent on the C-terminal domain of sGMRα[150] the domain on βc with which sGMRα associates is unknown. To determine if the ligand-independent association of sGMRα and βc was mediated through the extracellular domain of βc, conditioned media from the sGMRα/ECDβc co-expressing cell line was immunoprecipitated with anti-GMRα mAb 8G6 and anti-βc mAb 1C1 and analyzed by SDS-PAGE and Western blotting with 8G6 and 1C1. sGMRα was immunoprecipitated with both anti-GMRα mAb 8G6 and anti-βc mAb 1C1 (Figure 5.1, lanes 1 and 2, respectively) suggesting that sGMRα and ECDβc were associated in a complex in the absence of GM-CSF. The lower intensity of the sGMRα-protein band that was immunoprecipitated with anti-βc mAb is consistent with the ligand-independent sGMRα/βc complex being a minority species amongst a larger pool of free receptor subunits. To confirm that sGMRα forms a ligand-independent complex with ECDβc the reciprocal immunoprecipitations were performed. ECDβc was immunoprecipitated with both anti-βc mAb 1C1 and with anti-GMRα mAb 8G6 (Figure 5.1, lanes 3 and 4, respectively) confirming that sGMRα and ECDβc were associated in a complex in the absence of GM-CSF. Again the lower intensity of the ECDβc-protein band that was immunoprecipitated with anti-GMRα mAb suggests that the ligand-independent complex was a minority species. BHK-21/sGMRα conditioned media was also

immmunoprecipitated with anti-GMRα mAb 8G6 and probed with anti-βc mAb 1C1 to control for non-specific interactions between 1C1 and sGMRα (Figure 5.1, lane 5). These results demonstrate that the extracellular domain of βc is sufficient for the formation of a ligand-independent complex with sGMRα when the two subunits are co-expressed in the same cell line.

5.3.2 Determination of the GM-CSF binding abilities of the ligand-independent sGMRα/ECDβc complex. To determine if the preformed sGMRα/ECDβc complex observed in figure 5.1 could bind GM-CSF in solution, GM-CSF was directly conjugated to N-hydroxysuccinimide-activated agarose beads and used to try and co-adsorb sGMRα and ECDβc from media conditioned by the co-expressing BHK-21/sGMRα+ECDβc cell line.

The functional activity of the GM-CSF-beads was first confirmed by their ability to adsorb sGMRα from supernatant conditioned by the BHK-21/sGMRα cell line in a concentration-dependent manner (Figure 5.2A). Of interest, the GM-CSF beads could also adsorb a small amount of ECDβc from supernatant conditioned by the BHK-21/ECDβc cell line. However, the specificity of this interaction is questionable as a 100 fold excess of free GM-CSF could only partially inhibit this association (Figure 5.2B, lanes 1 and 2) and agarose beads conjugated with ethanolamine (sham-beads) were also able to adsorb a small amount of ECDβc (Figure 5.2B, lanes 3 and 4). Interestingly, the GM-beads adsorbed what appeared to be two different ECDβc protein bands while the sham-beads only adsorbed the larger molecular weight form. The reason for this is unknown.

To further explore the potential specificity of the GM-CSF interaction with ECDβc we also performed ^{125}I-GM-CSF soluble receptor binding assay using ECDβc. No specific binding of GM-CSF by ECDβc could be detected even at a concentration of 16 μM or nearly 2000 fold higher than the dissociation constant of the low affinity GM-CSF receptor[135] (data not shown). Finally, ECDβc conjugated to NHS-activated agarose beads was unable to adsorb ^{125}I-GM-CSF from solution (data not shown) further suggesting that no specific interaction between the GM-CSF-beads and ECDβc was occuring.

To determine if the sGMRα/ECDβc ligand-independent complex was capable of binding GM-CSF, we then incubated media conditioned by the co-expressing cell line BHK-21/sGMRα+ECDβc with GM-CSF-beads for 12 hours at 4°C. The beads were pelleted by centrifugation and washed extensively in PBS containing 0.02% Tween-20. The adsorbed protein were eluted from the beads by boiling them in SDS-PAGE loading buffer containing β-ME. The eluted protein were analyzed by reducing SDS-PAGE and Western blotting with the anti-GMRα mAb 8G6 or the anti-βc mAb 1C1. As can be seen from figure 5.3C the GM-CSF-beads were very effective at isolating sGMRα from solution (lane 2) when compared with immunoprecipitations performed with the anti-GMRα mAb 8G6 (lane 1). ECDβc was also adsorbed from BHK-21/sGMRα+ECDβc conditioned media with the GM-CSF beads but not from BHK-21/ECDβc media (Figure 5.2D, lanes 2 and 3, respectively) suggesting that the ligand-independent sGMRα/ECDβc complex could bind GM-CSF. However, these results do not exclude the possibility that the GM-CSF beads were adsorbing free sGMRα and ECDβc subunits that had assembled into a complex in the presence of GM-CSF. Indeed the weakness of the protein bands that were isolated in the absence of GM-CSF (Figure 5.1, lane 2) would suggest that the ligand-independent complex was a minority species amongst a much larger pool of free receptor subunits. To address this, we attempted to isolate the sGMRα/ECDβc complex from the free subunits by size exclusion chromatography. However the selectivity of the Superose 12/30 column was insufficient to effectively separate the preformed complex from the free receptor subunits (data not shown) and it remains uncertain whether the preformed sGMRα/ECDβc complex could indeed bind GM-CSF in solution.

5.3.3 An independent source of sGMRα can associate with ECDβc in the presence of GM-CSF. Previous work by our group[149] demonstrated that an independent (exogenous) source of sGMRα could not associate with βc on the cell surface even in the presence of GM-CSF. However, as we were unable to determine whether GM-CSF-beads had co-adsorbed the ligand-independent sGMRα/ECDβc complex or free sGMRα and ECDβc that had assembled into a GM-CSF binding complex, the possibility remained that in solution, an independent source of sGMRα could in fact associate with βc. To

explore this possibility further, we utilized independent sources of sGMRα and ECDβc supernatant for the adsorption assays. As expected, the GM-CSF beads adsorbed sGMRα from cell conditioned media that contained sGMRα alone or in conjunction with ECDβc (data not shown). ECDβc could not be readily adsorbed with the GM-CSF-beads beyond background levels resulting from the non-specific interaction between the agarose-beads and ECDβc (Figure 5.3, lane 1), consistent with ECDβc's lack of intrinsic affinity for GM-CSF. However, ECDβc was co-adsorbed with the GM-CSF-beads from supernatant containing admixtures of sGMRα and ECDβc (Figure 5.3, lane 2) suggesting that an independent source of sGMRα could indeed associate with ECDβc in the presence of ligand.

To confirm this observation the reciprocal experiment was performed using purified sGMRα immobilized on NHS-activated agarose beads. The activity of the sGMRα-beads was confirmed by their ability to specifically adsorb ^{125}I-GM-CSF from solution (data not shown). The sGMRα-beads were incubated with ECDβc in the absence and presence of GM-CSF, pelleted and washed. The adsorbed proteins were eluted off the beads by boiling in SDS-PAGE reducing buffer. The proteins were analyzed by SDS-PAGE and Western blotting with anti-βc mAb 1C1. In the absence of GM-CSF the sGMRα-beads could not adsorb ECDβc beyond background levels resulting from the non-specific interaction between the agarose-beads and the ECDβc subunit (Figure 5.3, lane 3). However in the presence of GM-CSF the sGMRα beads adsorbed a distinct protein band that corresponded to ECDβc (Figure 5.3, lane 4). Therefore while it remains unclear whether the ligand-independent sGMRα/ECDβc complex can bind GM-CSF in solution it is clear that independent sources of sGMRα and ECDβc can associate in the presence of GM-CSF.

5.3.4 The C-terminal domain of sGMRα does not mediate the interaction between independent sources of sGMRα and ECDβc. The C-terminal domain of sGMRα is necessary for the tethering of sGMRα to βc on the cell-surface[150]. To determine if this C-terminal domain was also responsible for the ability of an independent source of sGMRα to associate with ECDβc in the presence of GM-CSF we generated a

mutant isoform of sGMRα that was missing the 16 amino acid C-terminal domain (ECDα) and expressed it in the insect cell line Bm-5 (see Chapter 4). Affinity purified ECDα was immobilized on NHS-activated agarose beads. The activity of the beads was confirmed by their ability to specifically adsorb ^{125}I-GM-CSF from solution (data not shown). ECDα-beads were incubated with an independent source of ECDβc that was secreted by an insect cell line (Bm-5/ECDβc, see Chapter 4) in the absence and presence of GM-CSF. The beads were pelleted, washed and the adsorbed proteins were eluted off the beads by boiling them in SDS-PAGE reducing buffer. The eluted protein were were analyzed by SDS-PAGE and Western blotting with anti-βc mAb 1C1. ECDβc did not associate with the ECDα-beads in the absence of GM-CSF (Figure 5.4, lane 1). However in the presence of GM-CSF, ECDβc did associate with ECDα (Figure 5.4, lane 2) demonstrating that the C-terminal domain of sGMRα did not mediate the interaction between independent sources of sGMRα and ECDβc. To confirm these results we performed the reciprocal experiment using GM-CSF-beads. ECDβc conditioned media was incubated with GM-CSF-beads in the absence and presence of an independent source of ECDα. ECDβc did not associate with GM-CSF in the absence of ECDα (Figure 5.4, lane 3). However ECDβc did associate with GM-CSF in the presence of ECDα (Figure 5.4, lane 4) confirming that independent sources of ECDα and ECDβc can associate in the presence of GM-CSF and that the C-terminal domain of sGMRα does not mediate this interaction.

GMRα and βc form a high affinity GM-CSF binding complex on the cell surface[141], while GMRα alone binds GM-CSF with low affinity[135]. The βc receptor subunit mediates the conversion of the GMR from low to high affinity[142]. To determine whether ECDβc could also act as an affinity converting subunit in solution we performed ^{125}I-GM-CSF soluble receptor binding assays using media conditioned by the Bm-5/ECDα cell line in the presence and absence of media conditioned by the Bm-5/ECDβc cell line. In the absence of ECDβc, ECDα bound ^{125}I-GM-CSF with an affinity similar to that of the native cell surface GMRα (Kd= 2.8 nM, Chapter 4, Figure 4.2). However, upon addition of ECDβc to the reaction mixture, a 5-20 fold increase in ^{125}I-GM-CSF binding activity

was observed (Kd = 98 pM to 800 pM, Figure 5.5). This increase in GM-CSF binding affinity upon addition of ECDβc was consistent with the affinity shift that is observed on the cell surface suggesting that ECDβc did provide affinity converting activity. Furthermore, this result supports the observation that independent sources of ECDα and ECDβc can associate in solution in the presence of GM-CSF and that the subunits do not have to be co-expressed for this to occur.

5.3.5 ECDα can not associate with cell-surface expressed βc in the presence of GM-CSF. We performed flow cytometry to determine if ECDα and GM-CSF were also able to associate with cell-surface expressed βc. BHK-21 and BHK-21/βc cells were incubated with 50 nM ECDα in Bm-5/ECDα conditioned media and GM-CSF, or ECDα alone, and were analyzed for retention of ECDα on the cell surface by flow cytometry with the anti-GMRα mAb 8G6 and a FITC-labelled goat-anti mouse IgG secondary antibody. No signal was detected on the surface of BHK-21 (Figure 5.6A) or BHK-21/βc cells (Figure 5.6B) that were treated with either ECDα alone or ECDα and GM-CSF demonstrating that ECDα could not associate with cell-surface expressed βc even in the presence of GM-CSF. The lack of interaction of ECDα with βc was not due to a lack of βc receptors on the surface of BHK-21/βc cells as expression of βc was confirmed by flow cytometry using an anti-βc mAb (1C1, Figure 5.6C). Furthermore, the lack of interaction was not due to the inabilty of 8G6 to recognize receptors that were bound to GM-CSF as 8G6 recognized GMRα receptors with equal intensity on BHK-21/GMRα cells that were incubated in the absence and presence of GM-CSF (Figure 5.6D). Finally, the inability of ECDα and GM-CSF to associate with cell-surface expressed βc was confirmed by a radioligand binding assay that failed to detect the presence of ^{125}I-GM-CSF on the surface of BHK-21/βc cells that had been incubated with an admixture of ECDα and ^{125}I-GM-CSF (data not shown). Thus while ECDα and GM-CSF can clearly associate with ECDβc in solution they are unable to associate with cell-surface expressed βc.

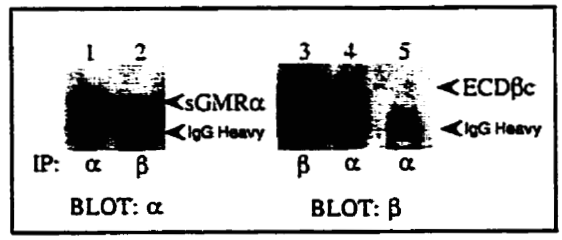


Figure 5.1. Co-immunoprecipitation of sGMRα and ECDβc in the absence of GM-CSF from a cell line that co-expressed both subunits. BHK-21/sGMRα+ECDβc (1-4) or BHK-21/sGMRα (5) conditioned media was incubated with anti-GMRα mAb 8G6 (α) or anti-βc mAb 1C1 (β) in the absence of GM-CSF. The precipitated proteins were eletrophoresed under reducing conditions by SDS-PAGE and visualized by Western blotting with the anti-GMRα mAb 8G6 or the anti-βc mAb 1C1. The positions of sGMRα and ECDβc are indicated with arrows, as is the position of the IgG heavy chain.

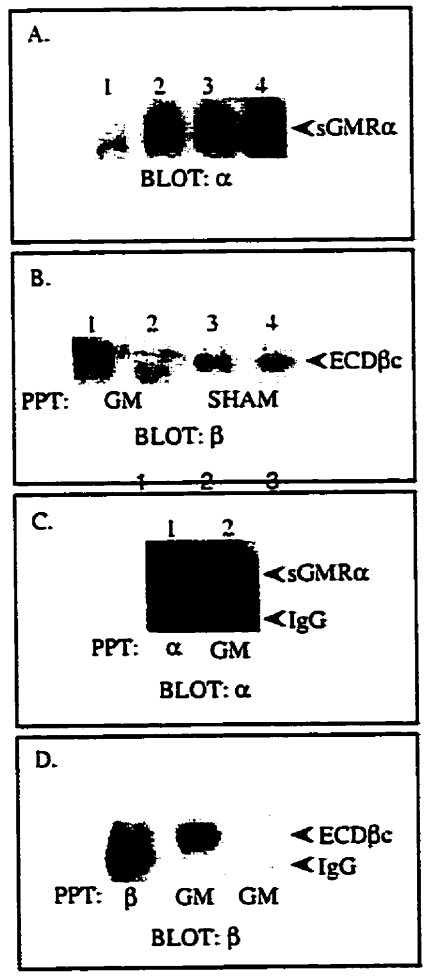

A.
1 2 3 4
sGMRα
BLOT: α
B.
1 2 3 4
ECDβc
PPT: GM SHAM
BLOT: β
1 2 3
C.
1 2
sGMRα
IgG
PPT: α GM
BLOT: α
D.
ECDβc
IgG
PPT: β GM GM
BLOT: β

Figure 5.2. Co-adsorption of sGMRα and ECDβc with GM-CSF from media conditioned by the co-expressing cell line BHK-21/sGMRα+ECDβc. A. GM-CSF-beads adsorbed sGMRα from solution in a concentration-dependent manner. BHK-21/sGMRα conditioned media was incubated with increasing amounts of a 50% slurry of GM-CSF-beads (1, 5, 10 or 20 μL, lanes 1-4) for 2 hours at room temperature. The bead were pelleted by centrifugation and washed in PBS containing 0.02% Tween-20. The adsorbed proteins were eluted from the beads by boiling in SDS-PAGE reducing buffer. The proteins were eletrophoresed under reducing conditions by SDS-PAGE and visualized by Western blotting with the anti-GMRα mAb 8G6. **B.** Non-specific interaction between GM-CSF-beads and ECDβc. BHK-21/ECDβc conditioned media was incubated with 100 μL of a 10% slurry of either GM-CSF-beads (GM) or sham-conjugated agarose beads (SHAM) in the absence (1,3) and presence (2,4) of 100 fold excess of free, unconjugated GM-CSF. The beads were pelleted and washed and the adsorbed protein were eluted from the beads by boiling in SDS-PAGE reducing buffer. The eluted proteins were fractionated by reducing SDS-PAGE and analyzed by Western blotting with the anti-βc mAb 1C1. C. GM-CSF-beads effectively adsorb sGMRα from BHK-21/sGMRα+ECDβc conditioned media. BHK-21/sGMRα+ECDβc conditioned media was incubated with anti-GMRα mAb 8G6 (α) and Protein-A Sepharose or 100 μL of a 10% slurry of GM-CSF-beads (GM). The beads were pelleted, washed and eluted from the beads by boiling in SDS-PAGE reducing buffer. The adsorbed proteins were electrophoresed under reducing conditions by SDS-PAGE and visualized by Western blotting with anti-GMRα mAb 8G6. **D.** ECDβc can be co-adsorbed with GM-CSF from media conditioned by the co-expressing cell line BHK-21/sGMRα+ECDβc but not from media conditioned by the BHK-21/ECDβc cell line. BHK-21/sGMRα+ECDβc (1,2) or BHK-21/ECDβc (3) conditioned media was incubated with anti-βc mAb 1C1 (β) and Protein A-Sepharose or 100 μL of a 10% slurry of GM-CSF-beads (GM). The beads were pelleted, washed and eluted by boiling in SDS-PAGE reducing buffer. The adsorbed proteins were electrophoresed under reducing conditions by SDS-PAGE and visualized by Western blotting with the anti-βc mAb 1C1.

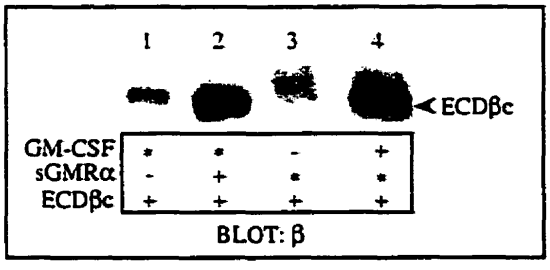


Figure 5.3. Admixtures of independent sources of sGMRα and ECDβc can associate in solution. BHK-21/ECDβc conditioned media was incubated in the presence and absence of different combination of BHK-21/sGMRα conditioned media and GM-CSF (or their bead-conjugated equivalents, represented by an asterix). The adsorbed protein complexes were pelleted, washed and eluted off the beads by boiling in SDS-PAGE reducing buffer. The eluted proteins were electrophoresed under reducing conditions by SDS-PAGE and visualized by Western blotting with the anti-βc mAb 1C1.

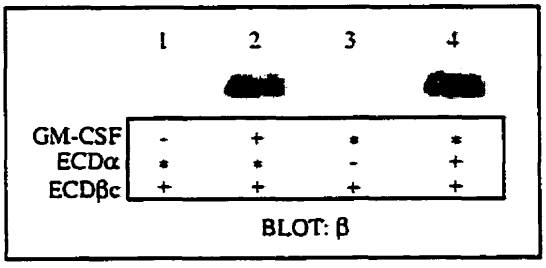


Figure 5.4. The C-terminal domain of sGMRα does not mediate the interaction between independent sources of sGMRα and ECDβc. Bm-5/ECDβc conditioned media was incubated in the presence and absence of different combinations of Bm-5/ECDα conditioned media and GM-CSF (or their bead-conjugated equivalent, represented by an asterix). The beads were pelleted, washed and the adsorbed proteins were eluted by boiling the beads in SDS-PAGE reducing buffer. The eluted proteins were electrophoresed under reducing conditions by SDS-PAGE and visualized by Western blotting with anti-βc mAb 1C1.

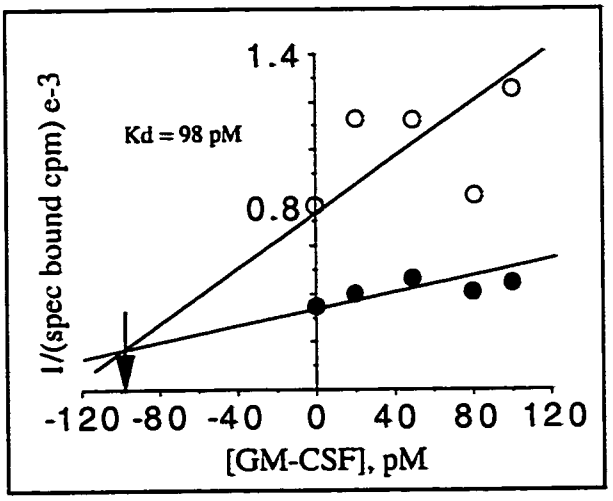


Figure 5.5. ECDβc increases GM-CSF binding affinity in solution. Dixon analysis of 125I-GM-CSF soluble receptor binding data for independent sources of ECDα and ECDβc. Cold-saturation binding assays were performed on Bm-5/ECDα and Bm-5/ECDβc conditioned media that was co-incubated. Data points were derived from binding assays performed with either 86 pM 125I-GM-CSF (open circles) or 260 pM 125I-GM-CSF (closed circles). The point of intersection of the two lines extrapolated to the x-axis gives the value of the dissociation constant. Shown is a representative of n=5 experiments in which the dissociation constants ranged from 98 pM to 800 pM. See figure 4.2 for comparison - the affinity of ECDα for GM-CSF in the absence of ECDβc was 2.8 nM.

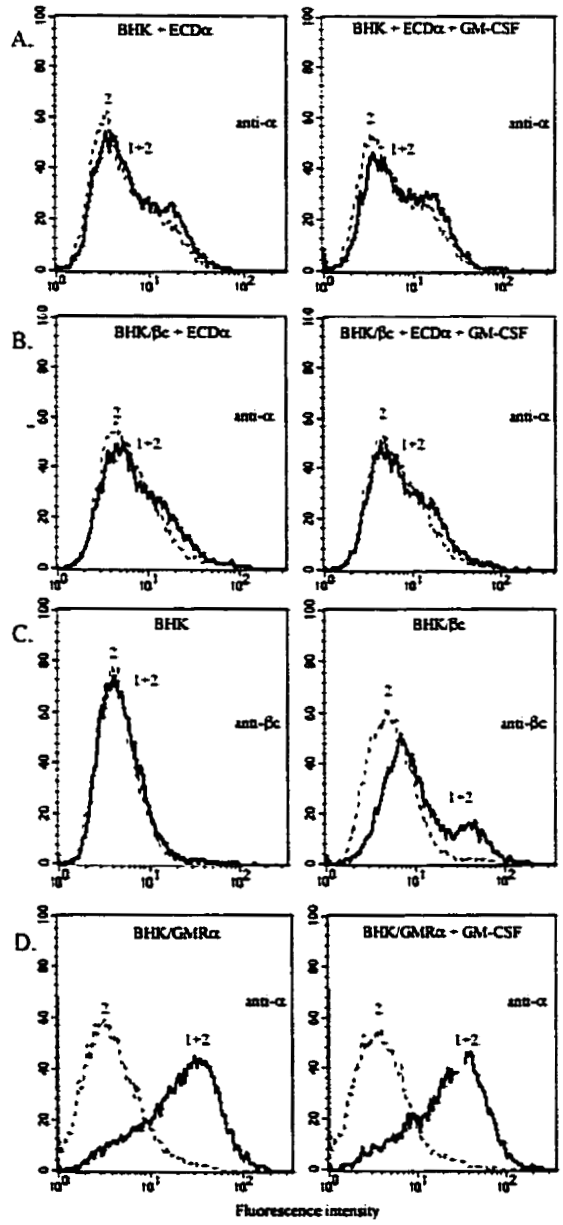


Figure 5.6. ECDα does not interact with cell-surface expressed βc in the presence or absence of GM-CSF. Flow cytometry was performed on cells that were pre-incubated for one hour with 50 nmol/L ECDα or 50 nmol/L ECDα and GM-CSF using anti-GMRα mAb 8G6 (anti-α, panels A, B and D) or anti-βc mAb 1C1 (anti-βc, panel C) and a FITC-labelled goat-anti mouse IgG secondary antibody. Fluorescence intensities of the cells incubated with both primary and secondary antibodies (1+2) was compared with the intensities of cells incubated with secondary antibody alone (2). A. ECDα is not retained on the surface of BHK-21 cells either in the presence or absence of GM-CSF. B. ECDα is not retained on the surface of BHK-21/βc cells in the presence or absence of GM-CSF. C. βc receptors are present on the surface of BHK-21/βc but not BHK-21 cells. D. mAb 8G6 recognizes GMRα receptors on the surface of BHK-21/GMRα cells in the presence and absence of GM-CSF.

5.4 Discussion

Soluble receptors exist for nearly all members of the cytokine receptor superfamily. Most are soluble variants of the ligand binding "α" chain that arise either by proteolytic cleavage or by an alternative splice event[144-146,189,232]. The function of a given soluble receptor is in part dependent upon its ability to associate with its cognate cell surface signaling subunit. For example the soluble IL-6 receptor can bind IL-6 in solution and associate with its cell surface signaling subunit, gp130, to form a functional receptor complex capable of signal transduction[233]. On the other hand while sGMRα in solution phase can bind GM-CSF it cannot interact with βc on the cell surface and is therefore believed incapable of forming a functional receptor complex with βc. Indeed sGMRα acts as an antagonist of GM-CSF function *in vitro* presumably by binding to and sequestering GM-CSF away from the cell surface receptor[149]. However, in contrast to exogenous sGMRα, sGMRα that is co-expressed in the same cell as βc is able to form a ligand-independent complex with βc that can also bind GM-CSF with high affinity[150,152]. Thus a paradox exists concerning the interaction of sGMRα with cell surface βc with the potential for interaction appearing to be critically dependent upon the source of sGMRα expression in relation to βc.

Previous work by our group demonstrated that sGMRα that was co-expressed with βc remained associated with the cell surface by direct interaction of its C-terminal domain with βc in the absence of GM-CSF[150]. The data in this manuscript demonstrates it is the extracellular domain of βc which interacts with the C-terminal domain of sGMRα and allows the formation of the ligand-independent complex (Figure 5.1):

$$\text{sGMR}\alpha + \text{ECD}\beta\text{c} \quad \leftrightarrow \quad \text{sGMR}\alpha/\text{ECD}\beta\text{c}$$

These data also demonstrate that an unidentified cell surface molecule is not mediating the interaction between βc and sGMRα as the subunits do not have to be tethered on the cell surface for the interaction to take place. These results are therefore

consistent with the extracellular domain of βc being sufficient for formation of the ligand-independent complex with sGMRα.

The sGMRα/ECDβc complex was a minority species in the cell conditioned media when compared with the much larger pool of free sGMRα and ECDβc subunits (Figure 5.1). This result was consistent with our earlier data which showed that the sGMRα/βc cell lines secreted ample sGMRα into the supernatant as well as having sGMRα on the surface[150,152]. The data also confirm that the ligand-independent complex only forms when the receptor subunits are co-expressed (Figures 5.3,4) suggesting that sGMRα and βc are assembling into a preformed complex prior to reaching the cell surface. Taken together these observations are in keeping with the interaction between sGMRα and ECDβc or βc being a low frequency event that occurs during co-transport of the receptor subunits through intracellular organelles.

Previous work by our group demonstrated that the ligand-independent cell surface complex consisting of sGMRα and βc could bind GM-CSF on the cell surface with high affinity[150]. There are constituents in the conditioned media of the sGMRα/ECDβc cell line which are also able to bind GM-CSF and which involve ECDβc as part of the soluble complex (Figure 5.2 C,D):

$$\text{sGMR}\alpha/\text{ECD}\beta\text{c} + \text{GM-CSF} \quad \leftrightarrow \quad \text{sGMR}\alpha/\text{ECD}\beta\text{c/GM-CSF}$$

However, we were unable to determine if the preformed sGMR α/ECDβc complex participated or whether it all arose from the ligand driven assembly of free sGMRα and ECDβc. This latter scenario, the assembly of GM-CSF, sGMRα and ECDβc into a complex in solution, clearly does occur and does not require co-expression of the receptor subunits (Figure 5.3).

These results contrast with previous findings where an independent source of sGMRα could not associate with cell surface βc even in the presence of GM-CSF[149,150]. It is possible that sGMRα interacts differently with ECDβc in solution than it does on the cell surface with βc. ECDβc may be in a less constrained configuration when in solution

allowing it to associate more readily with sGMRα and GM-CSF. Alternatively membrane-anchored βc may normally exist as part of a cell surface complex that is unable to associate with sGMRα and GM-CSF. There is evidence that at least a subpopulation of βc exists on the cell surface as a non-functional homodimer that is activated upon recruitment into a complex with GM-CSF and GMRα[159]. However as cell surface βc can clearly be recruited into a functional receptor complex with the membrane-spanning GMRα following ligand binding it seems unlikely that a homodimeric state of free βc on the cell surface would specifically preclude interaction with sGMRα and not GMRα.

While the ligand-independent interaction of co-expressed sGMRα with βc is mediated by the C-terminal domain of sGMRα, the ligand driven assembly of sGMRα and ECDβc does not require this C-terminal domain. A mutant sGMRα construct that was missing the C-terminal domain (ECDα) was also able to associate with ECDβc in the presence of GM-CSF (Figure 5.4) demonstrating that the ability of independent sources of sGMRα and ECDβc to associate in solution upon the introduction of GM-CSF is an intrinsic property of the extracellular domains of these receptor subunits. In contrast it has been previously reported that the extracellular domains of GMRα and βc can not assemble in solution unless co-expressed[200]. However, these experiments were performed in the presence of a high concentration of detergent (1% NP-40/PBS versus 0.02% Tween-20/PBS used here) suggesting that the detergent may have interfered with subunit association. Alternatively the wash buffers we used may have been too mild to disrupt non-specific subunit interactions. The latter scenario is unlikely as ECDα and ECDβc were able to bind GM-CSF with higher affinity than ECDα alone (Figure 5.5) suggesting that the association of independent sources of ECDα and ECDβc was specific. These results demonstrate that all of the domains necessary to form a functional high affinity GM-CSF receptor are present within the extracellular domains of the individual receptor subunits and that their association is not dependent upon co-expression.

There was an 8-fold range in dissociation constants observed for the binding of GM-CSF to ECDα/ECDβc. The variability in the estimated dissociation constants was likely

the result of using crude conditioned media of variable receptor concentration. Indeed, Wheadon et al.[205] have demonstrated that the molar ratio of GMRα:βc on the cell surface has a profound impact on the observed dissociation constant, where a shift in the molar ratio of GMRα:βc from 4:1 to 1:1 resulted in a 25 fold shift in high affinity binding from 15 pM to 364 pM. The range of estimated dissociation constants obtained for experiments reported in this manuscript (98 pM to 800 pM) is within the range that might be expected if the molar ratio of ECDα:ECDβc varied between experiments. However, we can not rule out the possibility that the range in dissociation constants observed was a direct result of the loss of the transmembrane domains of GMRα and βc, where loss of C-terminal anchorage of the receptor subunits somehow contributed to de-stabilization of the high affinity receptor complex.

The data in this chapter suggest that the GM-CSF receptor complex assembles in a stepwise manner that is initiated by the interaction of the extracellular domain of GMRα with GM-CSF, followed by the interaction of the GMRα/GM-CSF complex with the extracellular domain of βc:

ECDα + GM-CSF	↔	ECDα/GM-CSF
ECDα/GM-CSF + ECDβc	↔	ECDα/GM-CSF/ECDβc

That is, the extracellular domains of GMRα and βc have no apparent intrinsic affinity for one another, nor does the extracellular domain of βc have any intrinsic affinity for GM-CSF. On the other hand, the data presented in section 5.3.1 suggest that co-expression of the sGMRα subunit with the extracellular domain of βc can lead to the formation of a small pool of pre-formed GM-CSF receptors that might be able to bind GM-CSF:

sGMRα + ECDβc	↔	sGMRα/ECDβc
sGMRα/ECDβc + GM-CSF	↔	sGMRα/βc/GM-CSF

Therefore, it is conceivable that the different subunits that form part of the GMR complex can associate with one another in different ways depending upon whether they are co-expressed in the same cell or arise from different sources. The observation that GMRα and βc can form a small pool of pre-formed receptors on primary hematopoietic cells[200] demonstrates that the transmembrane and cytoplasmic domains of GMRα and βc and the plasma membrane likely play an important role in assembly of the GMR complex *in vivo*.

The results of our experimentation with ECDα have also demonstrated that it is not merely the presence of the C-terminal domain of sGMRα that precludes the interaction of sGMRα with βc on the cell surface. An exogenous source of ECDα is likewise unable to complex with cell-surface βc (Figure 5.6). Thus the paradox remains unresolved as to why sGMRα can interact with βc in a ligand-independent manner when the two are co-expressed and can interact with ECDβc in a ligand-dependent manner in solution but cannot interact with βc on the cell surface when introduced from an exogenous source. However, the recent identification of a soluble splice variant of βc in human mononuclear cells[234] introduces the possibility that sGMRα could also associate with a soluble βc variant *in vivo*. As neither sGMRα[152] nor ECDα were able to interact with cell-associated βc in the presence of GM-CSF (Figure 5.6) but readily associated with ECDβc in solution (Figures 5.3-5) it is likely that interaction with a soluble βc variant *in vivo* would result in antagonism of GM-CSF activity. Indeed, a soluble variant of the gp130 subunit has been identified *in vivo*[193] that is capable of antagonizing the activity of IL-6 responsive cells *in vitro*[193,197] by associating with IL-6 and the soluble IL-6 receptor. With the recent identification of a soluble GM-CSF binding protein that resembles sGMRα in human plasma and leukemic cell lines (Chapter 3) a unique opportunity now exists for exploring not only the molecular interactions of sGMRα but also its potential biological functions *in vivo*.

CHAPTER 6
PURIFICATION OF ECDβc

6.1 Introduction

The purpose of this study was to develop a purification protocol for the isolation of homogeneous ECDβc from media conditioned by the insect cell line Bm-5/ECDβc. Clinical grade GM-CSF was available for use and therefore obviated the need for purification and quantitation (section 2.15). A recently developed commercial ELISA for the sGMRα subunit allowed for accurate quantitation in crude conditioned media (section 2.16) while the previously described GM-CSF-affinity column allowed for purification of functional soluble GMRα variants (section 2.24). Therefore, the task of protein purification was limited to ECDβc.

The strategy for purifying ECDβc was first to identify protein purification methodologies that might be feasible for the purification of ECDβc. The second step was to devise a purification protocol for ECDβc that would allow for the purification of homogeneous ECDβc from cell conditioned media.

6.2 Materials and Methods

6.2.1 Size Exclusion Chromatography (SEC). SEC was performed using an FPLC Chromatography system (Pharmacia Biotech, Baie d'Urfe, PQ) and a Superose 12/30 size exclusion column (Pharmacia). All experiments were performed using phosphate-buffered saline (PBS, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, pH 7.4) as the mobile phase and elution of proteins from the column was monitored by UV absorption at 280 nm. Sample injections were limited to 1% of the column volume, or less than 200 μL. The void volume and total volume of the column were determined empirically by measuring the retention time of Blue dextran and acetone, respectively. The number of theoretical plates in the column, an indication of the selectivity of the column, was determined from analysis of the symetry of the peak of UV absorption for acetone. The estimated retention time for a molecule of a given molecular weight was

determined by analyzing the retention time of proteins of known molecular weight (bovine serum albumin (BSA), 66 kDa; ovalbumin, 48 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14.3 kDa). Experiments were performed at a flow rate between 200μL/min and 1mL/min. Fractions were automatically collected into 1.5mL Eppendorf tubes and stored at 4°C for analysis.

6.2.2 Flat bed isoelectric focusing (IEF). A bed of granular cross-linked agarose (Sephadex IEF, Pharmacia) was used as the stationary matrix for IEF. 58 mL of IEF buffer (8% glycerol, 4% ampholine solution pH 3.5-10, 60 μM EGTA pH 6.5, 8.65 mM β-mercaptoethanol) was used to reconstitute 2.64 g of Sephadex matrix. The matrix was spread to uniform thickness on a glass plate (approximately 20 cm by 12 cm) and was seated on a cooling tray. Electrode buffer strips (two 0.5 cm wide strips of Whatman paper) were soaked in either 2% ampholine solution or 1M H_3PO_4 and were placed on the gel bed at the anode and cathode positions, respectively. The pH gradient was formed prior to analyzing the Bm-5/ECDβc sample by electrophoresing the ampholyte mixture through the agarose bed for 1 hour at 500V. 2 mL of desalted Bm-5/ECDβc conditioned media was mixed with a strip of the agarose matrix that had been removed from the gel bed. The sample was then reapplied to the gel bed and electrophoresed for 12 hours at 500V. The voltage was increased to 1000V for 1 hours to sharpen the focusing of the bands. The gel bed was divided into 24 sections of approximately 1 mL of gel each and was harvested using a 0.5 cm wide plastic scraper. The gel matrix was transferred 10 mL platic tubes containing 1 mL of ddH_20 and the pH of each fraction was determined.

6.2.3 Reverse Phase Chromatography (RPC). All RPC was carried out on a Beckman System Gold HPLC system interfaced with a Dell 386 PC. A C4 analytical column (Biorad, Mississauga, ON) was used throughout. The mobile phase for all RPC consisted of 0.1% trifluoroacetic acid (TFA) in H_20. The elution phase consisted of 0.1% TFA in acetonitrile. All experiments were performed at room temperature and at a flow rate of 1 mL/min. Protein elution from the column was monitored by UV absorbance at 215 nm. Elution of adsorbed proteins from the C4 column was accomplished using a linear gradient (0-100%) of 0.1% TFA/acetonitrile. The slope of the gradient was varied

throughout experiments to optimize the specific elution of ECDβc. This was accomplished by lengthening the duration of the elution period from 30 minutes to 180 minutes. 1 mL fractions were collected manually into 1.5 mL Eppendorf tubes. The acetonitrile was immedietly evolved off each sample on a speed-vacuum and the fractions were frozen and lyophylized prior to analysis by reducing SDS-PAGE.

6.2.4 Concanavalin A-affinity chromatography. 1-10 mL of ConA-Sepharose (Pharmacia) was loaded into a 10 mL disposable plastic column and washed with running buffer (50 mM phosphate buffer pH 7.4, 0.5M NaCl). 2-30 mL of Bm-5/ECDβc conditioned media was loaded onto the column and allowed to flow through by gravity. The column was washed extensively with running buffer and eluted with 0.1M-0.5M α-D-methylmannoside in running buffer. Tightly adsorbed proteins were eluted by washing the column with 0.5M NaCl pH 4.5 alternated with 0.5M NaCl pH 8.5. The acid/base wash was also used to reconstitute the column (remove the adsorbed α-D-methylmannoside).

6.2.5 Anti-βc affinity chromatography. 1 mL of N-hydroxysuccinimide-activated sepharose 4B (Pharmacia) was washed three times with 10 mL of 0.01M HCl (pH 3) prior to conjugation. 300 μg of anti-βc monoclonal antibody 1C1 in 300 μL of PBS was incubated with the activated sepharose for 12 hours at 4°C. Remaining active sites were blocked by incubation with 1M ethanolamine for 1 hour at room temperature. The affinity matrix was loaded into a 10 mL disposable plastic column and washed extensively with PBS. 5 mL of Bm-5/ECDβc conditioned media was layered on the column and allowed to pass through by gravity (approximate flow rate of 300μL/min). The column was washed with 70 mL of PBS at maximal flow rate (approximately 3 mL/min). The adsorbed proteins were eluted with 0.1M glycine pH 2.5. 1 mL fractions were collected intro 1.5 mL Eppendorf tubes containing 20 μL of neutralization buffer (1M Tris pH 9.0) and stored at 4°C.

6.2.6 Anion-exchange chromatography (AEC). AEC was performed using the FPLC system connected to a quaternary amine derived anion exchange column (MonoQ, Pharmacia). Flow rates varied from 200 μL/min to 1 mL/min. The column was

equilibrated with binding buffer (50mM Tris, 137mM NaCl, pH 8.3) prior to use. 1 mL of Bm-5/ECDβc conditioned media was routinely injected over the column. The column was washed with buffer until the UV absorbance at 280 nm returned to baseline, at which time the adsorbed proteins were eluted with a linear gradient (0-1M) of NaCl in binding buffer. Fractions (200 μL to 1 mL) were collected into 1.5 mL Eppendorf tubes and stored at 4°C.

6.2.7 Protein assays for ECDβc. Samples from the various purification procedures were analyzed for the presence of ECDβc and other proteins by reducing SDS-PAGE (section 2.17), Western blotting with the anti-βc mAb 1C1 (section 2.18), dot blotting with the anti-βc mAb 1C1 (section 2.19), silver staining (section 2.20) and Coomassie blue staining of SDS-PAGE gels (section 2.21) and PVDF membranes (section 2.22). N-terminal protein sequencing and amino acid analysis was performed by the University of Calgary Protein Sequencing Facility at the Health Sciences Centre.

6.3 Results

6.3.1 Method Scouting

Size exclusion chromatography. 200 μL of Bm-5/ECDβc conditioned media was fractionated on the size exclusion column. 30 μL of each fraction was analyzed by reducing SDS-PAGE and Western blotting with mAb 1C1 and silver staining. A broad peak of UV absorbance was present in fractions 11-13 (Figure 6.1A). Western blotting revealed a 48 kDa protein band in fractions 11-14 with a peak of intensity in fraction 12 (Figure 6.1B). Silver staining of a parallel gel showed 66 and 55 kDa protein bands in all fractions with a peak of intensity in fraction 13 (Figure 6.1C). There was also a faint 50 kDa protein band present in fraction 14. A 48 kDa protein band corresponding in size to the putative ECDβc band that was visualized in figure 6.1B was present in fractions 14 and 15 (Figure 6.C). However the elution position of this band did not correspond with that of the band visualized by Western blotting.

Flatbed isoelectric focusing. A pH gradient (pH = 3.12 - 8.63) was established by electrophoresing a mixture of ampholytes through a 20 cm by 12 cm flat bed of granular

agarose. 2 mL of Bm-5/ECDβc conditioned media was desalted and loaded at the anode (Figure 6.2, fraction 24, pH = 8.63, not shown). Following electrophoresis, the gel bed was divided into 24 fractions of approximately 1 mL of gel each, harvested with a plastic scraper and mixed with 1 mL of dd H20. The pH of each fraction was determined. 30 μL of each of the 24 fractions was analyzed by reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1 (the Western blot for fractions 1-16 is shown in figure 6.2A). A 46 kDa protein band corresponding in size to the control ECDβc band was present in lanes 11-13 (pH = 4.29-4.36) with a peak of Western blot activity in lane 11 (Figure 6.2A). There were also a number of unique protein bands present in fraction 13 at 48 kDa and 66 kDa as well as 140 and 150 kDa (Figure 6.2A, lane 13). In addition there was a faint doublet present at the origin of the separation gel that probably consisted of large aggregates of ECDβc (Figure 6.2A, lane 13). 30 μL of each fraction was also analyzed by reducing SDS-PAGE and silver staining (again, only the portion of the gel representing fractions 1-16 is shown in Figure 6.2B). The silver stained gel showed a number of protein bands within the 48 kDa to 66 kDa range. The 66 kDa protein bands in fractions 10-16 were likely BSA, while the identity of the smaller molecular weight bands could not be confirmed. Further experiments to improve the separation of ECDβc from the other constituents of the conditioned media using different ampholyte mixtures did not improve the resolution of the procedure.

Reverse phase chromatography. 1 mL of Bm-5/ECDβc conditioned media was mixed with an equal volume of 0.1% TFA and injected over the column. The adsorbed proteins were washed with the running buffer and eluted with a linear gradient (0-100%) of acetonitrile/0.1% TFA over a 30 minute time period. 3 mL fractions were collected and analyzed by reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1 . A number of protein bands were visualized by Western blotting in fractions 24-30 (Figure 6.3). The 46 kDa protein band corresponded in size to ECDβc (Figure 6.3, lanes 24-30). The chromatograph revealed a complex pattern with substantial UV absorption throughout the elution period, particularly surrounding fraction 20-30, making the

chromatographic results uninterpretable. This suggests that there is a substantial concentration of proteins other than ECDβc in these fractions.

Concanavalin A-affinity chromatography. 2 mL of Bm-5/ECDβc conditioned media was resuspended in 8 mL of running buffer and applied to the column. The column was washed twice with 10 mL of running buffer and eluted twice with 10 mL of 0.1M, 0.3M and 0.5M α-D-methylmannoside. Tightly adsorbed proteins were removed by washing the column with 10 mL of 0.5M NaCl (ph 4.5) followed by 10 mL of 0.5M NaCl (pH 8.5). 30 μL of each 10 mL fraction, including the original material (Figure 6.4, lane 1), the column pass through (lane 2), the two wash steps (lanes 3 and 4), the column eluents (lanes 5-10) and the acid and base elutions (lanes 11 and 12, respectively), were analysed by reducing SDS-PAGE, Western blotting with the anti-βc mAb 1C1 and silver staining. Unfractionated ECDβc migrated as a 50 kDa protein band by Western blot (Figure 6.4A, lane 1). A small quantity of ECDβc passed through the column without adhering to the lectin (Figure 6.4A, lane 2) while an even smaller amount was washed off the column (Figure 6.4A, lanes 3 and 4). ECDβc eluted from the column with 0.1M α-D-methylmannoside along with three smaller molecular weight protein bands (Figure 6.4A, lanes 5 and 6). Lanes 7 and 8, representing elution of the column with 0.3M α-D-methylmannoside, were obscured during transfer by an air bubble and could not be visualized. However, 0.5M α-D-methylmannoside eluted a small amount of ECDβc from the column (Figure 6.4A, lanes 9 and 10). The first elution of the column with the pH 4.5 NaCl buffer also eluted ECDβc (Figure 6.4A, lane 11). Silver staining a parallel blot showed the presence of a large concentration of BSA in the starting material (Figure 6.4B, lane 1) and in the pass through (lane 2) with the majority being removed during washing (lanes 3 and 4). A small concentration of BSA was eluted with 0.1M α-D-methylmannoside (lanes 7,8) and with 0.3M α-D-methylmannoside (lanes 9,10). As for identification of ECDβc by silver stain, there were three distinct protein bands that eluted from the column with 0.1M α-D-methymannoside (60, 55 and 50 kDa, lane 5), one of which could have been ECDβc.

Anti-βc affinity chromatography. An anti-βc affinity column was constructed by coupling the anti-βc mAb 1C1 to N-hydroxysuccinimide activated sepharose as outlined in section 6.2.5. The efficiency of the conjugation of the anti-βc antibody to the NHS-activated sepharose was assessed by analyzing an aliquot of the reaction mixture before and after the conjugation reaction by reducing SDS-PAGE and silver staining. Lane 1 shows the presence of both the heavy and light chians of the 1C1 IgG in the reaction mixture prior to conjugation, while lane 2 shows the absence of the IgG following conjugation, demonstrating that the majority of the anti-βc antibody was coupled to the sepharose (Figure 6.5). The column was equilibrated with PBS and a sham elution was performed (0.1M glycine, pH 2.5) to determine if the antibody would remain associated with the sepharose. Reducing SDS-PAGE and silver staining of the eluent did not reveal the presence of any protein bands (data not shown) demonstrating that the anti-βc mAb 1C1 did not dissociate from the column in the presence of the 0.1M glycine buffer. The column was re-equilibrated with PBS and 5 mL of Bm-5/ECDβc conditioned media was loaded onto the column. The column was washed extensively with PBS and eluted with 0.1M glycine buffer (pH 2.5). The eluent was collected in individual 1 mL fractions. 30μl of each fraction was analyzed by reducing SDS-PAGE, Western blotting with the anti-βc mAb 1C1 and silver staining. A 50 kDa protein band corresponding in size to ECDβc was eluted from the column with the 0.1M glycine buffer and showed a peak of Western blot activity in fraction 2 (Figure 6.6). Silver staining of a parallel SDS-PAGE gel did not reveal the presence of any protein bands suggesting that only a small amount of ECDβc was purified (data not shown).

Two smaller molecular weight protein bands of approximately 40 and 35 kDa, seen previously in the elution product of the concanavalin A-sepharose column (see Figure 6.4), were also present in fraction 2 of the Western blot (see Figure 6.6). To determine if these smaller molecular weight bands were novel proteins or degradation products of ECDβc, aliquots of fraction 1, which did not initially contain the smaller molecular weight bands, were analyzed by reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1 after being stored for 7 days at either -20°C or 4°C. The smaller molecular

weight protein bands appeared in fraction 1 material that had been stored at 4°C (Figure 6.7, lane 2) but not -20°C (Figure 6.7, lane 1) suggesting that the smaller molecular weight bands represented ECDβc degradation products and not a unique protein species.

6.3.2 Purification of ECDβc. *Affinity-chromatography alone.* Affinity purification of Bm-5/ECDβc conditioned media using the anti-βc column was used as the first step in purifying ECDβc. 5 mL of Bm-5/ECDβc conditioned media was purified over the anti-βc column. The column was washed extensively and eluted with 0.1M glycine buffer (pH 2.5). The eluent was collected as individual 600 µL fraction. The eluted fractions were reduced in volume 25 fold using disposable ultrafiltration devices prior to reducing SDS-PAGE. 10% of the concentrated sample volume was analyzed by Western blotting with the anti-βc mAb 1C1 and the remaining 90% by silver stain. Western blotting revealed at least two distinct protein bands in fractions 2,3 and 4 (Figure 6.8A) with the 50 kDa band corresponding in size to ECDβc. The two smaller molecular weight degradation products seen previously were also present in fraction 3. Silver staining the parallel gel revealed a complex pattern of protein staining with the 40 kDa ECDβc degradation product being clearly visible in lane 3 (Figure 6.8B). By inference, the ECDβc protein bands seen in the Western blot probably corresponded to the faint 50 kDa protein band in lane 3. However BSA clearly co-migrated with ECDβc in fractions 1-4 (Figure 6.8B) making it difficult to identify ECDβc.

To reduce the non-specific adsorption of BSA to the affinity column, 0.05% of the non-ionic detergent Tween-20 was included in the PBS wash buffer (PBST). 5 mL of Bm5/ECDβc conditioned media was purified over the column. The column was washed extensively with PBST and PBS. The column was eluted as above, the eluent was volume reduced and the fractions were analyzed by reducing SDS-PAGE, Western blotting with the anti-βc mAb 1C1 and silver staining. A 48 kDa ECDβc protein band was present in fraction 2-4 (Figure 6.9A) while the other bands seen in Figure 6.8A were missing. The silver stained gel (Figure 6.9B) showed a 48 kDa protein band in fractions 2 and 3, a 40 kDa protein band in fraction 3 and a number of smaller protein bands. The

40 kDa band was likely a degradation product of ECDβc. A faint 62 kDa band was also visible in fractions 2-4 (Figure 6.9B) and was likely BSA.

Affinity-chromatography followed by size exclusion chromatography. The eluent in fraction 3, which contained the putative ECDβc protein, was fractionated on the Superose 12/30 size exclusion column. SDS-PAGE and Western blotting of the eluted fractions revealed a 48 kDa protein doublet and a 35 kDa protein band in fractions 58-66 (Figure 6.10A) that corresponded in size to ECDβc and the ECDβc degradation product, respectively. Silver staining showed the same staining pattern in fractions 58-66 (Figure 6.10B) suggesting that the 48 kDa doublet was indeed ECDβc.

Affinity-chromatography followed by ion exchange chromatography. Ion exchange chromatography was also tried as a second step in purifying ECDβc. 5 mL of Bm-5/ECDβc conditioned media was purified over the anti-βc affinity column in the presence of Tween-20 exactly as described above. The eluent was volume reduced by ultrafiltration and applied to a MonoQ anion exchange column. Following extensive washing of the MonoQ column with the Tris runing buffer, the adsorbed proteins were eluted with a linear gradient of 1M NaCl over a 60 minute period (Figure 6.11A). 200 μL of each fraction was adsorbed directly to PVDF membrane by vacuum filtration using a dot-blot apparatus. Western blotting of the membrane with the anti-βc mAb 1C1 showed the elution of a protein recognized by the anti-βc antibody starting at fraction 27 (Figure 6.11B, fractions 27-34). Reducing SDS-PAGE and silver staining of the same fractions revealed a single 66 kDa protein band in fractions 30-33 that corresponded in size to BSA but no protein band in fraction 27-30 (data not shown). Fractions 27 and 28, which showed significant ECDβc immunological activity by Western blot but appeared to be free of BSA by silver stain, were pooled and adsorbed to a piece of PVDF membrane and stained with Coomasie blue. The protein concentration was estimated to be approximately 5 μg. The membrane was washed extensively and subject to amino acid analysis. The amino acid composition of the hydrolyzed protein did not correspond to ECDβc suggesting that proteins other than ECDβc were co-eluting in fractions 27 and 28. This result was confirmed by analysis of the remainder of fractions 27 and 28 by

reverse phase chromatography, which showed multiple peaks of UV absorption (data not shown).

Affinity-chromatography followed by reverse phase chromatography. Reverse phase chromatography was also tried as a second step for purifying ECDβc. 5 mL of Bm-5/ECDβc conditioned media was purified over the anti-βc affinity column in the presence of Tween-20 as described above. The eluent was pooled, volume reduced, resuspended in 0.1% TFA and injected over the C4 RPC column. The column was washed with 5 mL of 0.1% TFA and eluted with a linear gradient of 0-100% 0.1%TFA in acetonitrile over 30 minutes. The chromatograph showed a broad peak of UV absorption in the 22-25 minute range (Figure 6.12A, panel A) indicating that ECDβc, which eluted in fraction 24 by reducing SDS-PAGE and Western blot with the anti-βc mAb 1C1 (data not shown), was co-eluting with other proteins. The elution profile was therefore extended to 60 minutes to improve the separation between eluting proteins. The broad peak in 6.12A (panel A) was resolved into three individual peaks when the column was eluted over a 60 minute period (Figure 6.12A, panel B). The separation between the peaks was improved further by lengthening the elution period to 90 minutes (Figure 6.12A, panel C). Reducing SDS-PAGE and silver staining of the fractions showed a distinct 48 kDa protein doublet corresponding in size to ECDβc in fraction 45, with a 66 kDa protein band that corresponded to BSA in fraction 46 (data not shown). The purification experiment was repeated with an elution period of 120 minutes to try and improve the resolution of the separation further. The 120 minute elution period resolved the single peak seen in fraction 46 (Figure 6.12A, panel C) into two individual peaks, one at 53 minutes and one at 54 minutes (Figure 6.12A, panel D). The broad peak that was shown in Figure 6.13A (panel A) to correspond to BSA now eluted at 56 minutes.

30 uL aliqouts of fractions 52-56 purified purified by reverse phase chromatography uisng the 120 minute elution profile (see Figure 6.12A, panel D) were analyzed by reducing SDS-PAGE and Western blotting with anti-βc mAb 1C1. A 45 kDa protein band eluted in fraction 54 and 55 by Western blot (Figure 6.12B, panel A). Reducing SDS-PAGE followed by silver staining of aliqouts of the same fractions showed the same

45 kDa protein band (Figure 6.12B, panel C). Importantly, BSA could be clearly differentiated from ECDβc by its elution in fraction 56 and 57 (Figure 6.12B, panel C). Analyzing the same fractions by non-reducing SDS-PAGE and Western blotting showed the same 45 kDa protein band (Figure 6.12B, panel B). Finally, analyzing the same fractions by non-reducing SDS-PAGE and silver staining also revealed the same 45 kDa protein band (Figure 6.12B, panel D) suggesting that the 45 kDa protein band was ECDβc. Importantly, the 75 kDa protein band seen by silver staining under non-reducing conditions (Figure 6.12B, panel D) did not disappear in the presence of β-mercaptoethanol (Figure 6.12B, panel B) suggesting that this protein did not correspond to a disulphide bonded multimer of ECDβc. The absence of a higher molecular weight band on the non-reduced Western blot (Figure 6.12B, panel C) also suggests that the 75 kDa protein bands in the silver stains were not oligomers of ECDβc. However, the possibility that the 75 kDa protein band was in fact a higher molecular weight aggregate of ECDβc that was not recognized by the anti-βc mAb 1C1 because the epitope was buried within the protein complex can not be ruled out.

To confirm the identity of the 45 kDa protein band, the experiment was repeated and fraction 54 and 55 were pooled and fractionated by reducing SDS-PAGE. The proteins were transferred to PVDF membrane and stained with Coomassie blue. The stained protein band corresponding in size to ECDβc was excised from the blot and subject to N-terminal sequencing. 8 rounds of N-terminal sequencing were performed generating a weak chromatographic signal corresponding to the following sequence:

Determined sequence:	eXeIPLQI
Expected sequence:	EETIPLQT

The low protein concentration, estimated to be less than 1 μg, was likely responsible for the incomplete and weak amino acid sequence. Nevertheless, the determined sequence corresponding to residues 9-16 of the mature ECDβc protein sequence was similar to the expected ECDβc sequence. Amino acid analysis was performed on the

remaining protein sample to confirm that the N-terminal sequence obtained was derived from ECDβc and not from another contaminanting. Amino acid analysis of the remainder of the hydrolyzed protein revealed an amino acid composition that was similar to but not entirely consistent with that of ECDβc:

Amino Acid	**Expected**	**Determined**
Asp + asn	36	39
Thr	30	23
Ser	44	67
Glu + gln	49	61
Pro	32	20
Gly	17	66
Ala	25	33
Val	29	18
Met	6	3
Ile	13	16
Leu	35	25
Tyr	12	13
Phe	10	8
His	14	17
Lys	11	37
Arg	32	15

The discrepency between the expected amino acid composition of ECDβc and the determined amino acid composition was likely due to the low protein concentration used and the resulting poor resolution on the chromatograph. However, the possibility that the observed amino acid sequence corresponded to a protein other than ECDβc can not be ruled out (see below).

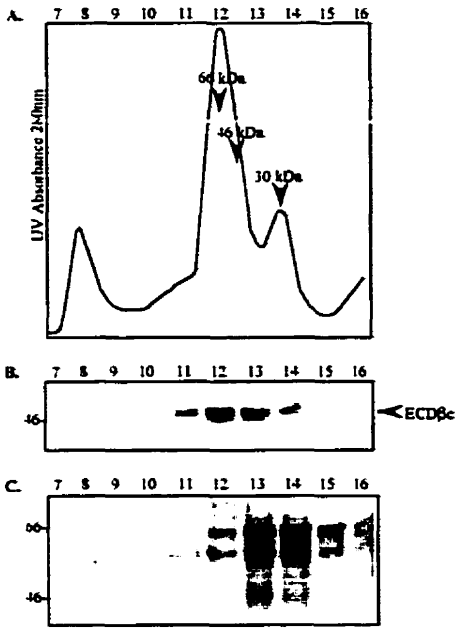


Figure 6.1. Size exclusion chromatography of Bm-5/ECDβc conditioned media. A. 200 µL of Bm-5/ECDβc conditioned media was applied to a Superose 12/30 size exclusion column equilibrated with PBS at 4C. The column was run at a flow rate of 1 mL/minute. The proteins were collected every minute as 1 mL fractions as they eluted off the column (the chromatograph for fractions 7-16 is shown). Elution of the proteins from the column was monitored by UV absorbance at 280 nm. The position of elution of various molecular weight markers are labelled on the chromatograph. B. 30 uL aliqouts of fractions 7-16 were analyzed by reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1. C. 30 uL aliqouts of fractions 7-16 were also analyzed by reducing SDS-PAGE and silver staining.

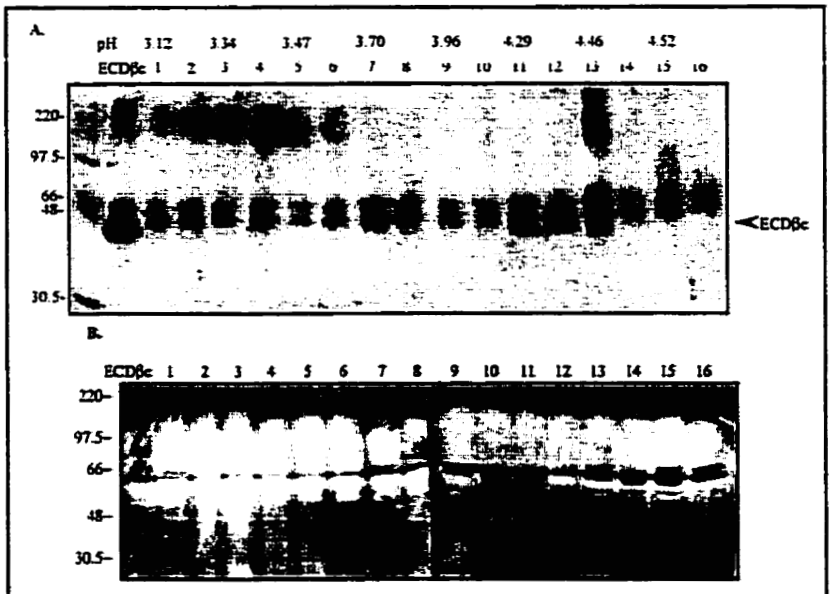


Figure 6.2. Isoelectric focusing of Bm-5/ECDβc conditioned media. (A) A pH gradient (pH = 3.12-8.63) was generated by electrophoresing a mixture of ampholytes through a 20 cm by 12 cm flat bed of agarose. 2 mL of desalted Bm-5/ECDβc conditioned media was loaded at the anode (where the pH = 8.63, corresponding to fraction 24, not shown) and subject to electrophoresis. The agarose bed was harvested into 24 individual fractions (labelled 1-24) of approximately 1 mL of gel each. The gel fractions were mixed with 1 mL of water and the pH of each fraction was determined. A 30 uL aliqout of each of fractions 1-24 was analyzed by reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1. The Western blot corresponding to fractions 1-16 is shown above. The pH of every second fraction is labelled above the fraction number. (B) A 30 uL aliqout of each of fractions 1-24 was also analyzed by reducing SDS-PAGE and silver staining. The silver stained gel for fractions 1-16 is shown above.

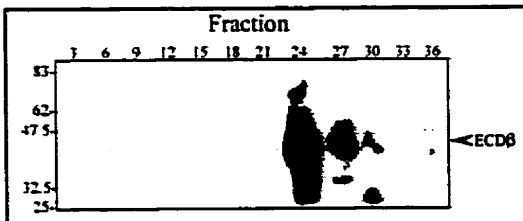


Figure 6.3. Reverse phase chromatography of Bm-5/ECDβc conditioned media. 1 mL of Bm-5/ECDβc conditioned media was mixed with 1 mL of 0.1% TFA and applied to a C4 reverse phase column that was equilibrated with 0.1% TFA at room temperature with a flow rate of 1 mL/minute. The column was washed with 5 mL of running buffer and the adsorbed proteins were eluted with a linear gradient (0-100%) of 0.1% TFA in acetonitrile over a 30 minute time period. Tightly adsorbed proteins were eluted from the column by holding the buffer concentration at 100% of the 0.1% TFA/acetonitrile buffer for 5 minutes. The eluent from the column was collected into individual 1 mL fractions. 30 uL aliquots from every third fraction (the numbering of the fractions includes those fractions collected during the 5 minute washing period. the 30 minute gradient elution and the 5 minute final elution period to give a total of 40 fractions) were analyzed by reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1.

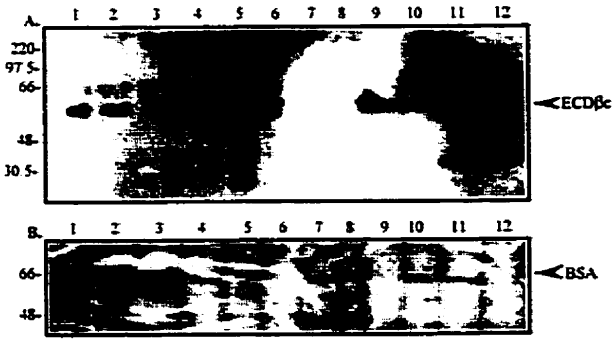


Figure 6.4. Concanavalin A-Sepharose affinity purification of Bm-5/ECDβc conditioned media. 2 mL of Bm-5/ECDβc conditioned media was resuspended in 8 mL of running buffer and applied to the column. The material that passed through the column was collected. The column was then washed twice with 10 mL of running buffer - this material was also collected. The column was next eluted twice with 10 mL of each of the following: 0.1M, 0.3M and 0.5M solutions of α-D-methylmannoside. The column was then eluted once with 10 mL of a 0.5M solution of NaCl (pH=4.5) and once with a 10 mL solution of 0.5M solution of NaCl (pH=8.5). 30 uL aliqouts of each of the 10 mL fractions that were collected (as described above) were analyzed by reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1 **(A)** and by silver staining **(B)**. 1: unpurified Bm5/ECDβc conditioned media; 2: column pass through; 3,4: column washes; 5,6: fractions eluted with 0.1M α-D-methylmannoside; 7,8: fractions eluted with 0.3M α-D-methylmannoside; 9,10: fractions eluted with 0.5M α-D-methylmannoside; 11: fraction eluted with 0.5M NaCl pH 4.5; 12: fraction eluted with 0.5M NaCl pH 8.5.

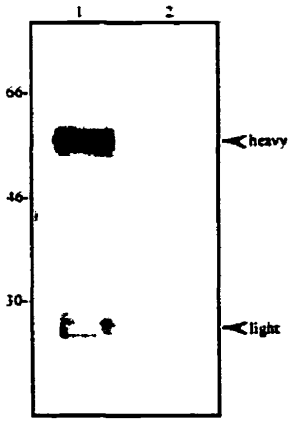


Figure 6.5. Conjugation of the anti-βc mAb 1C1 to NHS-activated sepharose. The efficiency of the conjugation reaction was measured by comparing the quantity of anti-βc antibody in the reaction mixture before and after conjugation by SDS-PAGE and silver staining. 1: before; 2: after.

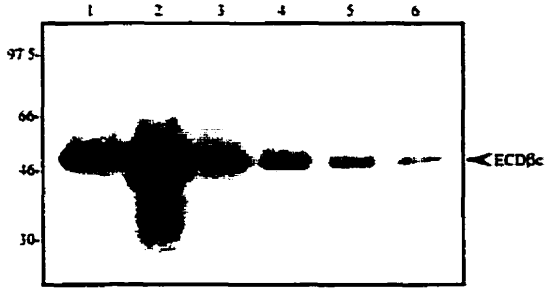


Figure 6.6. Anti-βc affinity purification of Bm-5/ECDβc conditioned media. 5 mL of Bm-5/ECDβc conditioned media was loaded on the affinity column. The column was washed extensively with PBS and the adsorbed proteins were eluted off the column with 6 mL of a 0.1M glycine buffer (pH 2.5). The eluent was collected into 6 individual fractions of 1 mL each (labelled 1-6). 30 uL aliqouts of each fraction were analyzed by reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1.

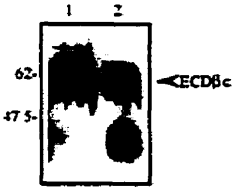


Figure 6.7. Degradation products of ECDβc. 30 uL aliqouts of anti-βc affinity purified material from fraction 1 (Figure 6.6) were stored at -20C (1) or at 4C (2) for 7 days and analyzed by reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1.

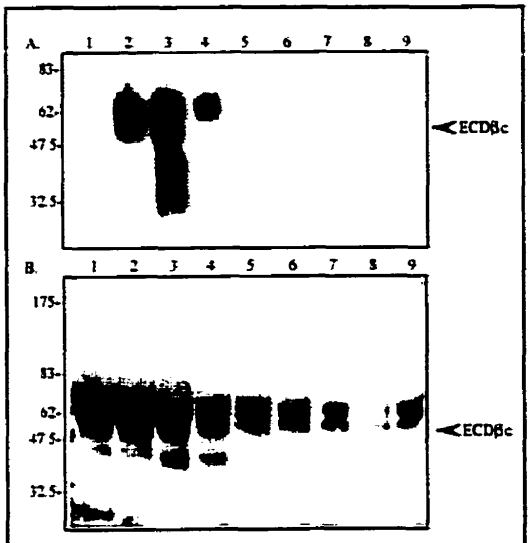


Figure 6.8. Anti-βc affinity purification of Bm-5/ECDβc conditioned media. 5 mL of Bm-5/ECDβc conditioned media was applied to the affinity column. The column was washed extensively with PBS and eluted with 6 mL of 0.1M glycine buffer (pH 2.5). The eluent was collected into 9 individual fractions (1-9) of 600 uL. Each fraction was volume-reduced 25 fold by ultrafiltration. 10% of the volume-reduced eluent from each fraction was analyzed by reducing SDS-PAGE and Western blotting with anti-βc mAb 1C1 (A) and the remaining 90% of each fraction was analyzed by reducing SDS-PAGE and silver staining (B).

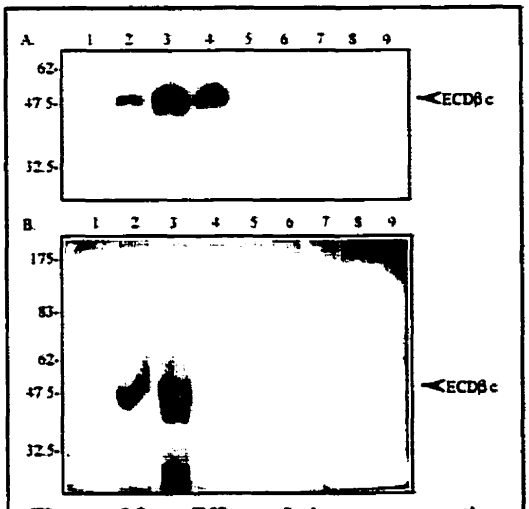


Figure 6.9. Effect of detergent on the stringency of the anti-βc affinity purification of Bm-5/ECDβc conditioned media. 5 mL of Bm-5/ECDβc conditioned media was purified and volume reduced exactly as in Figure 6.8 except that 0.05% Tween-20 was included in the wash buffer. The adsorbed proteins were eluted and analyzed exactly as in Figure 6.8. (A) Western blot with anti-βc mAb 1C1; (B) Silver stain.

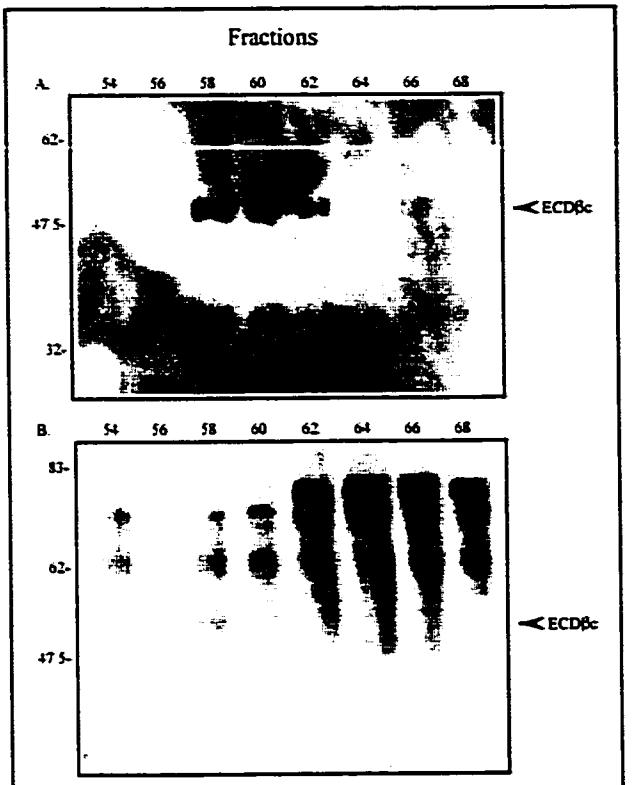


Figure 6.10. Size exclusion chromatography of anti-βc affinity purified ECDβc. 5 mL of Bm-5/ECDβc conditioned media was applied to the anti-βc affinity column. The column was washed extensively with PBS and the adsorbed proteins were eluted off the column with 6 mL of 0.1M glycine buffer (pH 2.5). The 6 mL eluent was volume reduced to approximately 200 uL and was injected onto a Superose 10/30 size exclusion column that was equilibrated with PBS at 4C. The column flow rate was 200 uL/minute. The column flow through was collected every minute for 90 minutes to give 90 fractions of 200 µL each (shown are fractions 54 to 68). 30 uL aliqouts from every other fraction were analyzed by reducing SDS-PAGE and Western blotting with the anti-bc mAb 1C1 (A) and by silver staining (B).

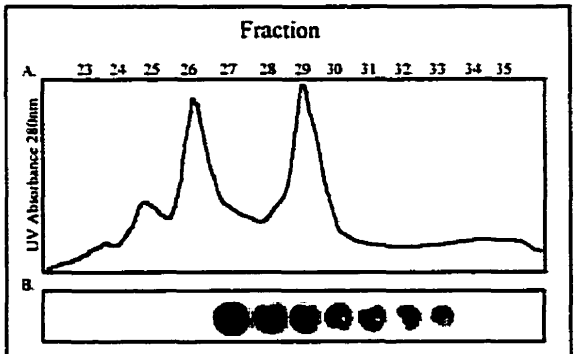


Figure 6.11. Ion exchange chromatography of affinity purified ECDβc. 5 mL of Bm-5/ECDβc conditioned media was applied to the anti-βc affinity column. The column was washed extensively with PBS and the adsorbed proteins were eluted off the column with 6 mL of 0.1M glycine buffer (pH 2.5). The 6 mL eluent was volume reduced to approximately 200 uL and was injected onto a MonoQ anion exchange column that was equilibrated with running buffer (50 mM Tris, 137 mM NaCl, pH 8.3) at 4C and at a flow rate of 200 uL/min. The column was washed with running buffer until the UV absorbance returned to baseline at which time the adsorbed proteins were eluted with a linear gradient (0-100%) of 1M NaCl buffer. The elution of the protein from the column was monitored by UV absorbance at 280 nm (A). The eluent was collected into 60 individual fractions of 400 uL each. 200 uL of each fraction (shown above are fractions 23-35) were adsorbed directly to PVDF membrane using a dot-blot apparatus. The membrane was subject to Western blotting with the anti-βc mAb 1C1 (B).

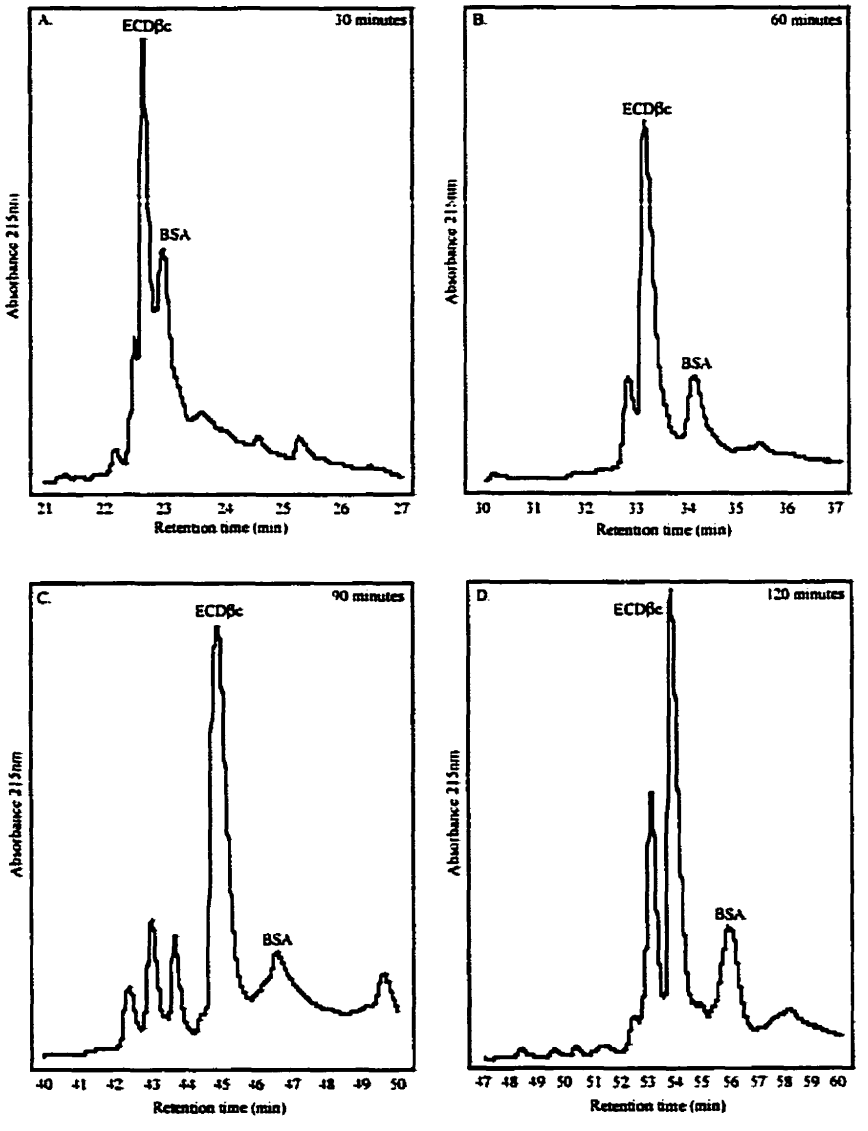


Figure 6.12A. Optimization of RPC of anti-βc purified ECDβc. 5 mL of Bm-5/ECDβc conditioned media was applied to the anti-βc affinity column. The column was washed extensively with PBS and the adsorbed proteins were eluted off the column with 6 mL of 0.1M glycine buffer (pH 2.5). The 6 mL eluent was volume reduced to approximately 200 uL and was mixed with an equal volume of 0.1% TFA and injected onto a C4 reverse phase column that was equilibrated with 0.1% TFA at a flow rate of 1 mL/minute. The column was washed with running buffer and eluted with a 0-100% linear gradient of 0.1%TFA/acetonitrile. The elution time was varied from 30 minutes (A) to 120 minutes (D) in order to achieve baseline resolution of each peak of UV absorbance. The elution position of ECDβc and BSA are indicated on the representative chromatographs.

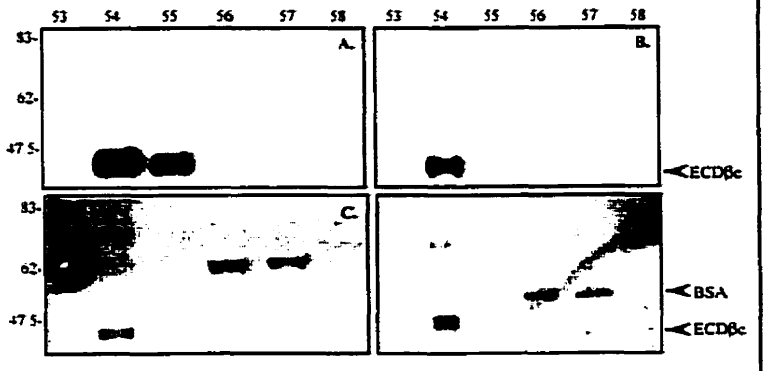


Figure 6.12B. 30 uL aliqouts of fractions 53-58 (from Figure 6.12A, panel D) were analyzed by reducing and non-reducing SDS-PAGE and Western blotting with the anti-βc mAb 1C1 and by silver staining. The positions of ECDβc and BSA are indicated on the right. A: Reducing SDS-PAGE and Western blotting; B: Non-reducing SDS-PAGE and Western blotting; C: Reducing SDS-PAGE and silver staining; D: Non-reducing SDS-PAGE and silver staining.

6.4 Discussion

6.4.1 Method scouting. Five protein purification techniques were investigated for their potential use in purifying ECDβc from crude Bm-5/ECDβc conditioned media. Size exclusion chromatography (SEC) is a gentle procedure that can be performed under physiological buffer conditions. SEC is however limited in its ability to separate proteins of similar molecular weight and shape, with a minimum requirement for good resolution being at least a two-fold difference in size between the protein of interest and the contaminants. Since ECDβc exists as a 45-50 kDa protein it is very similar in size to BSA and the other constituents of the tissue culture media making it difficult to separate it from the contaminants. ECDβc eluted from the size exclusion column as a 48 kDa protein band over a narrow range of fractions (Figure 6.1B, lanes 11-14) with a peak of intensity in fractions 12 and 13. A similar sized protein band was also present by silver staining (Figure 6.1C) but its peak of intensity was in fractions 13 and 14 rather than 12 and 13. It is likely that the 48 kDa protein band visualized by silver staining in fractions 13 and 14 was not ECDβc but was instead a constituent of the cell conditioned media as it was also clearly visualized by SDS-PAGE and silver staining of crude Bm-5/ECDβc conditioned media (Figure 6.4B, lane 1) and is also present in mock-transfected Bm-5 cell conditioned media (data not shown). Therefore, no clear correspondence could be made between the identity of the 48 kDa protein visualized by silver staining and Western blotting. The 66 kDa protein band seen in all lanes (Figure 6.1C) was likely BSA.

Flatbed isoelectric focusing is a simple yet effective method of protein purification that separates proteins based on their net charge. A 46 kDa protein band corresponding in size to ECDβc migrated with an apparent isoelectric point of 4.3-4.5 (Figure 6.2A, lanes 11-13). This pH range is similar to the pI estimated from the amino acid content of ECDβc (pI=5.6). Interestingly, a number of other proteins were also visualized by Western blotting with the anti-βc mAb 1C1 in addition to the putative ECDβc band (Figure 6.2A, lane 13). Whether these bands represented non-specific binding of the 1C1

antibody to the blot, differentially glycosylated forms of ECDβc or higher molecular weight aggregates was not determined. No distinct 46 kDa protein band could be visualized by silver staining making it impossible to clearly identify ECDβc (data not shown).

Reverse phase chromatography separates proteins according to their affinity for a hydrophobic stationary matrix. The presence of an ion coupling agent such as trifluoroacetic acid (TFA) in the running buffers neutralizes the charge of the protein allowing the hydrophobic regions of the peptide to interact with the hydrophobic alkyl chains of the column matrix. An organic solvent such as acetonitrile is used to disrupt the hydrophobic interaction and elute the adsorbed proteins. The unique hydrophobic properties of peptides that may differ little in other physical characteristics gives RPC extraordinary resolution and makes it a highly useful technique for separating proteins. However, the use of ion pairing reagents and organic buffers can result in permanent denaturation of the purified protein and subsequent loss of activity. Therefore, while a useful technique for the purification of proteins for sequencing or use as a concentration standard, RPC may not be the purification method of choice for isolating ECDβc for functional studies. ECDβc was adsorbed to the C4 column and eluted at an acetonitrile concentration of 45%. Similar to the results seen in the IEF experiments a number of protein bands were visualized by Western blot in addition to the expected 48 kDa ECDβc (Figure 6.3). The identity of these bands was not determined but again could suggest the presence of differentially glycosylated isoforms. Importantly, the chromatograph revealed that ECDβc did not elute on its own in these fractions but was accompanied by a large concentration of other constituents (data not shown) demonstrating that ECDβc could not be purified in a single step by RPC.

The ability of ECDβc to be specifically adsorbed and eluted from Concanavalin A-Sepharose (Chapter 4.3.1) suggested that this might be a useful technique for purification. Concanavalin A binds terminal mannose residues in the presence of Ca^{2+} and Mn^{2+} ions. Competitive elution with either mannose, glucose or methylated derivatives of these two simple sugars is a gentle method of protein elution that should not affect the proteins

activity. ECDβc was adsorbed to the Concanavalin A-sepharose matrix and eluted with a step gradient of 0.1M to 0.5M α-D-methylmannoside (Figure 6.4). The majority of ECDβc was eluted as a 50 kDa protein with 0.1M α-D-methylmannoside but also to a lesser extent with 0.3M, 0.5M α-D-methylmannoside and with 0.5M NaCl pH 4.5 (Figure 6.4A). The majority of the constituents in the conditioned media, including a large portion of BSA, did not adsorb to the Concanavalin A-column resulting in significant purification of ECDβc (Figure 6.4B). However ECDβc could not be clearly identified in the column eluent following purification.

The final purification method attempted was affinity chromatography using an affinity column contsructed with the anti-βc mAb 1C1. 1C1 was effectively linked to the activated sepharose (Figure 6.5) and remained associated with the matrix following elution of the column (data not shown). ECDβc in the Bm-5/ECDβc conditioned media was adsorbed to the column and eluted as a 50 kDa protein band, with a peak of Western blot intensity in fraction 2 (Figure 6.6). The smaller molecular weight bands in fraction 2 (approximately 35 kDa and 40 kDa) were degradation products of ECDβc as the 50 kDa protein in lane 1 also migrated as two distinct bands if stored at 4°C but not -20°C (Figure 6.7). Silver staining of the gel did not reveal the presence of any protein bands suggesting that more sample would have to be analyzed if ECDβc were to be visualized by silver staining.

6.4.2 Purification of ECDβc. *Affinity-chromatography alone.* The method scouting exercise identified a number of useful methodologies for the purification of ECDβc. However, affinity purification of ECDβc using the anti-βc Sepharose column was by far the most efficient method of purifying and enriching ECDβc and was therefore chosen as the first purification step. The following two modifications to the anti-βc affinity purification protocol outlined in section 6.3.1 were made. First, to better visualize ECDβc by silver staining, the volume of the individual fractions eluted from the affinity column were reduced 25 fold using disposable ultrafiltration devices. This allowed analysis of an entire fraction by SDS-PAGE and silver staining, substantially improving the sensitivity of the assay. Western blotting of the concentrated column fractions with

the anti-βc mAb 1C1 showed four independent protein bands ranging in size from 35 to 62 kDa (Figure 6.8). The 50 kDa protein band corresponded in size to ECDβc. The 62 kDa protein band, while apparently recognized by the anti-βc mAb 1C1, did not represent a different isoform of ECDβc since similar sized protein bands have also been visualized when other ECDβc-containing Western blots have been probed with secondary antibody alone. Furthermore, the 62 kDa protein band was absent when the purification procedure was performed in the presence of a small amount of detergent suggesting that protein was adsorbed non-specifically to the column (see below). The 40 kDa and 35 kDa protein bands were likely the previously described degradation products of ECDβc. Silver staining of a parallel blot showed a large quantity of BSA co-eluting with ECDβc which made it difficult to visualize ECDβc on the silver stain (Figure 6.8B, lanes 1-3). Presumably, ECDβc was present above the 40 kDa ECDβc degradation product buried beneath the mass of co-eluting proteins.

The second modification to the affinity purification protocol was to increase the stringency of the wash step by incorporating a small amount of nonionic detergent in the wash buffer. The addition of 0.05% Tween-20 to the wash buffer removed the majority of the proteins that had previously adsorbed to the affinity column, leaving only a 48 kDa protein band that corresponded in size to ECDβc (Figure 6.9A). However, the presence of the detergent in the wash buffer also resulted in a reduction in the yield of ECDβc (compare Figure 6.8A with 6.9A). Of interest, it was primarily the unidentified 62 kDa protein and ECDβc degradation products that were washed from the column with the detergent containing wash buffer. Importantly, what appeared to be ECDβc by Western blot could also be visualized by silver staining when purified in the presence of Tween-20 (Figure 6.9B).

Affinity purification of ECDβc using the anti-βc column was an efficient purification step. However, it was clearly insufficient to completely separate ECDβc from the other constituents of the conditioned media. Size exclusion chromatography, ion exchange chromatography and reverse phase chromatography were all shown to be efficient

techniques for the capture and partial purification of ECDβc and were therefore re-investigated as potential secondary purification steps for isolating ECDβc.

Affinity-chromatography followed by size exclusion chromatography. Size exclusion chromatography of affinity-purified ECDβc increased the separation of ECDβc from other proteins sufficiently so that a 48 kDa protein doublet corresponding in size to ECDβc could be clearly visualized by both Western blot and silver staining (Figure 6.10). The 35 kDa protein that co-migrated with the putative ECDβc band likely corresponded to the previously identified ECDβc degradation product.

Affinity-chromatography followed by ion exchange chromatography. Determination of the isoelectric point of ECDβc by IEF permitted experimentation with ion exchange chromatography (IEX) as a secondary purification technique. Ion exchange chromatography is an effective means of fractionating charged particles such as proteins by exploiting the subtle differences in net charge of different proteins under specific buffer and salt conditions. Proteins are adsorbed from solution to either a positively (anion exchange chromatography) or negatively (cation exchange chromatography) charged matrix and are eluted from the column with an ionic buffer such as NaCl or a change in pH. The IEF experiments showed that ECDβc was a negatively charged protein at neutral pH with an estimated isoelectric point of 4.3-4.5. Indeed, ECDβc adsorbed strongly to the positively charged sepharose matrix of the MonoQ column and required a strong NaCl solution (approximately 0.6M) to elute (Figure 6.11B). This single purification step resulted in removal of a large portion of unwanted molecules from the fractions containing ECDβc including BSA. However, amino acid analysis of fractions 27 and 28, which contained ECDβc by Western blot but appeared to be free of other protein constituents by silver stain, showed that ECDβc was not the only constituent in these fractions. Indeed, reverse phase chromatography of the pooled fractions showed multiple peaks by UV absorption demonstrating that anti-βc affinity chromatography followed by IEX would not be sufficient to purify ECDβc to homogeneity.

Affinity-chromatography followed by reverse phase chromatography. Reverse phase chromatography was also attempted as a second step in the purification of ECDβc. Affinity purified ECDβc was applied to the C4 column and eluted with a linear gradient of acetonitrile. The purification of ECDβc was optimized by increasing the time of the elution from 30 minutes to 120 minutes (Figure 6.12A). ECDβc eluted as a single peak of UV absorbance at 54 minutes which corresponded with an acetonitrile concentration of 45% (Figure 6.12A, panel D). A 45 kDa protein doublet was visualized by both Western blotting and silver staining of fraction 54, both in the presence and absence of β-mercaptoethanol (Figure 6.12B). The higher molecular weight bands that co-eluted with ECDβc in fraction 54 did not correpond to multimers of ECDβc as they were not recognized by the anti-βc mAb 1C1 (Figure 6.12B, panels A and C) and there was no change in their migration pattern by silver stain in the presence or absence of β-mercaptoethanol (Figure 6.12B, panels B and D).

The purification protocol outlined above provided what appeared to be a homogeneous source of ECDβc. To confirm this, the putative ECDβc protein band was isolated by anti-βc affinity chromatography, reverse phase chromatography and reducing SDS-PAGE and was immobilized on PVDF membrane by electroblotting. The immobilized protein was visualized by Coomassie blue staining and the concentration was estimated to be less than 1 μg of protein. The protein band was excised from the blot and subject to N-terminal amino acide sequencing. The low protein concentration provided a weak chromatographic signal that corresponded to residues 9-16 of the mature ECDβc protein. Amino acid analysis of the putative ECDβc protein band revealed an amino acid composition that was similar but not identical to that of ECDβc. The low protein concentration of the putative ECDβc protein band was likely to blame for the poor correlation between the predicted and determined amino acid composition of ECDβc. On the other hand, the possibility that a protein other than ECDβc was mistakenly purified using the anti-βc affinity chromatography/reverse phase chromatography procedure can not be ruled out.

For another protein to have been mistakenly purified and identified as ECDβc, all of the following variables would have had to been in place. First, if the hypothetical contaminating protein was derived from the Bm-5/ECDβc conditioned media it would have to have a strong affinity for the anti-βc mAb 1C1 that was immobilized on the affinity-column since this purification step was performed under relatively stringent conditions (0.02% Tween-20 was included in the wash buffer). Second, the hypothetical contaminating protein would also have to be similar in size to ECDβc (approximately 45-48 kDa) for it to be mistakenly identified as ECDβc. Third,the contaminating protein band would have also had to share an epitope for either the anti-βc mAb 1C1 or the polyclonal rabbit-anti mouse antibodies that were used for Western blotting. Fourth, the hypothetical contaminating protein band would also have to have an N-terminal amino acid sequence that corresponded to that of ECDβc.

On the other hand, one could argue that the hypothetical contaminating protein did not arise from the Bm-5/ECDβc conditioned media but instead from the anti-βc affinity column itself. That is, the contaminating protein could have been the anti-βc 1C1 antibody that had leached off the affinity-column during elution with the acidic glycine buffer. However, when the affinity column was sham-eluted with glycine buffer alone (pH 2.5) no protein bands were ever visualized in the eluent. Further, there was a direct correlation between the concentration of ECDβc in a given batch of crude Bm-5/ECDβc conditioned media and the concentration of ECDβc purified from that media using the anti-βc affinity column. Since there should be no relationship between the concentration of ECDβc in the Bm-5/ECDβc crude conditioned media and the amount of anti-βc mAb 1C1 that may have leached off the affinity-column, it seems highly unlikely that the putative ECDβc protein band that was isolated using the affinity-column could have instead been the anti-βc mAb 1C1. Therefore, the data in this chapter suggest that ECDβc can be purified from Bm-5/ECDβc conditioned media by affinity-chromatography using the anti-βc mAb 1C1 immobilized on a sepharose matrix, followed by reverse phase chromatography using a C4 analytical column.

CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Identification of the naturally occuring sGMRα protein. sGMRα is a naturally occuring protein that is expressed by normal and leukemic hematopoietic cells and can be detected in human peripheral blood (Chapter 3). Recent work by our group has confirmed and extended my preliminary findings[235]. The sGMRα protein expressed by leukemic cell lines and in human plasma was shown to bind ^{125}I-GM-CSF with a similar affinity as recombinant sGMRα (Kd=2-7 ηM). sGMRα was also shown to be expressed by the leukemic cell lines and in human plasma at very low concentrations (5-50 pM). Importantly, recent work by our group has observed a substantial increase in sGMRα expression (300-500 pM vs 30-50 pM) in plasma from individuals with a subtype of acute myelogenous leukemia suggesting that sGMRα expression may be upregulated during leukemogenesis. Finally, preliminary investigation into the mechanism of regulation of expression of sGMRα has demonstrated that certain inflammatory stimuli can increase the expression of sGMRα by human mononuclear cells.

7.2 Engineering, expression and characterization of soluble isoforms of the GMRα and βc receptor subunits. ECDα was expressed as a functional soluble protein by both mammalian and insect cells (Chapter 4). The loss of the 16-amino acid C-terminal domain of sGMRα did not affect the activity of this protein demonstrating that the extracellular domain of GMRα is sufficient for ligand binding. Of interest, the lower level of glycosylation of insect-expressed ECDα did not alter its ability to bind GM-CSF in solution demonstrating that complex glycosylation of GMRα is not required for activity.

ECDβc was expressed by both mammalian and insect cells as a monomer and as what appeared to be a disulphide-bonded homodimer (Chapter 4). As βc has been shown to exist in part as a non-functional homodimer on the cell surface[159,195] it would be of interest to use ECDβc to map the location of the sulfhydryl groups that mediate this interaction.

This might help explain why homodimerization of βc can, under certain circumstances, lead to constitutive activation. I would hypothesize that the interactions that mediate the formation of non-functional versus constitutively active homodimers of βc would map to very different regions of the receptor subunit since activation of βc is probably not mediated by facile dimerization but is instead dependent upon bringing the cytoplasmic domains of two βc subunits into close enough proximity for the associated JAK2 molecules to transphosphorylate one another. Therefore I would predict that the dimerization of βc and ECDβc that leads to the formation of non-functional homodimers would be mediated through the N-terminal region of the subunits while the homodimerization of βc that leads to constitutive activation would result in the approximation of the membrane proximal domains of the two receptor subunits.

7.3 Determinants of the functional interaction between the soluble GM-CSF receptor and βc. Co-expression of sGMRα with ECDβc results in their constitutive association (Chapter 5). It would be of interest to determine if this complex can bind GM-CSF in solution, and if so, if it occurs with higher affinity that sGMRα alone. It would also be of interest to determine if a naturally occurring soluble isoform of βc exists and if it can form a similar complex when co-expressed with sGMRα. The recent isolation of a soluble splice variant of βc from mononuclear cells suggests that a soluble βc receptor may indeed exist in vivo. The existence of a soluble βc subunit with the potential to convert sGMRα to a higher affinity receptor complex (see below) would also be of particular interest in understanding how sGMRα, which is expressed at very low concentration *in vivo*, can effectively antagonize the activity of the high affinity cell surface GM-CSF receptor.

The extracellular domains of GMRα and βc can associate in solution in the presence of GM-CSF and can bind GM-CSF with higher affinity than ECDα or sGMRα alone (Chapter 5). The range of dissociation constants determined for this higher affinity complex (98 pM-800 pM) suggest however that other factors might be involved in mediating high affinity binding. I would hypothesize that, as demonstrated on the cell surface[205], the molar ratio of ECDα to ECDβc can influence the affinity of the receptor

complex for GM-CSF. With the acquisition of a quantitative assay for sGMRα and the purification of ECDβc (see 7.4) it is now possible to test this hypothesis.

7.4 Purification of ECDβc. ECDβc was purified to homogeneity from cell conditioned media by anti-βc affinity chromatography and reverse phase chromatography (Chapter 6). The purified receptor subunit can now be quantified and used as a concentration standard in a Western blot assay to determine the concentration of ECDβc in crude conditioned media. The purified receptor can also be used for generating anti-βc antibodies that could be used to scale up the purification protocol. It would also be highly useful to develop a gentler protocol for purifying ECDβc that was not dependent upon reverse phase chromatography. Preliminary results have shown that a multi-step protocol using anion-exchange chromatography, hydrophobic interactive chromatography, conA-sepharose affinity chromatography followed by size exclusion chromatography can effectively purify substantially larger concentrations of ECDβc under much gentler conditions. The purification of larger quantities of functional ECDβc would allow us to expand our investigation into the structure and function of the GM-CSF receptor complex.

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