# CONSTRUCTION AND CHARACTERIZATION OF A CHIMERICAL CYTOKINE WHICH BINDS TO CELLULOSE: FUSION OF A BACTERIAL CELLULOSE-BINDING DOMAIN TO STEEL FACTOR.

by

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**Abstract.**

The carboxy-terminal cellulose-binding domain (CBD) from the bacterial cellulase Cex was fused to the amino-terminal extracellular domain from the murine growth factor steel factor (SLF) to create a chimeric growth factor (SLF-CBD) which binds specifically to cellulose. The biological activity of this fusion protein was tested using the murine bone marrow cell line B6SUtA. The biological activity of the fusion protein, when free in solution, was found to be similar to that of steel factor which lacked a cellulose-binding domain. Cellulose, being inert to mammalian cells, was then used as a matrix for the immobilization of the fusion protein *in situ*. The adsorption of the fusion protein to cellulose *in situ* was accompanied by an enhancement of its biological activity.

The SLF-CBD fusion protein was developed as a prototype cellulose-binding growth factor. The reasons for developing a cellulose-binding-cytokine technology were threefold. Firstly, the availability of growth factors which bind to cellulose would greatly simplify the cultivation of bone marrow cells *ex vivo*. The adsorption of growth factors to cellulose *in vitro* would provide a concentrated source of these growth factors, and reduce the net amounts required for the efficient cultivation of these cells. Secondly, growth factors which can be immobilized on the outer surfaces of cellulose-coated microcarrier beads, or on the inner surfaces of cellulose-coated rollerbottles could be used for the large scale cultivation of transformed mammalian cell lines used to produce recombinant proteins. Thirdly, cellulose as an artificial extracellular matrix, could simplify the study of receptor-cytokine interactions and signal transduction pathways by eliminating the need for specially engineered stromal cells to present immobilized growth factors to target cells.

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| | |
|---|---|
| amp. | Ampicillin |
| BSA | Bovine serum albumin |
| BMCC | Bacterial microcrystalline cellulose. |
| CBDcex | Cellulose-binding domain of Cex |
| cDNA | DNA transcribed from messenger RNA |
| CMC | Carboxymethyl cellulose |
| DEAE | Diethylaminoethyl |
| ECM | Extracellular matrix |
| EDTA | Ethylenediamine tetra-acetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EPO | Erythropoietin |
| FBS | Fetal bovine serum |
| FCS | Fetal calf serum (fetal bovine serum) |
| G-CSF | Granulocyte-colony stimulating factor |
| GM-CSF | Granulocyte-macrophage colony stimulating factor |
| H-SFM | Hybridoma serum free medium |
| IL-2 | Interleukin 2 |
| IL-3 | Interleukin 3 |
| IL-4 | Interleukin 4 |
| IL-6 | Interleukin 6 |
| IL-11 | Interleukin 11 |
| IL-12 | Interleukin 12 |
| IPTG | Isopropyl-b-D-thiogalactoside |
| kan | Kanamycin |
| kb | Kilobase pairs (DNA) |
| kDa | KilloDalton (molecular weight) |
| LB | Luria-Bertani |
| MCAC | Metal chelate affinity chromatography |
| M-CSF | Macrophage-colony stimulating factor |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| rpm | Revolutions per minute |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SLF | Steel factor |
| SLF-CBD 1.0 | Steel factor-CBD fusion protein exported to the periplasm of *E. coli*. |
| SLF-CBD 1.1 | Steel factor-CBD fusion protein produced as inclusion bodies in *E. coli*. |
| Xa | Cleavage site for activated blood factor X |

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The funding for this research was generously provided by the people of British Columbia through the **British Columbia Health Research Foundation**, and by the people of Canada through the **Natural Sciences and Engineering Research Council**.

**Dedication.**

I dedicate this thesis to my beloved parents **Irene** and **Rodney** to whom I owe all that I am and all that I have, and to my dear brother **Pip (J.S.P. Esq.)**. I can never repay the happy debt of love I owe my family.

also,

The proteins created for this thesis were originally designed to facilitate bone marrow stem cell research and transplantation. If there is anything contained here that is of value to humanity I dedicate it to **Dr. Marianne Huyer**, a fellow apprentice and traveler through the dark woods of science. She was the best of us, and she became the first of us to conquer completely the secrets of life.

*AD MAJOREM DEI GLORIAM.*

# 1. INTRODUCTION.

## 1.1. Hematopoiesis, cytokines, and the extracellular matrix.

For the average person the demand placed on the hematopoietic system for the replacement of blood cells is believed to be in excess of one trillion cells per day, including 200 billion erythrocytes (Ogawa 1994, Erslev *et al.* 1983) and 70 billion neutrophilic leukocytes (Ogawa 1994, Dancey *et al.* 1976). This demand is met through the tightly regulated metamorphosis and maturation of a population of immature precursor cells known as hematopoietic stem cells. These cells can differentiate into any of a vast number of lympho-myeloid lineages. Staggering in both magnitude and complexity, the hematopoietic process is orchestrated by a balance of stimulatory and inhibitory cytokines (Ogawa 1994, Williams *et al.* 1991, Broxmeyer 1986). In the steady state, most stem cells are resting in the $G_0$ stage, where they can remain for long periods of time before either dividing or differentiating in response to signals from these cytokines.

The growth factors which coordinate the hematopoietic process can be divided into three broad categories: 1) early acting growth factors, which affect the cell cycle kinetics of the most primitive and uncommitted cells, 2) intermediate acting growth factors, which exert their influence on intermediate, but still lineage nonspecific cells, and 3) late acting growth factors, which act on monopotent (committed) progenitor cells. There is considerable functional redundancy among the early and intermediate acting growth factors, as well as a great deal of synergy. Generally speaking, growth factors which are active on more primitive cells can also influence more committed cells. This is to be expected, since many committed cells continue to express receptors for the more general growth factors which regulated their growth kinetics earlier on.

Early acting growth factors fulfill one of their most important roles by bringing dormant progenitor cells out of the stationary $G_0$ phase. The growth factors interleukin-6 (IL-6), granulocyte-colony stimulating factor (G-CSF), interleukin-11 (IL-11), interleukin-12 (IL-12), and steel factor (SLF) are examples of this, as illustrated by their ability to stimulate blast cell colony formation from dormant human progenitors (Ogawa 1994). It is common for

these early growth factors to have the ability to synergize with intermediate acting growth factors, such as interleukin-3 (IL-3). Steel factor is, however, unique in its ability to synergize with many of the late acting growth factors as well (Dai *et al.* 1991). It is also believed that steel factor functions not only as a stimulating growth factor for early progenitor cells, but also as a survival factor for these cells, thus stimulating their proliferation as well as enhancing their longevity (Katayama *et al.* 1993).

Cytokines such as IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin-4 (IL-4), can be classified as intermediate acting, lineage-nonspecific factors, which support the proliferation of multipotential progenitors, but only after they have been brought out of dormancy (Ogawa 1994). These intermediate factors can often synergize with the later acting growth factors (Ogawa 1994, Sonoda *et al.* 1988). Later acting growth factors, such as erythropoietin (EPO), interleukin-5 (IL-5), and macrophage-colony stimulating factor (M-CSF), by contrast, are only capable of influencing committed progenitors.

Pluripotent stem cells exist in a complex hematopoietic stem cell microenvironment, composed of an intimate mixture of stem cells, stromal cells, growth factors, and extracellular matrix (ECM) molecules, all of which are in a constant state of interaction. The extracellular matrix is the complicated firmament in which this cellular galaxy is fixed; and it is thought that cytokines act together with ECM molecules to anchor stem cells within this microenvironment, and modulate their function. The ECM is composed of collagens, proteoglycans and glycoproteins, the components of which may vary throughout the body (Broxmeyer *et al.* 1991). It is believed that many of these components are capable of binding hematopoietic growth factors which, in turn, bind stem cells through receptor-ligand interactions. Indeed, it has been shown that the glycosaminoglycan side chains of proteoglycans, such as heparin sulfate, can bind hematopoietic growth factors (Roberts *et al.* 1988, Williams *et al.* 1991, Moore and Warren, 1987). Therefore, it is reasonable to assume that one of the functions of the ECM is to bind and localize hematopoietic growth factors, which in turn bind and localize hematopoietic stem cells. By extension, therefore, it is also reasonable to assume that matrix mediated localization of growth factors can augment growth factor function, and that growth factor mediated localization of stem cells can augment hematopoiesis (Roberts *et al.* 1988).

Reconstruction of the ECM microenvironment *in vitro* could be useful for stem cell research. The complexity of this microenvironment could, however, make this difficult. It would perhaps be more expedient to engineer an artificial ECM, in which growth factors could be anchored and tested. The contributions of each growth factor to the hematopoietic process could then be studied individually or in combination.

Rudimentary attempts have already been made to create artificial ECM's through the nonspecific attachment of growth factors and ECM molecules to plastic surfaces. In one such study growth factors, such as IL-3 and steel factor, and ECM proteins, such as thrombospondin, were adsorbed to plastic, and used to stimulate primary cells. This study showed that a combination of plastic-adsorbed growth factors and ECM proteins had a greater than expected ability to stimulate primary cell division (Long *et al.* 1992). Evidently the process of localizing both growth factors and stem cells to the same region served to increase cell proliferation, presumably because the immobilization of growth factors in a specific area increased their local concentration.

Surfaces, such as surface hydrolyzed poly(methyl methacrylate) films (Ito *et al.* 1992a), and glass beads (Ito *et al.* 1992b) have also been used as artificial ECMs through the covalent attachment of growth factors. Insulin (Liu *et al.* 1992), transferrin (Liu *et al.* 1993), and a combination of insulin, transferrin, and collagen (Ito *et al.* 1991) were immobilized in this way, with some retention of activity. Such methods of immobilization, however, cannot ensure that the growth factor is immobilized in the correct orientation. It would be preferable, therefore, to use a polar affinity tag for attachment, but most of the affinity tags commonly used, such as polyhistidine (M.C. Smith *et al.* 1988), streptavidin (Kasher *et al.* 1986), or glutathione-S-transferase (D.B. Smith and Johnson 1988) rely on matrices which could interfere with the *in situ* cellular environment. For instance, glutathionine-S-transferase, being a protein, could potentially contain combinations of amino acids which could coincidentally stimulate cells. Polyhistidine, as another example, must be adsorbed to nickel-sepharose for immobilization. Binding does not take place unless the pH of the solution is greater than 7.9, which is not optimal for most cell types. Furthermore, the presence of large concentrations of nickel ions could also be harmful to most mammalian cells.

Cellulose, on the other hand, is an ideal choice for an artificial ECM, since it is completely inert to mammalian systems, and cannot interfere with or

enter into the metabolic pathways involved. Furthermore, unlike flat surfaces, crystalline cellulose particles are capable of surrounding the target cells on all sides. Crystals also possess a larger surface area per unit than do flat surfaces, so that a greater concentration of growth factor could be immobilized in a smaller volume.

If cellulose is to be used as an artificial ECM for the immobilization of cytokines, then a cellulose-binding affinity tag is needed. Such tags can be obtained from cellulases, such as the exoglucanase Cex (O'Neill *et al.* 1986a). The binding domains from these enzymes have already been isolated, at the genetic level, and used to make cellulose-binding fusion proteins (section 1.10). The present study describes the construction and testing of a prototype growth factor-cellulose-binding fusion protein, for use in cellulose-based artificial ECMs. Steel factor was chosen as a growth factor fusion partner because of its versatility.

## 1.2. Discovery of the *c-kit* receptor and its ligand, steel factor.

The tyrosine kinase receptor c-kit was first discovered in 1986 by Besmer (Besmer *et al.*, 1986) through the initial isolation of the transforming gene*v-kit*, borne by a strain of feline sarcoma virus. Several groups then demonstrated that this gene was the naturally occurring product of the *W* (white spotting) locus in mice (Chabot *et al.*, 1988; Geissler *et al.*, 1988). Mice with mutations at this locus displayed hematopoietical defects similar to mice with mutations at another locus, designated as the *SL* (steel dickie) locus, located on chromosome 10 of the mouse genome (Zsebo *et al.*. 1990a). Bone marrow transplantation studies demonstrated that the hematopoietic defects of the *W* locus were intrinsic to the pluripotent stem cell (Fletcher and Williams 1992, Russell and Bernstein 1968), while the hematopoietic defects at the *SL* locus were stromal in nature (Fletcher and Williams 1992, Fried *et al.* 1973), and that there was a cross complementary relationship between the two mutations. This cross complimentivity suggested a receptor/ligand relationship. A putative ligand for the c-kit receptor was identified simultaneously by three different groups (Williams *et al.* 1990, Zsebo *et al.* 1990a, and Nocka *et al.* 1990), and was simultaneously named mast cell growth factor, stem cell

factor, kit ligand, and steel factor by the various researchers. The latter name will be used in this thesis.

### 1.3. Structure and biological properties of steel factor.

Steel factor is the ligand of a tyrosine kinase receptor encoded by the proto-oncogene c-kit (Geissler 1988, Chabot 1988). This pleiotropic growth factor exhibits profound effects on the early stages of the hematopoietic process, and serves as a general augmentor of stem cell proliferation (for reviews see Morrison-Graham and Takahashi 1993, Moore 1991). It also synergizes with IL-6, IL-11, GM-CSF, G-CSF, and EPO to stimulate an increase in the production of myeloid and erythroid lineage colony forming units(Miura *et al.* 1993, Du *et al.* 1993, McNiece *et al.* 1991, Briddell *et al.* 1993). Table 1.1 summarizes the current state of knowledge regarding the tissue specific expression of steel factor. For a recent comprehensive revies see Galli (Galli *et al.* 1994)

The wide ranging effects of steel factor on early hematopoietic progenitors have implicated it as a key factor in the efficient operation of the hematopoietic system, and an important constituent of the bone marrow stem cell microenvironment. By studying this protein it should be possible to gain some insight into the role of the bone marrow stem cell microenvironment in hematopoiesis. Also, as an expander of the primitive cellular components of bone marrow, steel factor is of potential use as a therapeutic for stem cell transplantation.

Steel factor can be either secreted from or bound to the surfaces of stromal cells. The protein, as encoded by the *sl* gene, consists of a 25 amino acid signal peptide, a 185 amino acid extracellular domain, a 27 amino acid hydrophobic membrane anchor, and a 36 amino acid cytoplasmic domain(Anderson *et al.* 1990) (Fig. 1.1 A i). Although the exact structure of steel factor has not yet been determined, a putative tertiary structure for the protein has been proposed based on sequence homology to M-CSF (Bazan, 1991) (Fig. 1.1 B i). According to this model, the protein forms four $\alpha$-helices which are arranged into two pairs, each pair being linked by a disulfide bridge. This proposed structure is tentative, and is only presented here for illustration purposes.

A proteolytic cleavage site separates the extracellular domain from the transmembrane anchor, so that the whole protein (KL-1) can be cleaved at the transmembrane anchor to generate the secreted form (Fig. 1.1 B ii). The second of the two isoforms is created by alternative mRNA splicing, to remove

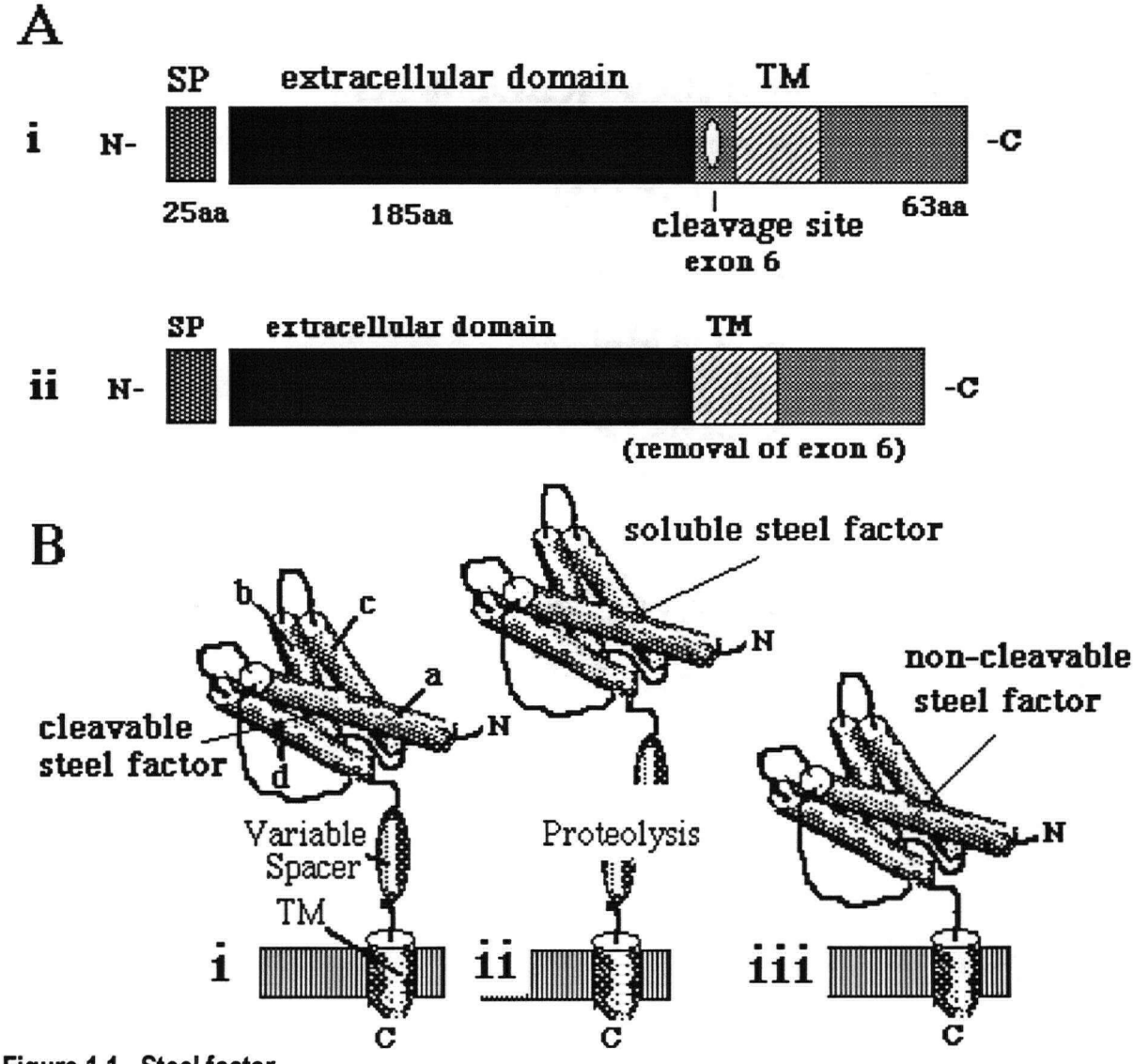



**Figure 1.1. Steel factor.**

**A) i.** Composition of the cleavable form of steel factor (KL-1). **ii.** The non-cleavable form of steel factor (KL-2). **B) i.** Putative tertiary structure of the cleavable form of steel factor. **ii.** Proteolytic cleavage of KL-1 to release the soluble form of steel factor. **iii.** The non-cleavable form of steel factor. (Putative structure adapted from Bazan 1991).

the exon which encodes the cleavage site (Figure 1.1 A ii and Fig. 1.1 B iii). The resulting protein (KL-2), which cannot be cleaved, remains bound to cell surface at all times (Huang *et al.* 1992, Pandiella *et al.* 1992). It is this form which is believed to be the more potent of the two isoforms.

**Table 1.1:**
**Steel Factor Sources, Cellular Targets, and Hematopoietic Effects:**

| Cells Producing Steel Factor: | Cells Responding To Steel Factor: | Factors That Act Synergistically with Steel: | Cell Type Production Boosted By This Combination of Cytokines: |
|---|---|---|---|
| **Bone marrow Stromal Cells** | Early Hematopoietic Progenitors (yolk sac, liver, bone marrow) | **SLF + IL-6** | Blasts, Mega-Karyocytes, Early Myeloid Progenitors, and Early Lymphoid Progenitors. |
| | | **SLF + IL-3** | Mixed colonies, Megakaryocytes, Macrophages, Blasts, and Granulocytes. |
| | | **SLF + IL-7** | Pre-B-Cells. |
| | | **SLF + GM-CSF** | Granulocytes and Macrophages. |
| | | **SLF + CSF-1** | Macrophages. |
| | | **SLF + G-CSF** | Granulocytes. |
| | | **SLF + Erythropoietin** | Erythrocytes. |
| **T-Cells** | Intermediate Hematopoietic Progenitors (yolk sac, liver, bone marrow) | **SLF + IL-6** | Blasts, Mega-Karyocytes, Early Myeloid Progenitors, and Early Lymphoid Progenitors. |
| | | **SLF + IL-3** | Mixed colonies, Megakaryocytes, Macrophages, Blasts, and Granulocytes. |
| | | **SLF + IL-7** | Pre-B-Cells. |
| | | **SLF + GM-CSF** | Granulocytes and Macrophages. |
| | | **SLF + CSF-1** | Macrophages. |
| | | **SLF + G-CSF** | Granulocytes. |
| | | **SLF + Erythropoietin** | Erythrocytes. |
| Fibroblasts | Primordial Germ Cells (Genital Ridge, Testes, Ovaries) | ----- | ----- |
| Selected Mesodermal and Ectodermal Cells During Embryogenesis | ---?---- | ----- | ----- |
| Brain Cells | ---?---- | ----- | ----- |

(adapted from Sigma ImmunoNotes, No. 8, 1992)

## 1.4. Membrane bound (immobilized) vs. free steel factor.

The purpose of having two isoforms of steel factor is not yet fully understood. It is possible that the two forms play different roles in the regulation of hematopoiesis. There is evidence to suggest that the membrane bound form is the more potent of the two. This is demonstrated by the severe hematological defects exhibited by mutant mice, which express only the secreted form of steel factor (Godin *et al.* 1991, Brannan *et al.* 1991, Dolci *et al.* 1991). Furthermore, *in vitro* studies of steel have shown that the membrane bound form is capable of supporting long term bone marrow culture systems for greater lengths of time than is the soluble form (Toksoz *et al.* 1992). The bound form was also found to cause more persistent tyrosine kinase activation of the c-kit receptor than the soluble form (Miyazawa *et al.* 1995). It is possible, therefore, that hematopoiesis is partially regulated by a variable ratio of soluble to immobilized steel factor.

These increases in growth factor potency could be the result of an increased concentration of the growth factor at the point of contact between the stromal cell and the target cell. It is possible that a certain localized concentration of ligand, presented to the target cell surface by another cell, could encourage dimerization and subsequent capping of the receptors. It is also possible that an increased concentration of ligand leads to a local compression of receptors which allows them to dimerize more easily and set off the signal transduction cascade.

Unfortunately, such *in vitro* studies of steel factor have always relied upon specially designed stromal cells to present the protein to its target cells while in the immobilized state, and it is difficult to differentiate between the effects of the protein itself and the effects of the cells which present it. It is possible that some of the observed effects were the result of synergy between steel factor and other proteins present on the surfaces of the stromal cells. In any case, it should be possible to gain insight into this controversy by creating an artificial model of immobilized steel factor. As mentioned previously, a cellulose based artificial ECM would have the advantage of being able to present immobilized cytokines to various cell types without interfering with

any of the metabolic processes involved. Cytokines could then be immobilized in such a matrix, and the effects on target cells measured, without any danger of the matrix itself influencing the results.

### 1.5 . Polar affinity tags and fusion proteins.

When recombinant proteins are produced in heterologous hosts they are sometimes produced as fusion proteins. Fusion proteins are created by the splicing together of two unrelated genes. When this hybrid gene is transcribed and translated, a chimerical protein is produced. Although numerous types of fusion proteins have been created (Fig. 1.2) almost all have been created for one of two purposes: 1) To increase the production of recombinant protein in a heterologous host (Fig. 1.3 A), or 2) to simplify the purification of recombinant protein from such a host (Fig. 1.3 B).

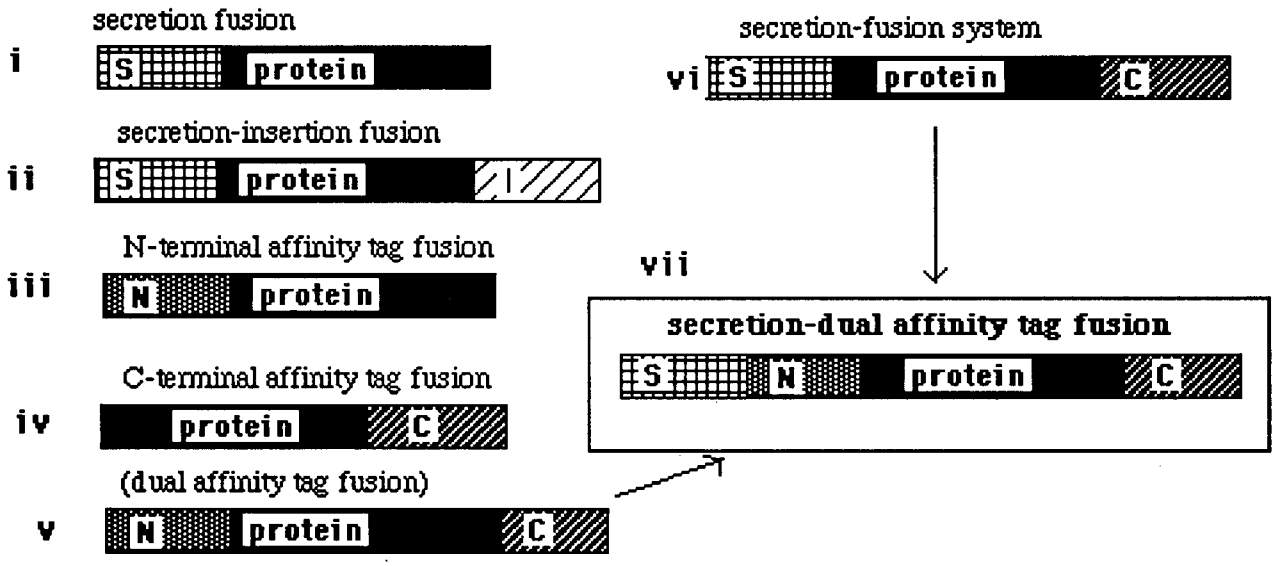



**Figure 1.2 Commonly used types of fusion proteins.**

**i.** N-terminal fusion for production and secretion. **ii.** Secretion-insertion fusion. **iii.** N-terminal affinity tag. **iv.** C-terminal affinity tag. **v.** Dual affinity tag. **vi.** Combined affinity tag/secretion system. **vii.** Dual affinity tag, secretion combination.

As an example, recombinant proteins produced in *Escherichia coli* may use promoters which are not recognized by the host, or codons which are not frequently used by the host, and underexpression may be the result. Furthermore, since the protein is usually foreign to the host cell, some proteolytic degradation is to be expected. In order to circumvent these problems, highly expressed *E. coli* proteins are sometimes fused to the amino-termini of heterologous proteins (Fig. 1.2i). The *lacZ* fusion system (see Table 1.2) is an example of such a strategy. In this case the β-galactosidase enzyme, which is native to *E. coli*, and highly expressed therein, is fused to the amino-terminus of a recombinant protein. Since this enzyme uses an *E. coli* promoter and *E. coli* preferred codons translation is usually initiated more efficiently. Also, β-galactosidase, being highly soluble, can help to prevent the formation of inclusion bodies. A side benefit to such a fusion is that the protein can subsequently be identified using antibodies specific for the fusion tag. This is especially important if the desired protein has not yet been characterized, and antibodies specific to it are not available. The formation of inclusion bodies is not always undesirable however, and in some cases precipitation and aggregation are encouraged by flanking the protein of interest with insoluble peptide sequences. This deliberate formation of inclusion bodies is sometimes used to evade proteolytic degradation. The *trpE* and λcII systems (see Table 1.2) are examples of fusions designed to form inclusion bodies.

Removal of the recombinant protein from the cytoplasm is another strategy commonly employed to evade proteolysis (Fig. 1.3 A). Fusion of an export signal peptide to the amino-terminus of a recombinant protein will often result in export to the periplasm, or to the culture supernatant, where fewer proteases are present (Fig. 1.2 i). This can also be used as a first step in protein purification. The export leader peptide from the cellulase Cex can be used for protein export (O'Neill *et al.* 1986c). This signal peptide directs export of Cex from *Cellulomonas fimi,* and is removed from the amino-terminus during translocation across the cytoplasmic membrane.

In some cases, a hydrophobic protein segment is fused to the carboxy-terminus of the protein to cause export followed by insertion into the host cell membrane (Fig. 1.2 ii). This method is usually only used for functional studies, where proteins are to be expressed on the surfaces of cells.

Polar affinity tags are sometimes spliced onto proteins of interest, at the genetic level, to facilitate subsequent purification (Fig. 1.2). The resulting fusion protein binds to a matrix which it did not previously have an affinity for, and this quality is exploited to simplify purification of the protein. As Figure 1.4 illustrates, the fusion protein is first recovered in a cellular extract from a host cell such as *E. coli*. This mixture is then sent through an affinity column packed with a matrix for which only the fusion protein has an affinity. After all other proteins are washed from the column, the fusion protein is then eluted under specific conditions which allow the affinity tag to be released from its matrix.

A proteolytic cleavage site can be placed between the protein of interest and the affinity tag to facilitate removal of the tag. When this mixture of cleaved proteins is sent through the affinity column a second time, only the

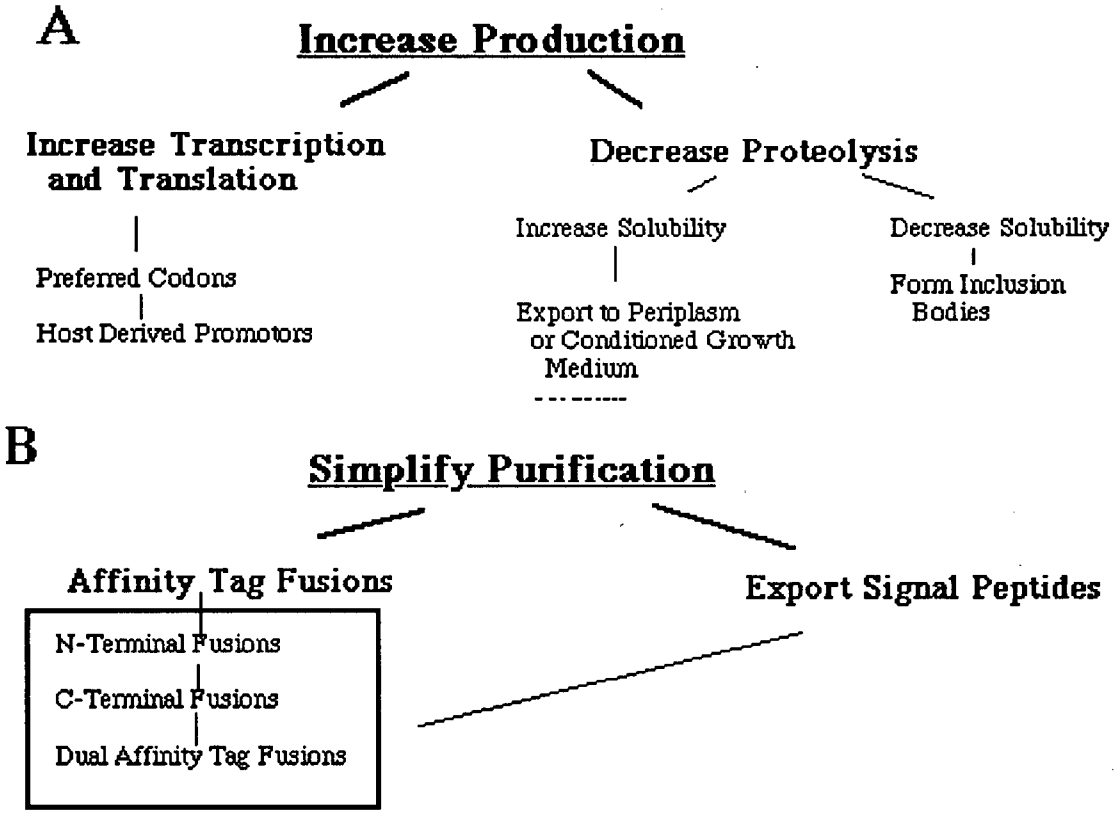



**Figure 1.3 Fusion proteins for increased production and purification efficiency.**

**A)** Fusion proteins designed to increase net protein yield. **B)** Fusion proteins designed to simplify purification and recovery.

# Table 1.2:

## Commonly Used Affinity Tags and Their Matricies:

| Affinity Tag | Molecular Size(kDa) | Secretable system | Affinity Matrix | Reference |
|---|---|---|---|---|
| | | | **Soluble Fusion Protein Systems** | |
| β-Galactosidase | 116 | n | TPEG,APTG | (Ullman 1984) |
| Protein A | 31 | y | IgG | (Nilsson et al. 1985) |
| Protein G | 28 | y | Albumin | (Nygren et al. 1988) |
| Poly(His) | 1-7 | y | $Zn^{2+}$, $Cu^{2+}$, (Immidazole) | (Smith et al. 1988)) |
| Z protein | 7 | y | IgG | (Moks et al. 1987) |
| Cysteine | <1 | y | Thiol | (Carter et al. 1987) |
| MBP (Maltose Binding Protein) | 40 | y | Starch | (diGuan et al. 1988) |
| Mannose Binding Protein) | 17 | y | Mannose-sepharose | (Taylor and Drickamer 1991) |
| GST(Gltathione S-transferase) | 26 | n | Glutathione | (Smith and Johnson, 1988) |
| Flag Peptide | 2-5 | y | Specific Monoclonal Antibodies | (Hopp et al., 1988a) |
| **Cellulose Binding Domains** | **13-14** | **y** | **Cellulose** | **(Greenwood et al., 1989; Ong et al., 1989)** |
| | | | **Inclusion Body Forming Fusion Systems** | |
| cII | 13aa | n | Monoclonal Antibodies etc. | (Nagai and Thorgersen 1984) |

# Table 1.3
## Common Chemical and Enzymatic Methods of Cleaving Fusion Proteins

**Enzymatic Methods:**

| Enzyme (Protease) | Cleavage site | Reference |
|---|---|---|
| Collagenase | Pro Val / Gly Pro | (Germino and Bastia 1984) |
| Enterokinase | Asp Asp Asp Lys / | (Hopp *et al.* 1988b) |
| Trypsin | Arg / | (Shine *et al.* 1980) |
| Clostripain | Arg / | (Lowe *et al.* 1987) |
| Plasmin | Gly Ala Arg / | (Forsberg *et al.* 1992) |
| Urokinase | Pro Gly Arg / | (Forsberg *et al.* 1992) |
| V8 Protease | Asp / or Glu / | (Gentz *et al.* 1989) |
| **Factor Xa** | **Ile Glu Gly Arg /** | **(Nagai and Thogersen 1987)*** |
| **Thrombin** | **Gly Pro Arg /** | **(Smith and Johnson 1988)*** |

**Chemical Methods:**

| Chemical | Cleavage site | Reference |
|---|---|---|
| Cyanogen Bromide | Met / | (Itakura et al. 1977) |
| Formic Acid | Asp / Pro | (Szoka et al. 1986) |
| Hydroxylamine | Asn / Gly | (Moks et al. 1987) |

affinity tag will be retained, leaving the purified protein free to be collected in the flow through. Table 1.2 is a compilation of some of the more commonly used purification tags. Table 1.3 is a compilation of some of the more common proteolytic agents used to cleave fusion proteins.

The affinity tags listed in Table 1.2 are polar in the sense that they are fused to either the N-terminus (Fig. 1.2 iii) or the C-terminus (Fig. 1.2 iv) of the protein of interest, and therefore will immobilize the protein at one end only. Most of these

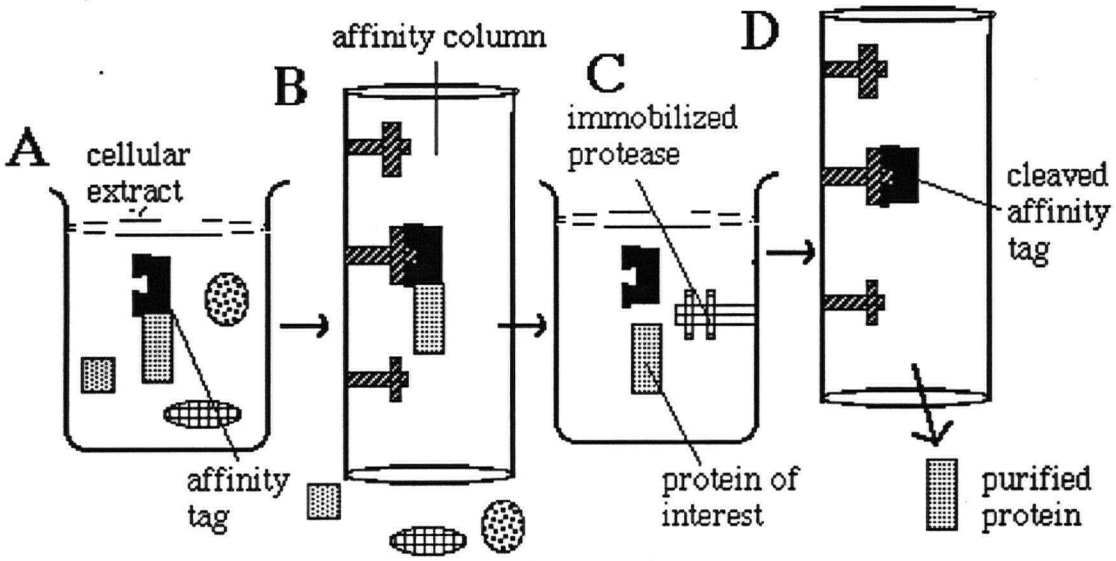



**Figure 1.4 Schematic diagram for the purification of a recombinant protein as a fusion protein.**

**A)** Recovery of the fusion protein in a cellular extract. **B)** Purification of the fusion using an affinity column packed with a matrix specific for the affinity tag. **C)** Cleavage by an immobilized protease. **D)** Removal of the severed affinity tag by passing the solution through the same column.

tags have a functional preference for either the N or the C-terminus of the fusion protein. In rare cases, a dual affinity tag fusion system is used, where a different tag is attached to each end of the protein (Fig. 1.2 v). Such a protein might be constructed when N-terminal proteolytic degradation is a problem. An initial purification step using the C-terminal affinity tag, followed by a second purification step using the N-terminal affinity tag could then ensure that only the intact protein has been recovered.

Needless to say, purification of fusion proteins in this way is not practical unless the binding process is reversible. It is essential that the protein of

interest be eluted from the affinity column under conditions which do not denature it, or change its conformation. It is for this reason that the matrices used in affinity columns are usually not the matrices for which the affinity tag has its greatest affinity. Fusion proteins can then be eluted from their affinity columns by a soluble substrate for which the affinity tag has an even greater affinity. For example, in the maltose-binding protein system (Kellerman and Ferenci 1982) fusion proteins bind to solid starch inside an affinity column, and are subsequently eluted by soluble maltose.

The secretion system (Fig. 1.2 i) can also be combined with the affinity tag system. In such a case (Fig. 1.2 vi), the recombinant protein is exported to the periplasm as an initial purification step, and subsequently purified using affinity chromatography. In the present study, all of the above methods were employed to create an exported fusion protein which can be purified by affinity chromatography using either of two affinity tags (Fig. 1.2 vii).

## 1.6. Immobilized enzymes, cytokines, and other biologically important proteins.

Immobilized enzymes have certain advantages over their soluble counterparts. They are more resistant to proteolysis, and they do not contaminate the protein mixtures in which they operate. Although the immobilization of industrially important enzymes has become more common in recent years, the employment of affinity tagged fusion proteins as immobilized enzymes is still somewhat rare. Immobilization of enzymes by conventional methods usually relies on either non-specific adsorption (hydrophobicity for example), ionic interaction, or covalent attachment mediated by chemical agents. In the former two cases, the strength of the bond between the protein and the support becomes the limiting factor, as these interactions are not very strong. Binding is stronger in the latter case, but there is often a reduction in enzymatic activity due to the harshness of the immobilization process. Some of the more successfully immobilized enzymes are listed in Table 1.4 along with their matrices and methods of immobilization.

Most proteins will adsorb to plastics, such as polystyrene. The attraction is usually due to weak hydrophobic or ionic interactions between the protein and the matrix. Since the matrix is relatively unobtrusive and simple to use, however, this has become the method of choice for *in situ* experiments. Covalent methods of attachment, by contrast, rely on the oxidation of certain amino acid side chains, and their subsequent attachment to activated matrices.

Although fusions are often created to simplify the purification of recombinant proteins, there are also a few examples of enzymes which have been linked to affinity tags for purposes of immobilization. Table 1.5 is a list of enzymes which rely on affinity tags for immobilization. Affinity tag immobilization is not as strong as covalent attachment. The redeeming feature of such a system, however, is that the enzyme usually retains more of its functional activity, having circumvented the need for harsh, chemically mediated immobilization.

# Table 1.4
## Immobilized Enzymes:

### Non-specific Adsorption

| Enzyme | Matrix | Reference |
|---|---|---|
| β-galactosidase | hydrophobic cotton | Sharma and Yamazaki 1984. |
| β-glucosidase | ConA-Sepharose | Lee and Woodward 1983. |
| β-glucosidase | hydrophobic cotton | Sharma and Yamazaki 1984. |
| β-glucosidase | ConA-Sephadex | Husain and Saleemuddin 1989. |
| Cellulase enzymes | ConA-Sepharose | Woodward *et al.* 1982. |
| Glucose isomerase | glass beads | Chen *et al.* 1981. |

### Ionic Interactions

| Enzyme | Matrix | Reference |
|---|---|---|
| β-amylase | polystyrene/$Al^{3+}$ | Roy and Hegde 1987. |
| Glucoamylase | DEAE-cellulose | Tomar and Prabhu 1985. |
| Invertase | poly(ethylene-vinyl alcohol) | Imai *et al.* 1986. |

### Covalent Attachment

| Enzyme | Matrix | Reference |
|---|---|---|
| β-galactosidase | alumina | Nakanishi *et al.* 1983. |
| β-galactosidase | alginate | Dominquez *et al.* 1988. |
| β-glucosidase | Sepharose | Kierstan et al. 1982. |

| | | |
|---|---|---|
| β-glucosidase | alginate | Hahn-Hagerdal 1984. |
| β-glucosidase | alginate | Fujikawa e*t al.* 1988. |
| β-glucosidase | Sepharose | Roy *et al.* 1989. |
| β-xylosidase | alumina/$TiCl_4$ | Oguntimein and Reilly 1980. |
| Glucose isomerase | glass beads | Strandgerg and Smiley 1972. |

Biologically important proteins such as hormones and antibodies have also been immobilized using the same methods, although cytokine fusion proteins designed for immobilization have not previously been reported. When a biologically important protein is immobilized, it is helpful to have an intimate knowledge of the protein's structure. In some cases this has allowed a rational strategy for covalent immobilization to be designed, which maintains the correct orientation of the protein. For example, immobilization of the steroid hormone 17β-Estradiol via the hemisuccinate derivative to a diaminodipropylamine (DADPA) matrix leads to the phenolic portion of the molecule facing away from the matrix surface (Parikh *et al.*, 1974). Immobilization of the regular molecule leads to the reverse orientation.

# Table 1. 5

## Fusion Proteins Constructed for the Immobilization of Enzymes:

| Enzyme | Affinity tag | Matrix | Reference |
|---|---|---|---|
| β-lactamase | Protein A | IgG/Sepharose | Baneyx *et. al.* 1990. |
| **β-glucosidase** | **$CBD_{Cex}$** | **cellulose** | **Ong. et al. 1989a,b;1991.** |
| **alkaline phosphatase** | **$CBD_{CenA}$** | **cellulose** | **Greenwood *et. al.* 1989.** |
| **blood Factor $X_a$** | **$CBD_{Cex}$** | **cellulose** | **Assouline *et. al.* 1994.** |

As mentioned previously, surface hydrolyzed poly(methyl methacrylat) films, and glass beads have also been used as surfaces for immobilization. Various combinations of insulin, transferrin and collagen have been immobilized on such surfaces. It is rare, however, that such methods of attachment and immobilization can guarantee the correct orientation of the growth factor or cytokine being tested. An affinity tag fused to the cytokine

at the correct terminus would be preferable. Although a cytokine fusion protein has never been constructed for this purpose, cytokine fusions have been created for other purposes.

### 1.7. Cytokine fusion proteins.

To date only a few cytokine fusion proteins have been created. They fall into two broad categories: 1) those that promote primary cell growth and differentiation through the fusion of two synergistic cytokines, and 2) those that kill rapidly dividing cancer cells through the fusion of a cytokine to an endotoxin. The most conspicuous representative of the former category is the so-called "second-generation" cytokine PIXY321 (Curtis *et al.*, 1991). This hybrid cytokine was constructed by fusing together the genes encoding the growth factors GM-CSF and IL-3. The fusion protein was found to be ten fold more potent than either of the two proteins alone or in combination when assayed for colony formation..

The fusion of GM-CSF to tumor-cell idiotype-specific antigens, in order to increase the antigenicity of tumor cells, is an example of the latter category (Chen *et al.* 1994). Fusions of Pseudomonas exotoxin A (PE) to transforming growth factor alpha (TGF-alpha), insulin-like growth factor (IGF-I), and acidic fibroblast growth factor (FGF) have also been constructed to treat glioblastoma multiforme, since many malignant gliomas overexpress receptors for these growth factors (Kunwar *et al.*, 1993). To date, the only cytokine fusion protein constructed for *in situ* immobilization has been the $CBD_{CenA}$/IL-2 fusion (Ong *et al.* 1995, Greenwood 1993), where a cellulose binding domain was fused to the amino-terminus of IL-2.

### 1.8. The cellulose-binding domain ($CBD_{Cex}$).

In order to adsorb growth factors to cellulose, a cellulose-binding affinity tag must be used as a fusion partner. The Gram-positive bacterium *Cellulomonas fimi* produces a number of cellulases which bind to and digest

cellulose. The genes for the major cellulases of the *C. fimi* cellulase system have been cloned and expressed in *E. coli* (O'Neill *et al.*, 1986b; Wong *et al.*, 1986; Owolabi *et al.*, 1988; Coutinho *et al.*, 1991; Meinke *et al.*, 1991). One of these enzymes is the exoglucanase Cex (Fig. 1.5) which has a molecular mass of 49.3 kDa when produced in the glycosylated form in *C. fimi*, and a non-glycosylated mass of 47.3 kDa when produced in *E. coli*. This enzyme is composed of two functionally independent domains, one of which mediates binding to cellulose, while the other mediates the catalytic digestion of cellulose.

Both the Cex catalytic domain and the Cex cellulose binding domain (the $CBD_{Cex}$) have been cloned and expressed separately, and their tertiary structures examined (Figures 1.5 A and 1.5 B respectively) (White *et al.*, 1994; Xu *et al.*, 1995). $CBD_{Cex}$ is composed of 108 amino acids, and is separated from the catalytic domain by a 19 amino acid linker made up of repeating prolyl and threonyl residues. Two of the amino acids in the CBD are cysteines, which form a disulfide bond. The five tryptophan residues contained within the CBD are believed to be important for cellulose binding, since conserved tryptophan residues are a common feature among proteins which interact with polysaccharides (Svensson *et al.* 1989; Drickamer, 1988). The aforementioned structural analysis of $CBD_{Cex}$ indicated that three of these tryptophans were on the same plane, and would be exposed to the cellulose substrate during binding. It is likely, therefore, that the orientation of these three aromatic residues is critical for proper binding. The parameters of $CBD_{Cex}$ mediated binding have been examined in some detail (Gilkes *et al.* 1992).

## 1.9. The cellulose matrix.

Cellulose is a principal component of plant cell walls, and forms a major portion of the Earth's biomass. It is a linear polysaccharide composed of β-d-glucopyranosyl units linked by β-1,4-glucosidic bonds. (Fig. 1.6A) The majority of cellulose found in nature exists in one of two forms: cellulose I or cellulose II. The native form, cellulose I, has a highly ordered parallel-chain structure (Fig. 1.6B) in which each repeating cellobiosyl unit forms two hydrogen bonds with its neighbors.

When the ordered structure of cellulose I is swelled by acid or alkali, or is otherwise disrupted, an antiparallel cellulose structure results (cellulose II). This structure is more stable, and lower in potential energy than is cellulose I.

Cellulose is readily available in a number of inexpensive forms. Since it is relatively inert to most systems, it constitutes an ideal matrix for the binding of novel fusion proteins. Furthermore, since mammalian systems do not possess any enzymes which can digest or alter cellulose, it represents an ideal matrix for the immobilization if cytokine fusion proteins which are to be tested *in situ*. Cellulose is both inert and innocuous to mammalian systems, and is already used in hollow-fiber and microcarrier cell culture systems.

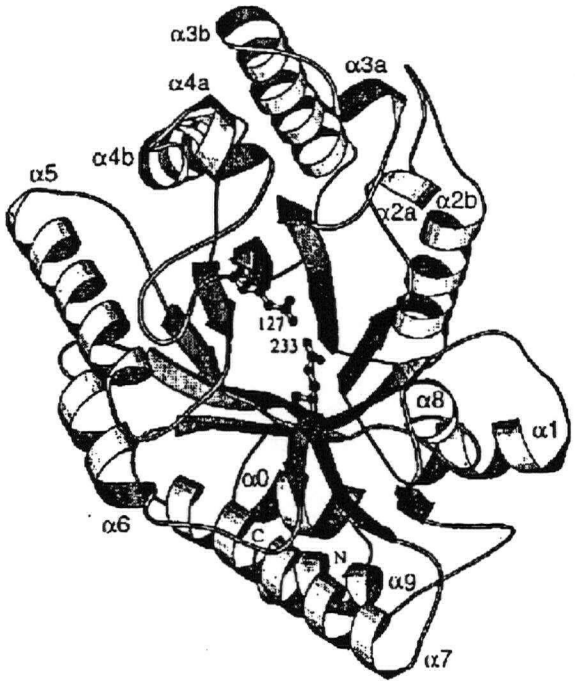
### 1.10. CBD fusion proteins.

A number of novel CBD based fusion proteins have already been constructed. Alkaline phosphatase and β-glucosidase have both been successfully fused to CBDs with retention of their respective activities (Greenwood *et al.* 1989; Ong *et al.* 1991). These fusions were created to both simplify the purification of these enzymes and to immobilize and localize their catalytic activity. For example, blood Factor $X_a$ was fused to a cellulose binding domain in order to simplify the purification of this protease (Assouline *et al.*. 1993) and to allow its activity to be immobilize on a cellulose matrix (Assouline *et al.* 1994).

### 1.11. Objectives of the present study.

The specific objectives of the present study are threefold. Firstly, a steel factor-$CBD_{Cex}$ fusion protein will be created and tested for biological activity while immobilized *in situ*. Secondly, the effects of growth factor immobilization on biological activity will be examined. The biological activity of the immobilized fusion protein will be compared to the biological

A)




B)

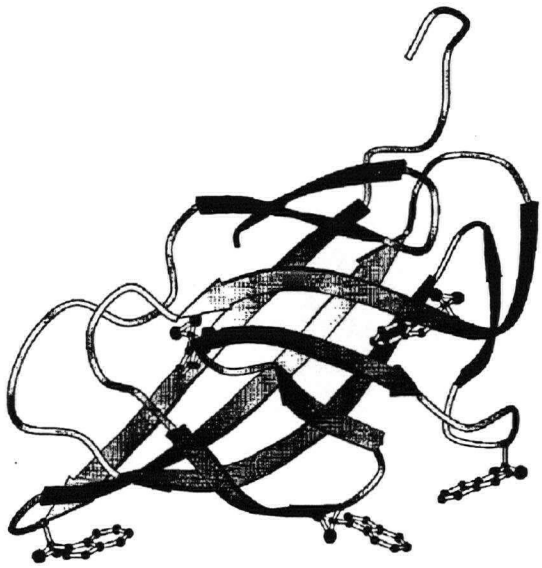
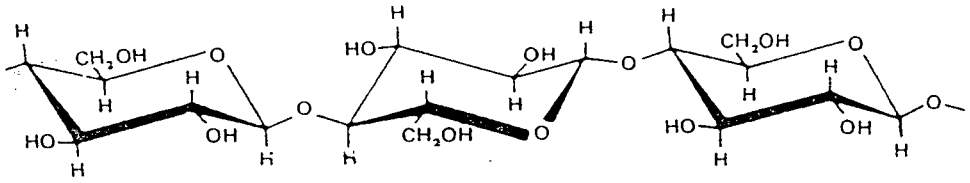


**Figure 1.5. Catalytic domain and cellulose-binding domain of Cex.**

**A)** Tertiary structure (X-ray crystallography) of the amino-terminal catalytic domain of Cex (White *et al.* 1994). **B)** Tertiary structure (Nuclear magnetic resonance) of the carboxy-terminal cellulose-binding domain of Cex (Xu *et al.* 1995).

A)



B)

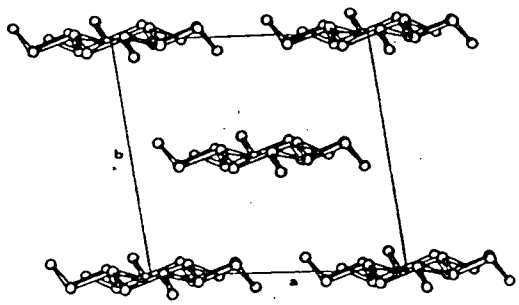


**Figure 1.6. Cellulose.**

**A)** Cellulose molecule **B)** Cellulose I. (Adapted from Blackwell 1987).

activity of the free protein, in an artificial system. Thirdly, the relationship of the immobilized growth factor surface density to the magnitude of the induced proliferative response will be examined.

## 2. MATERIALS AND METHODS.

### 2.1. Chemicals, media components, buffers, and enzymes.

All chemicals used were of HPLC grade. Water for solutions and buffers was single glass distilled. Buffers were prepared according to Sambrook *et al.* (1989) and Perrin and Dempsey (1974). Media components were from Difco. Enzymes and enzyme buffers were purchased from Boehringer Mannheim and Gibco BRL, and used according to the manufacturers' instructions.

### 2.2. Bacterial strains and cell lines.

*Escherichia coli* was the host organism used throughout the present study for both DNA manipulation and gene expression. Strains are listed in Table 2.1. The strain DH5α was used exclusively for DNA work; JM101 was used for production of the exported form of the fusion protein; BL21 was used for the production of inclusion bodies. DH5α was used for cloning because it was recombination defficient, and would not have the ability to delete gene segments or alter DNA sequences.

**Table 2.1.**

**List of *E. coli* strains.**

| *E.coli* strain | Genotype | Reference |
|---|---|---|
| **JM101** | F' *tra*D36 *pro*A+ *pro*B+ *lac*I$^q$ *lac*ZΔM15 /*sup*E *thi* Δ(*lac-pro*AB) | Yanisch-Perron *et al.* 1985. |
| **DH5αF'** | F'/*end*A1 *hsd*R17($r_k^-M_k^+$) *sup*E44 *thi*-1 *rec*A1 *gyr*A (Nar$^r$) *rel*A1 Δ(*lac*ZYA-*arg*F)U169 (m80*lac*ZΔM15) | Hanahan 1983. |
| **JM110** | ***dam dcm*** *sup*E44 *hsd* R17 thi leu rpsL *lac*Y *gal*K *gal*T *ara* tonA thr tsx Δ(*lac-pro*AB) F' (*tra*D36 *pro*AB+ *lac*I$^q$ *lac*ZΔM15) | Yanisch-perron 1985. |
| **XL-1 Blue** | *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi* *rel*A1 lac$^-$ F'(*pro*AB+ *lac*I$^q$ *lac*ZΔM15 Tn10(tet$^r$)) | Bullock *et al.* 1987. |
| **BL21(λDE3)** | *hsd*S *omp*T (λDE3) | Studier and Moffatt 1986. |

## 2.3. Media and growth conditions.

LB medium (Miller 1972) was used for propagation of DH5α while TYP broth (16 g tryptone, 16 g yeast extract, 5 g NaCl, 2.5 g $K_2HPO_4$ per liter) was used for JM101 and BL21. In all cases, cells were transformed with DNA (Sambrook *et al.* 1989) immediately before use, and not stored as glycerol stocks. Either ampicillin (100 $\mu g\ mL^{-1}$) or kanamycin (50 $\mu g\ mL^{-1}$) was used for selection and maintenance of plasmids. Liquid cultures were grown at either 30°, and 150 rpm shaking, or 37° , and 150 rpm shaking, as specified. Cell density was measured as the optical density at 600 nm ($OD_{600}$) in a Hitachi U-2000 spectrophotometer. Both *lac* and *tac* promoters were induced with isopropyl-β-D-thiogalactoside (IPTG; Sigma) at a concentration of 0.1 mM unless otherwise indicated. Solid media contained 1.5 % (w/v) agar.

Mammalian cells were maintained in Hybridoma serum free medium (H-SFM) (Gibco -BRL) supplemented with 10 % (v/v) fetal bovine serum (FBS; Gibco) and 5 % spleen cell conditioned medium (STEMCELL TECHNOLOGIES INC., cat. no. HCC-2100). Cells were propagated in 5 mL tissue culture flasks (Falcon) at 37° and 5% $CO_2$. Cell proliferation assays were carried out in unsupplemented H-SFM.

## 2.4. Recombinant DNA techniques.

Standard techniques were used for all DNA manipulations (Sambrook *et al.* 1989). DNA restriction and modification enzymes were used according to manufacturers' instructions. DNA fragments were excised from agarose gels and purified using the QIAEX gel extraction kit (QIAGEN). Oligonucleotides were synthesized using an Applied Biosystems automated DNA synthesizer model 380A (University of British Columbia Oligonucleotide Synthesis Lab). Site directed mutagenesis was carried out as previously described (Zhou *et al.* 1990, Zoller and Smith 1982). Restriction sites and factor Xa cleavage sites were introduced into the steel factor gene using PCR mediated mutagenesis (Kaufman and Evans 1990; Saiki *et al.*

1988). DNA was sequenced by the dideoxy chain termination method (Sanger 1977) using the Sequenase version 2.0™ kit (United States

**Table 2.2.**

**List of plasmids and constructs.**

| Plasmid | Promoter | Marker | Reference |
|---|---|---|---|
| **pSLF/CBD 1.0*** | tac | Kan. | This study. |
| **pSLF/CBD 1.1†** | λT7 | Kan. | This study. |
| **pBS K/S II+(SLF)** | lac | Amp. | (Dr. D. Williams, Immunex Corp., Seattle, Wa.) |
| **pBS K/S II+** | lac | Amp. | Short *et al.* 1988. |
| **pSL1180** | -none- | Amp. | (Stratagene inc.) |
| **pTUg AS** | tac | Amp. | Graham *et al.* 1995. |
| **pTUg 3SN8** | tac | Kan. | R. Graham (unpublished). |
| **pTUg CBD$_{CenB}$** | tac | Kan. | C. Ramirez (unpublished). |
| **pET28a** | λT7 | Kan. | (Novogen corp.) |

* For export of SLF-CBD to periplasm and culture supernatant.
†For production of SLF-CBD as cytoplasmic inclusion bodies.

Biochemical). Plasmids and constructs used in the present study are listed in Table 2.2. The vector construct pSLF/CBD 1.0 was designed to produce an SLF-CBD fusion protein which would be exported to the periplasm of *E. coli*, while the vector construct pSLF/CBD 1.1 was designed to produce a SLF/CBD fusion protein which would form inclusion bodies in the cytoplasm of *E. coli*. The proteins encoded by these two vectors have slightly different amino termini (appendix 4), and the proteins produced by pSLF/CBD 1.0 and pSLF/CBD 1.1 will be referred to as SLF-CBD 1.0 and SLF-CBD 1.1 respectively.

### 2.4.1. PCR mediated mutagenesis.

$10^{10}$ copies of the plasmid pBS K/S II+(SLF), containing the murine SLF gene (as cDNA) were combined with primers 1 and 2 (appendix 1) in an initial reaction volume of 50 µL, with primers being present at a concentration of 200 pM. The reaction was heated to 95° C in a TwinBlock™ System PCR thermocycler (Ericomp, Inc., San Diego, CA.) and 50 µL of a reaction master mix was then added such that the reaction would contain *taq* ™ DNA polymerase reaction buffer (supplied by the enzyme manufacturer), 1 unit of *taq* DNA polymerase, MgCl at a final concentration of 5 mM, and each of the four deoxynucleotides at a final concentration of 200 µM. The reaction was then carried through 30 cycles, with denaturation at 94° C for 1 minute, primer annealing at 45° C for 1 minute, and primer extension at 72° C for 1 minute and thirty seconds. The PCR product was then extracted twice with phenol chloroform and used for subsequent cloning steps.

## 2.5. Preliminary expression and binding test of exported SLF-CBD 1.0.

Preliminary expression, secretion, and binding tests were carried out on pSLF/CBD 1.0 clones prior to optimization of protein production. Optimum protein production was first established for the plasmid pSLF/CBD 1.0, and then used for large scale protein production with plasmid pSLF/CBD 1.1.

Plasmids construction was carried out with DH5α, with subsequent transformation into JM101 for protein expression. JM101/pSLF/CBD 1.0 cells were plated on $LB^{Kan}$ and grown at 37° C for 24 hours. Individual colonies were then inoculated into 250 mL shake flasks containing 50 mL $TYP^{Kan}$ and grown for 8 hours at 37° with 250 rpm shaking. Protein production was then induced with 0.1 mM IPTG for a further 4 hours, after which cells were harvested by centrifugation. Periplasmic proteins were isolated by osmotic shock (Nossal and Heppel 1966) and cytoplasmic proteins were extracted by rupturing cells in a French pressure cell (Aminco) at 17 000 psi.

A 100 mg $mL^{-1}$ slurry of Avicel™ (FMC International), an amphorous cellulose powder, was prepared in phosphate buffered saline (PBS) and

sterilized. To screen for cellulose-binding proteins, of the predicted size, in the culture supernatant a 10 mL aliquot of supernatant was transferred to a 15 mL plastic Falcon$^{TM}$ tube, and 0.5 mL of the Avicel slurry was added to it. The tube was agitated for 10 minutes at room temperature and then the Avicel was removed by centrifugation, washed in 1 mL PBS, and boiled in 40 µL loading buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Laemmli 1970) to visualize any proteins which had adsorbed to the cellulose. 5 mg of Avicel were also added to 1 mL of periplasmic extract and treated in the same manner. 20 µL from each preparation were analyzed by SDS-PAGE.

The above samples were also analyzed by western blot (Towbin *et al.* 1979, Burnette 1981) using goat IgG α-steel factor primary antibodies (R&D corp. cat. no. AB-455-NA) at a dilution of 1/500, and alkaline phosphatase conjugate rat α-goat IgG secondary antibodies (Sigma) at a dilution of 1/10,000. Blots were also analyzed using polyclonal rabbit α–CBDcex primary antibodies at a dilution of 1/5 000 and goat α-rabbit IgG alkaline phosphatase conjugate secondary antibodies at a dilution of 1/10 000.

## 2.6. Determination of optimum conditions for gene expression.

### 2.6.1. Conditions for optimum cell growth.

A 3 mL test tube culture of JM101/pSLF/CBD 1.0 was grown for four hours at 37$^{o}$ and then used to inoculate three 2 L flasks containing 500 mL of TYP$^{Kan}$. One of these flasks was placed in a 37$^{o}$ shake flask incubator, another in a 30$^{o}$ shake flask incubator, and the third in a room temperature shake flask incubator. All were shaken at 250 rpm and allowed to grow, with samples being removed periodically for optical density measurements.

A second test was carried out to determine the effect of protein production on cell growth. Two parallel 300 mL cultures were grown at 30$^{o}$ C, with samples being removed every 30 min. for optical density measurements. One culture was induced with 0.1 mM IPTG at mid log phase while the other was not, and the optical densities of the two cultures were measured for an additional five hours.

### 2.6.2. Optimal induction period.

A 300 mL culture of JM101/pSLF/CBD 1.0 was grown to mid log phase and then induced with 0.1 mM IPTG. Cell culture samples were removed every hour for eight hours, and culture supernatants were examined for cellulose-binding proteins as described in section 2.5.

## 2.7. Affinity chromatography of SLF-CBD 1.0 using cellulose.

### 2.7.1. Desorption of SLF-CBD 1.0 from Avicel.

A series of experiments was conducted to determine the conditions required for desorption of the fusion protein from Avicel. 300 mg of Avicel was added to 300 mL of culture supernatant and stirred for 10 minutes to allow SLF-CBD 1.0 to adsorb. The Avicel was then recovered by centrifugation and washed in 20 mL of PBS. A small sample of this Avicel was analyzed by SDS-PAGE, as described in section 2.5, to confirm adsorption of the fusion protein to the cellulose. The remaining Avicel was then divided into 5 mg aliquots, each of which was washed with a different solution or reagent in an attempt to desorb the fusion protein. Bound protein samples were washed with either 2 mL or 10 mL of these solutions for 10 minutes. The Avicel was then boiled in SDS-PAGE loading buffer as before and the buffer loaded onto an SDS-PAGE gel for analysis as described in section 2.5.

### 2.7.2. Affinity chromatography on cellulose: desorption with guanidinium hydrochloride.

A small amount of protein was purified from an Avicel affinity column using 6 M guanidinium hydrochloride for elution. 5 g of Avicel was added to 500 mL of culture supernatant, stirred at room temperature for 10 minutes, removed by centrifugation, and washed in 500 mL of PBS/100 mM NaCl for a further 10 minutes. The resulting Avicel slurry was poured into a Pharmacia XK 26/20 column and allowed to drain. The column was then connected to an FPLC system (Pharmacia LKB Biotechnology) and the protein was eluted with a 100 mL gradient ranging from 0 M to 6 M guanidinium chloride at a flow

rate of 1 mL min.$^{-1}$. 10 mL fractions were collected and analyzed by SDS-PAGE. Fractions containing the fusion protein were pooled, and the guanidinium hydrochloride exchanged for PBS, over a period of 12 hours, using high pressure ultrafiltration.

### 2.7.3. Affinity chromatography on cellulose: desorption with ethylene glycol.

A small scale purification of the fusion protein was carried out, as described above, using an ethylene glycol gradient. 5 g of Avicel was added to 500 mL of culture supernatant and stirred at room temperature for 10 minutes. Avicel was then recovered by centrifugation, re-suspended in 500 mL of PBS/100 mM NaCl , and washed for a further 10 minutes. The slurry was then poured into a Pharmacia 26/20 FPLC column, and the protein eluted using a 100 mL ethylene glycol gradient, ranging from 0% to 100% (v/v) ethylene glycol, at a flow rate of 1 mL min.$^{-1}$. 10 mL fractions were collected and analyzed by SDS-PAGE. Fractions containing the fusion protein were combined and the ethylene glycol was exchanged for PBS as described above. The resulting sample was reduced to a final volume of 3 mL by ultrafiltration, filter sterilized, and stored at -70$^{0}$ C for activity testing. The final protein concentration was determined using both the ultraviolet absorbency at 280 nm wavelength (Sambrook *et al.* 1989, Scopes 1974) and the Bradford method (Bradford 1976), with bovine serum albumin (BSA) and recombinant CBDcex as standards. Calculation of the theoretical extinction coefficient of SLF-CBD 1.0 is given in appendix 2.

## 2.8. Purification of secreted SLF-CBD 1.0 by metal chelate affinity chromatography (MCAC).

### 2.8.1. Purification of secreted SLF-CBD 1.0 from cell periplasms.

JM101 was transformed with the plasmid pSLF/CBD 1.0 and plated on LB$^{Kan}$ solid medium. Five isolated colonies were used to inoculate each of five 2 L shake flasks containing 400 mL each of TYP$^{Kan}$ liquid medium. Cultures were grown at 37$^{0}$ C with 250 rpm shaking to an optical density of

2.0. Flasks were then shifted to 30° and 150 rpm, and protein production was induced with 0.1 mM IPTG for a further eight hours. Cells were then separated from culture supernatant by centrifugation and subjected to osmotic shock (Nossal and Heppel 1966). Proteins from the periplasmic compartment were then collected. The periplasmic extract was buffered to pH 8.0 with 50 mM Tris-base (Boehringer-Mannheim) and the volume reduced to 2 mL by ultrafiltration. SLF-CBD 1.0 was subsequently purified using metal chelate affinity chromatography (MCAC) (Ausubel *et al.* 1994a, Hochuli 1990) and a Pharmacia XK 16 column packed with 50 mL of His-Bind™ resin (Novagen inc., cat. no 69670). An imidazole gradient, ranging from 0 mM to 500 mM imidazole, was used to elute the protein from the column. Peak fractions were pooled and the elution buffer was exchanged for PBS by ultrafiltration. The sample was then reduced to a volume of 5 mL by ultrafiltration, and filter sterilized for use in subsequent activity tests. The purity of the final sample was evaluated by SDS-PAGE and western blotting. Protein concentration was determined as described in section 2.7.3.

### 2.8.2. Purification of secreted SLF-CBD 1.0 from culture supernatant.

Protein from the culture supernatant was purified as described above after an initial ammonium sulfate precipitation was used to reduce the sample volume. The supernatant was chilled to 0° C and brought to 80% of saturation with ammonium sulfate, while maintaining the pH at 6.05 (the predicted isoelectric point of the fusion protein) with 1N $NH_4OH$. The solution was then centrifuged at 10 000 g and the resulting protein precipitate was re-dissolved in 10 mL of MCAC loading buffer. Purification was then carried out as described above.

## 2.9. Purification and solid phase re-naturation of SLF-CBD 1.1 inclusion bodies using MCAC.

JM101/pSLF/CBD 1.1 prepared as described in section 2.8. Cells were then harvested and re-suspended in MCAC loading buffer containing 6M guanidinium hydrochloride, and ruptured in a french pressure cell. Inclusion bodies were then solubilized by stirring at 0° C for one hour in the presence of

the 6M guanidinium hydrochloride, after which any remaining insoluble cell components were removed by ultra centrifugation. The viscosity of the sample was then reduced by forcing the solution through a 25 gauge needle to shear the DNA. The re-solubilized SLF-CBD 1.1 inclusion bodies were purified and re-natured while bound to nickel sepharose by gradual, stepwise removal of the 6M guanidinium chloride (Ausubel *et al.* 1992b). Peak fractions of SLF-CBD 1.1 were again pooled and the elution buffer was exchanged for PBS as before. Proteins were analyzed as described above.

### 2.10. Western blotting analysis of purified protein.

Western blot analysis was carried on purified SLF-CBD 1.0 (periplasmic) (as described in section 2.5).

### 2.11. MTT assay (without cellulose).

The steel factor dependent bone marrow stem cell line B6SUtA (Greenberger *et al.* 1983) was used as a target cell for testing the biological activity of the recombinant growth factors. As mentioned above, cells were maintained in H-SFM supplemented with 10 % (v/v) FBS and 5 % (v/v) murine spleen cell conditioned medium. Cell proliferation assays were carried out in unsupplemented H-SFM.

Samples of purified SLF-CBD 1.0 (periplasmic) protein were diluted in H-SFM. 70 µL samples of test protein in H-SFM were placed into the wells of sterile 96 well tissue culture plates (Costar corp. cat. no. 3595). Protein was then incubated for 12 hours at 37° C with 5 % $CO_2$ before 100 µL of B6SUtA cells (which had been grown to one day past confluence and then re-suspended in H-SFM at a concentration of 2 X $10^5$ cells $mL^{-1}$) were added for a final assay volume of 170 µL. Test samples were then incubated for 48 hours at 37° C and 5 % $CO_2$ before cell proliferation was measured either by direct cell count in a standard 1 $mm^2$ hemocytometer, or by the MTT assay (Denizot and Lang 1986). Data points were collected in duplicate for MTT tests, and in quadruplicate for direct counts. These points were then plotted graphically as the average of the replicates, with error bars being equal to one

standard deviation above and below the mean before subtraction of the baseline activity. Equimolar concentrations of CBDcex were again used as negative controls. Activity measurements were taken from several wells containing CBDcex, and the average of these points was used as the baseline value for cell proliferation in the absence of growth factors. This baseline value was then subtracted from all test protein data points.

Recombinant steel factor (R&D systems, Minneapolis, MN, cat. no. 455-MC) was used as a positive control, while recombinant CBDcex was used as the negative control. A limited amount of glycosylated SLF, produced in *Saccharomyces cerevisiae,* was also available (Intermedico., cat. no. 1832-01), and was used in the initial test of activity to compare the glycosylated protein to the non-glycosylated protein. SLF-CBD 1.0 eluted from cellulose by ethylene glycol and SLF-CBD 1.1 re-natured from inclusion bodies were also tested for activity. The various proteins were tested on the same day using the same cells, and therefore the results were directly comparable.

## 2.12. Neutralization of SLF-CBD 1.0 by neutralizing polyclonal antibodies.

500 pM of SLF-CBD 1.0 was incubated with various concentrations of goat α-SLF polyclonal neutralizing antibody (R&D systems cat. no. AB-455-NA) for one hour prior to the addition of the B6SUtA cells. Cell proliferation was then measured as before.

## 2.13. Preparation of bacterial micro crystalline cellulose (BMCC).

Bacterial micro crystalline cellulose (BMCC) is a highly crystalline form of cellulose produced as a pelicle by an aquatic bacteria. BMCC was selected as an immobilization matrix because of its high degree of crystallinity. BMCC was prepared† at an initial concentration of 3 mg $mL^{-1}$ as previously described (Gilkes *et al.* 1992), sterilized, and re-suspended in H-SFM at various concentrations by serial dilution.

†BMCC was prepared by Mrs. Emily Kwan (Cellulase Laboratory).

## 2.14. Determination of an optimal BMCC concentration for immobilization of SLF-CBD 1.0.

### 2.14.1. Variation of BMCC concentration in the presence of a constant amount of growth factor.

A series of preliminary tests was constructed to determine the optimal concentration of BMCC to use for immobilization of SLF-CBD 1.0. In these cell proliferation tests the concentration of growth factor was held constant while the concentration of BMCC was varied. The entire test was then repeated three times with three different concentrations of growth factor.

BMCC suspended in H-SFM was diluted serially by the addition of 1 mL of BMCC suspension to 0.5 mL of H-SFM. 20 μL recombinant SLF and SLF-CBD 1.0 test samples were added to wells as described above, and then 50 μL samples of the BMCC, at various concentrations, were added to the wells for a final volume of 70 μL. The recombinant protein and the BMCC were then incubated together at 37° C and 5% $CO_2$ for 12 hours before 100 μL of B6SUtA cells were added, and the cell proliferation assay carried out as before. Data points were collected in duplicate. Recombinant SLF and recombinant CBDcex were again used as the positive and negative controls.

### 2.14.2. Separation of adsorbed activity from free activity: (variable BMCC concentration).

The BMCC serial dilution experiments described above were repeated. In this case, however, 50 μL BMCC samples were allowed to bind to 20 μL protein samples for 12 hours at 37° C and 5% $CO_2$ in Eppendorf tubes rather than in tissue culture wells. BMCC was then recovered by centrifugation, and the supernatant was transferred to a new tube. The BMCC was then re-suspended in 70 μL of fresh H-SFM, and all samples were tested as before. Data points were again collected in duplicate.

## 2.15 . Effect of immobilization on SLF-CBD 1.0 activity: (variable SLF-CBD 1.0 concentration).

A set of experiments, converse to that listed in section 2.14, was carried out in which the amount of BMCC present was held constant, while the concentration of SLF-CBD 1.0 was varied. The activity of SLF-CBD 1.0 was measured in the presence of, and in the absence of BMCC. As before, SLF was used as a positive control while CBDcex was used as a negative control. Activity was measured using both MTT and direct cell counts in a standard 1 $mm^2$ field hemocytometer as described below.

### 2.15.1. Direct cell count activity test.

Activity tests were carried out as described in section 2.11. Data points were collected in quadruplicate, with four fields of a standard haemocytometer being counted four each of the four data points. In cases where the cell number was low sufficient fields were counted to include a minimum of 50 cells. Two parallel sets of data were generated: one set in which BMCC (in H-SFM) had been added to the reaction mixture, and one set in which H-SFM alone had been added to the reaction mixture. For tests containing BMCC, 50 µL aliquots of BMCC (in H-SFM) were added to 20 µL samples of test protein (in H-SFM) and allowed to incubate for 12 hours before addition of test cells. The final concentration of BMCC was approximately 1 µg $mL^{-1}$ after the addition of B6SUtA cells, in a final test volume of 170 µL. For samples where no BMCC was present 50 µL of H-SFM alone was added to the wells.

All of the direct cell count data collected for this section was generated from the same cells on the same day, and therefore the results were comparable. Activity trends were assessed with regression analysis of the curves generated from the direct cell count data. Best fit curves were calculated for these data, in two iterations, using the non-linear regression analysis program GraFit version 3.0 (Erithacus Software Ltd.). This program supplied both the theoretical cell maximum and the theoretical $ED_{50}$ (the growth factor concentration required to generate half the maximum cell density) based on these regression curves.

### 2.15.2. MTT activity test data.

MTT activity tests were carried out as described in section 2.11, except that parallel tests were also carried out in which BMCC was added to the test samples before the addition of B6SUtA cells. Final BMCC concentrations were as indicated. Not all MTT tests were carried out on the same day with the same cells, and therefore the data are not necessarily comparable.

## 2.16. Separation of adsorbed activity from free activity: (variable SLF-CBD 1.0 concentration).

The activity of immobilized SLF-CBD 1.0 was separated from the activity of free SLF-CBD 1.0 as described in section 2.14.2 except that in this case the concentration of BMCC present was held at 1 $\mu g\ mL^{-1}$ while the concentration of SLF-CBD 1.0 was varied.

## 2.17. *In situ* cleavage of adsorbed SLF-CBD 1.0 by Factor Xa.

Adsorbed SLF-CBD 1.0 was released from BMCC by cleavage of the CBDcex affinity tag with Factor Xa protease *in situ*. Tests were carried out as described in sections 2.11 and 2.15 except that 2.5 ng of Factor Xa (Boehringer-Mannheim, cat. no. 1179 888) was added to each of one set of test samples during the 12 hour incubation period prior to the addition of the B6SUtA cells.

## 2.18 . Preparation of cellulose coated tissue culture plates (regenerated cellulose surfaces).

A 1% (w/v) solution of cellulose acetate was prepared by adding one gram of cellulose acetate (Kodak Inc.) to 99 mL of acetic acid. 100 µL of this solution was added to each well of the standard 96 well tissue culture plates used above, and the acetic acid allowed to evaporate in a standard fume hood.

100 µL of 50 mM NaOH was then added to each well and left for 20 minutes to neutralize any remaining acetate. The NaOH was then drained off, and the wells rinsed three times with PBS. 300 µL of 70 % (v/v) ethanol (for sterilization) was then added to each well, the plate lid replaced, and the ethanol allowed to evaporate.

### 2.19. Activity tests of SLF-CBD 1.0 adsorbed to a regenerated cellulose surface.

Activity tests were carried out as previously described in sections 2.11 and 2.15 except that a regenerated cellulose surface was used to immobilize SLF-CBD 1.0. 20 µL protein test samples were put into cellulose acetate coated wells. 50 µL of H-SFM was then added to the wells, and the plate was incubated for 12 hours, as before, prior to the addition of 100 µL of B6SUtA cells for the standard final volume of 170 µL. Data points were collected in quadruplicate with activity being measured by MTT. A control group of cells was added to each of four wells in a cellulose acetate coated plate and to each of four wells in a non-coated plate. These cells were then counted directly after a 48 hour incubation, in the presence of SLF, to determine whether or not any toxic effects could be attributed to the cellulose acetate.

#### 2.19.1. Separation of adsorbed activity from free activity: (variable SLF-CBD 1.0 concentrations adsorbed to a regenerated cellulose surface).

Protein samples were added to cellulose acetate coated wells and allowed to incubate for 12 hours at 37° C and 5% $CO_2$. 55 µL of the 70 µL supernatant was then transferred to a new well in a non-cellulose acetate coated plate, and 55 µL of fresh H-SFM was then added to the original well. At least 15 µL was left in the original well at all times in order to avoid leaving the surface, and any proteins immobilized thereon, exposed to the air. The cell proliferation assay was then carried out as before. SLF was used as a control protein.

### 2.19.2. Re-use of SLF-CBD 1.0 adsorbed to a cellulose surface.

The cellulose-coated tissue culture plates from section 2.19.1 were re-seeded with fresh B6SUtA cells (at a concentration 2 X $10^5$ cells $mL^{-1}$) for a total of four rounds of cell proliferation, with proliferation being measured as before. As an additional control the conditioned cell culture supernatant from the SLF plate was re-cycled for four rounds of cell culturing. Cells grown in the presence of SLF were first transferred from the tissue culture wells to Eppendorf tubes. Cells were then separated from conditioned culture supernatant by centrifugation, re-suspended in fresh H-SFM, and tested by MTT. 150 µL of the conditioned culture supernatant was then returned to the original well and re-seeded with 20 µL of B6SUtA cells (at a concentration of 1.5 X $10^6$ cells $mL^{-1}$ for a final concentration equivalent to that used above). For consistency, B6SUtA cells grown in the presence of immobilized SLF-CBD 1.0 were also separated from their supernatant by centrifugation and re-suspended in fresh H-SFM prior testing. Cells were transferred to non-cellulose plates before proliferation was measured.

# 3. RESULTS.

## 3.1. Construction of SLF/CBD Fusion Genes.

### 3.1.1. Construction of the fusion protein expression plasmid pSLF/CBD 1.0.

A cDNA copy of the murine SLF gene was obtained from Dr. R. K. Humphries (via Dr. D. E. Williams, Immunex Corp., Seattle Wa.) as a 2.0 kb Sma I insertion into pBS K/S II+ (Stratagene) (Fig. 3.1 A). Using this plasmid (pBS K/S II (SLF)) as a template the polymerase chain reaction (PCR) was carried out (as described in section 2.4.1) to create a synthetic version of the gene segment encoding the SLF extra-cellular domain (Fig. 3.1 A insert). The PCR product was ligated into the non-expression plasmid pSL1180 as a 0.542 kb Nco I - Hind III fragment (Fig. 3.1 A ii), with the resulting plasmid being designated pSL/SLF (PCR) (Fig. 3.1 A iii). Since the PCR reacation is known to introduce mutations into gene segments the majority of the PCR product was removed from this clone and replaced with wild type DNA as outlined below.

The majority of the PCR product, containing mutations introduced by the PCR process, was then replaced with DNA from the original pBS K/S II (SLF) clone. In order to confirm that the exchange had taken place a 2.0 kb fragment of "filler" DNA was first inserted into the PCR product to increase its size (Fig. 3.1 B i). The resulting hybrid was designated pSL/SLF/Cex (due to the fact that the "filler" DNA was actually a segment of the *Cex* gene). The bulk of this hybrid gene was then replaced with DNA from the original clone (Fig. 3.1 B ii), with the return of the Nco I - Hind III fragment to its original size confirming the exchange (Fig. 3.1 B iii). The remaining sections of PCR product were sequenced, with no PCR introduced mutations being found.

The expression vector pTug A (Graham *et al.* 1995) was modified to encode the amino-terminal Cex signal peptide (Cex LP), a hexahistidine affinity purification tag (H6), and kanamycin resistance (Dr. R. Graham, unpublished construct). This plasmid (pTug3SN8) was designed for high level recombinant protein expression, with export to the periplasm (mediated by the Cex signal peptide) followed by metal chelate affinity chromatography

(MCAC) purification of the protein. A variation of this plasmid (containing the cellulose-binding domain from the cellulase CenB) was obtained from Dr. C. Ramirez (Dr. C. Ramirez, unpublished construct), and used as the

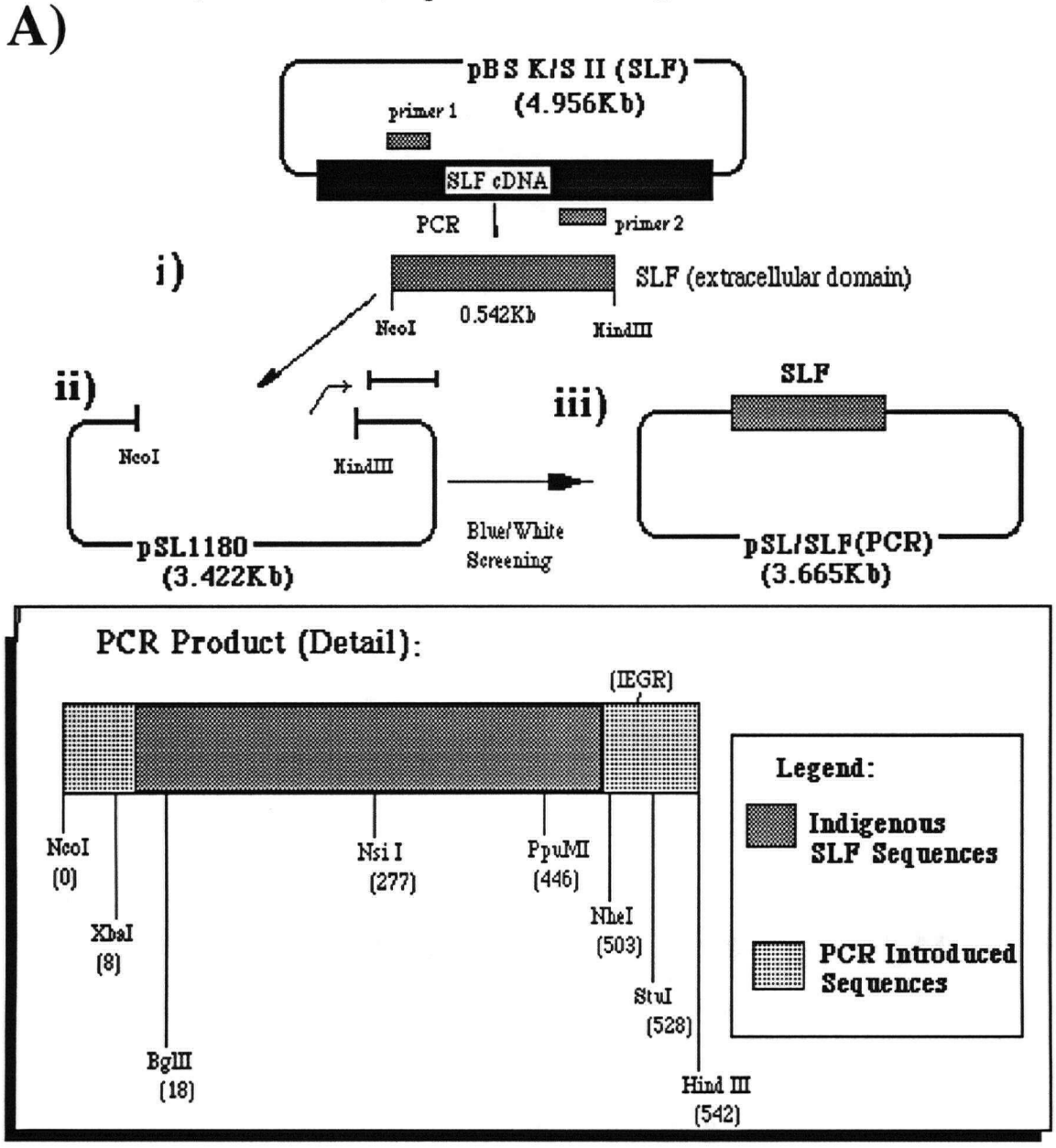



**Figure 3.1 A. Construction of a synthetic SLF gene using the polymerase chain reaction.**

**i)** The polymerase chain reaction was used to create a synthetic version of the gene fragment encoding the extracellular domain of murine SLF. **ii)** The PCR product was then inserted into the non-expression plasmid pSL1180. **iii)** Blue/white colony screening followed by restriction digest analysis confirmed the presence of the insert in the plasmid pSL1180 to create the intermediate plasmid pSL/SLF (PCR).

**B)**

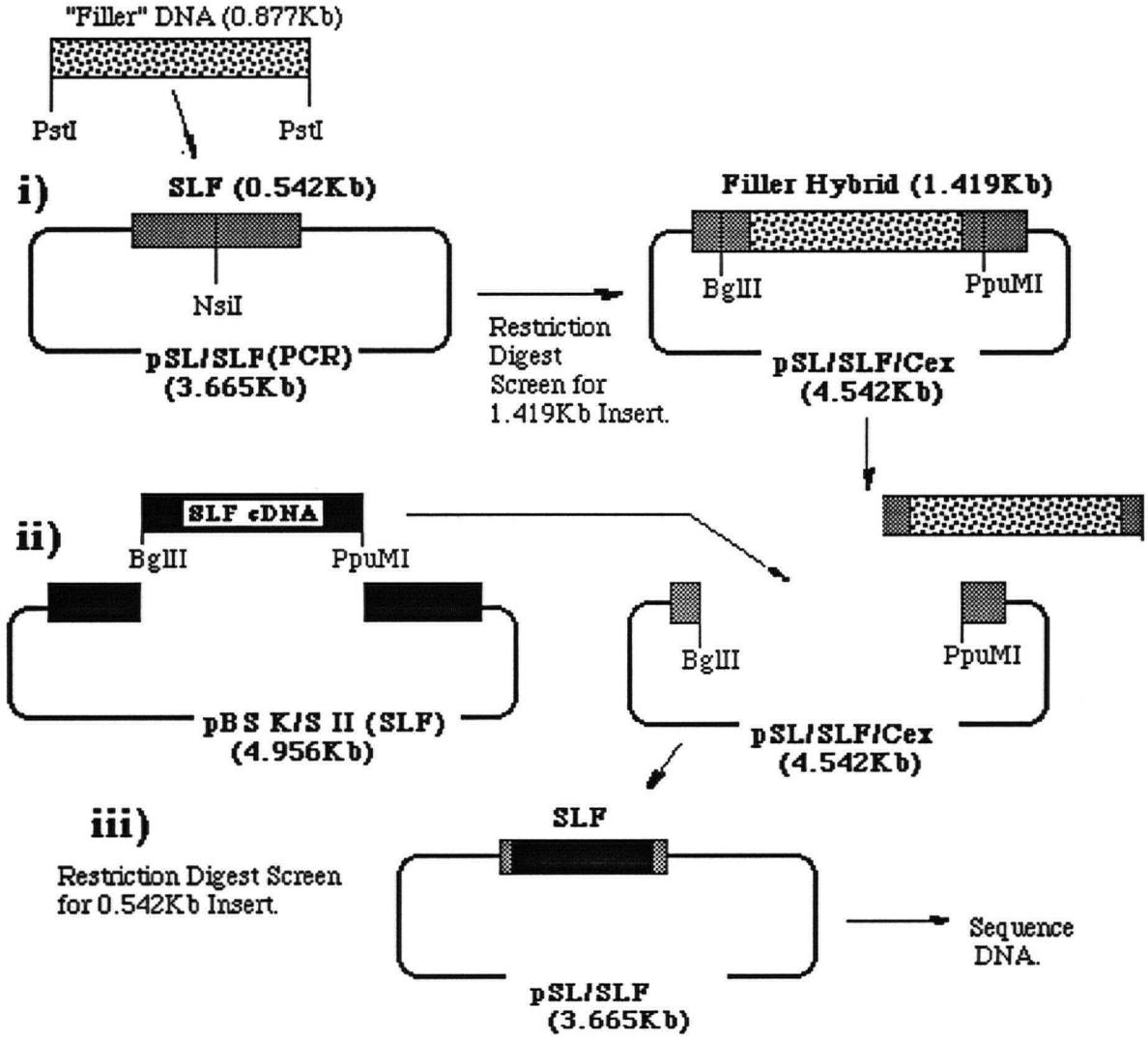



**Figure 3.1 B. Replacement of PCR product with original SLF DNA.**

**i)** Insertion of "filler" DNA into the PCR product causing an increase in the size of the Nco I - Hind III fragment. **ii)** Replacement of the PCR product with the original gene segment from pBS K/S II (SLF), causing the Nco I - Hind III fragment to return to its original size. **iii)** Insertion of original DNA created the plasmid pSL/SLF.

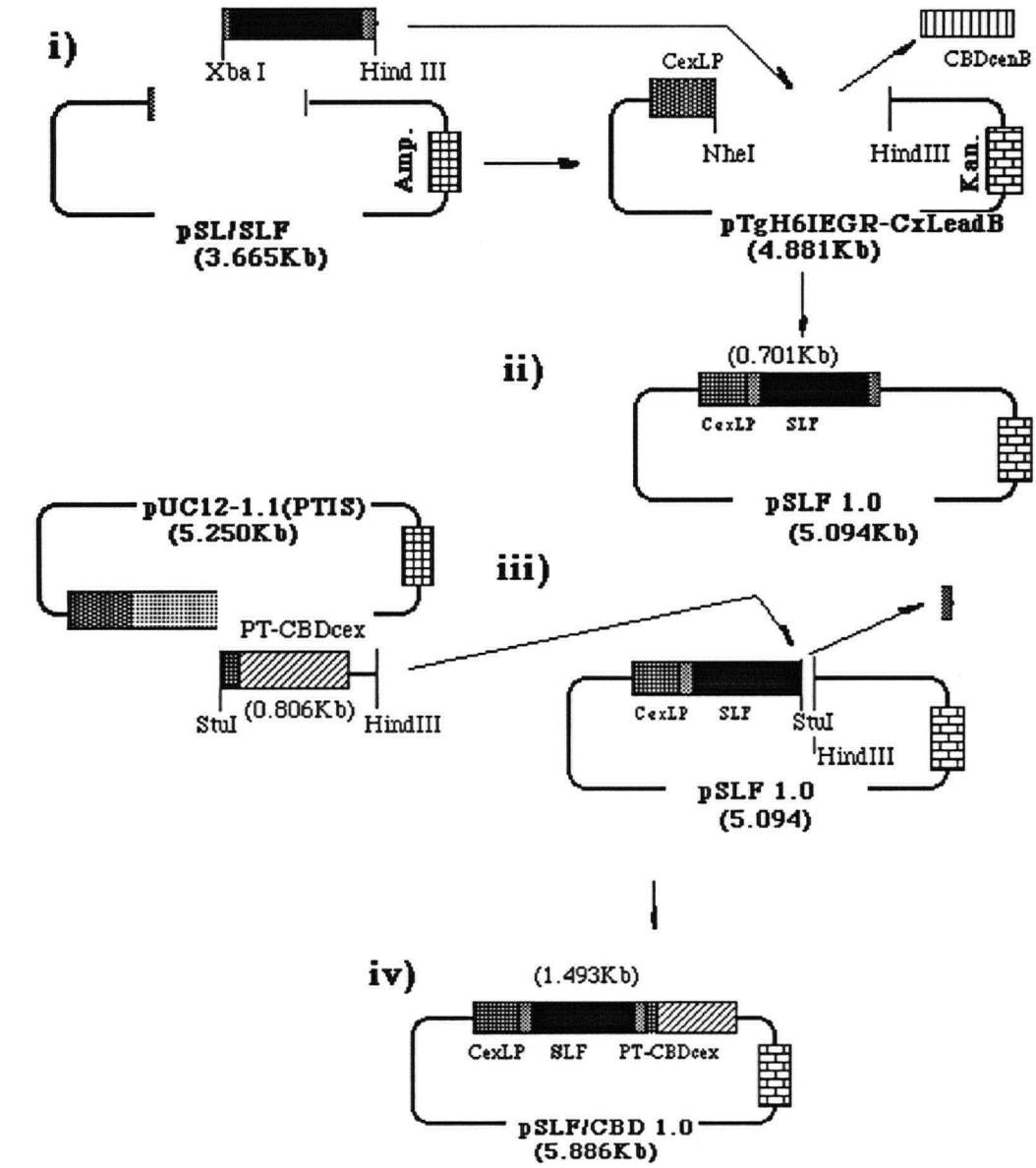
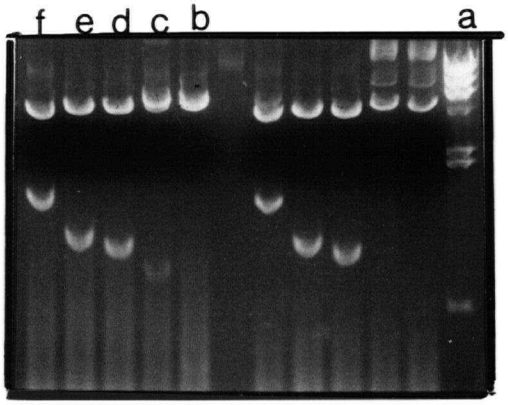



**Figure 3.1 C. Creation of the fusion plasmid pSLF/CBD 1.0.**

**i)** The gene segment encoding the SLF extracellular domain was excised from the plasmid pSL/SLF and inserted into the expression plasmid pTgH6IEGR-Cx Lead B, downstream of the Cex signal peptide. **ii)** The gene encoding the SLF extracellular domain was preceeded by the Cex export signal peptide in the intermediate plasmid pSLF 1.0. **iii)** The gene segment encoding the cellulose binding domain of Cex was then excised from the Cex gene (O'Neill *et al.* 1986a) and inserted downstream of the gene segment encoding the SLF extracellular domain. **iv)** The resulting gene fusion plasmid was designated pSLF/CBD 1.0.

**A)**




**a) standards (λ/Hind III)**
**b) Sma I-Hind III**
**c) Nhe I-Hind III**
**d) EcoRI-Hind III**
**e) Nsi I-Hind III**
**f) Avr II-Hind III**

| (standards kb) |
|---|
| 23.13 |
| 9.42 |
| 6.56 |
| 4.36 |
| 2.32 |
| 2.03 |
| 0.56 |
| 0.13 |

**B)**

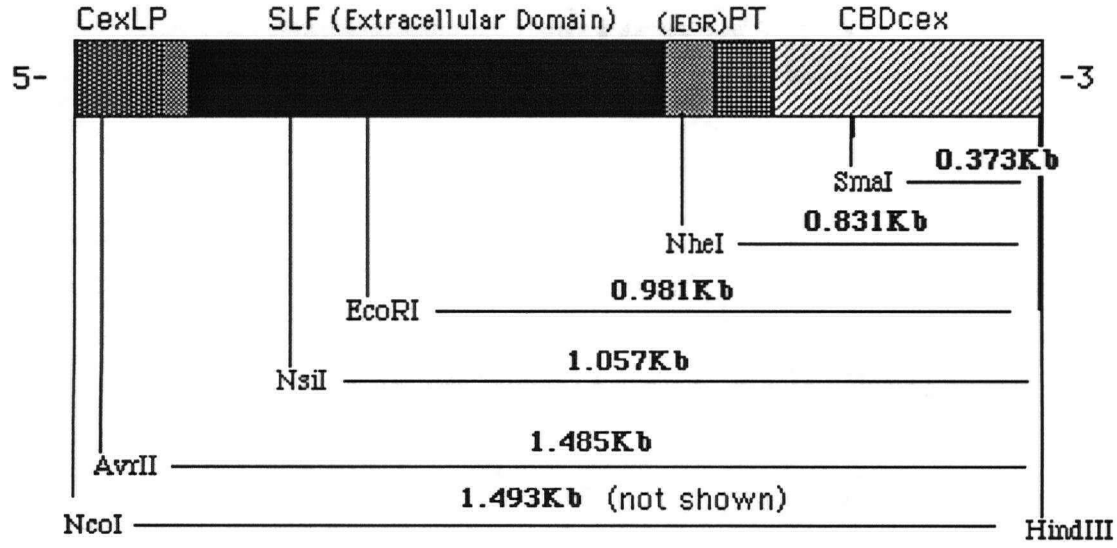



**Figure 3.2. Restriction digest identity test of pSLF/CBD 1.0.**

**A)** Restriction digestion fragments visualized on an agarose gel. **B)** Predicted sizes of restriction fragments.

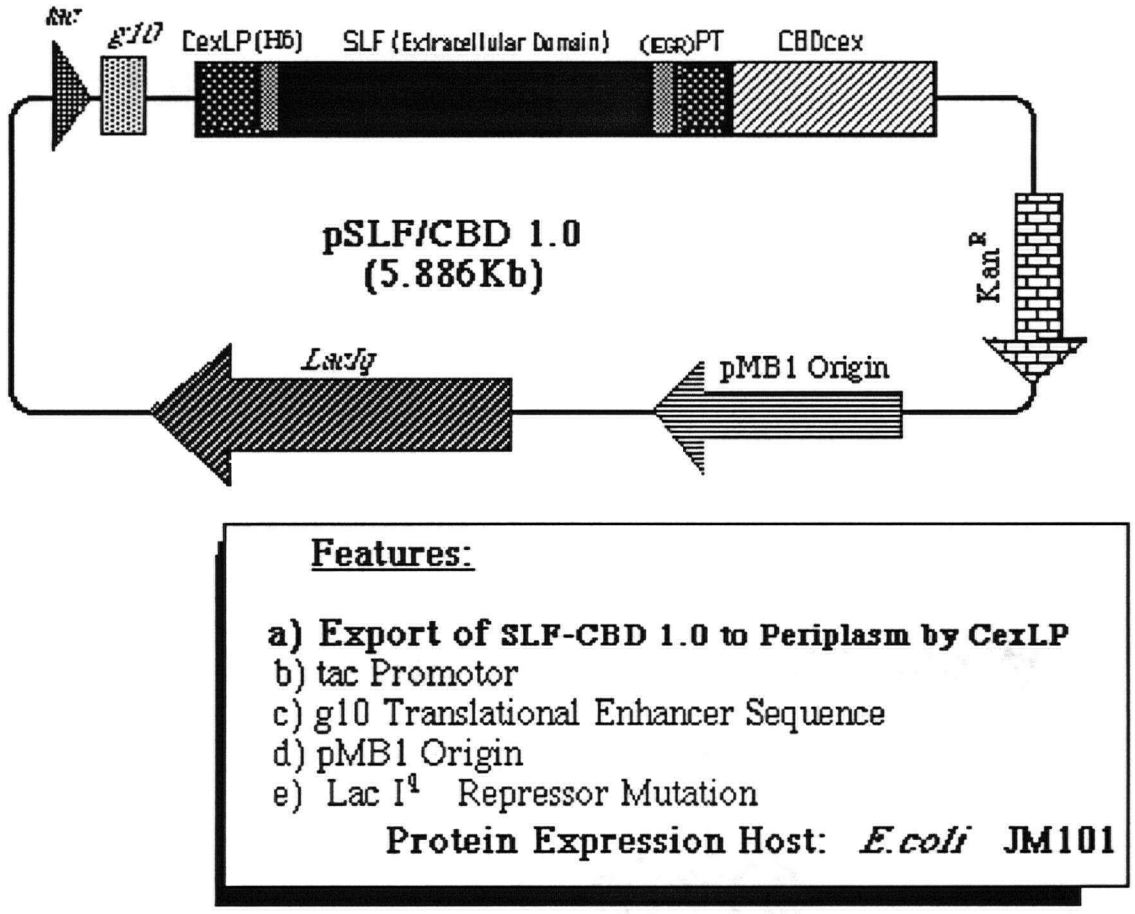



**Figure 3.3. Schematic diagram of the plasmid pSLF/CBD 1.0.**

expression vector for the present study. The gene segment encoding $CBD_{CenB}$ was removed (Fig. 3.1 C i) and replaced by the gene segment encoding the modified SLF extracellular domain (Fig. 3.1 C ii). The gene segment encoding the cellulose-binding domain (and proline-threonine linker) from the cellulase Cex was then inserted downstream of the modified SLF gene segment (Fig. 3.1 C iii) to create the plasmid pSLF/CBD 1.0 (Figures 3.1 C iv and 3.3). The identity of this plasmid was confirmed by restriction digest (Fig. 3.2).

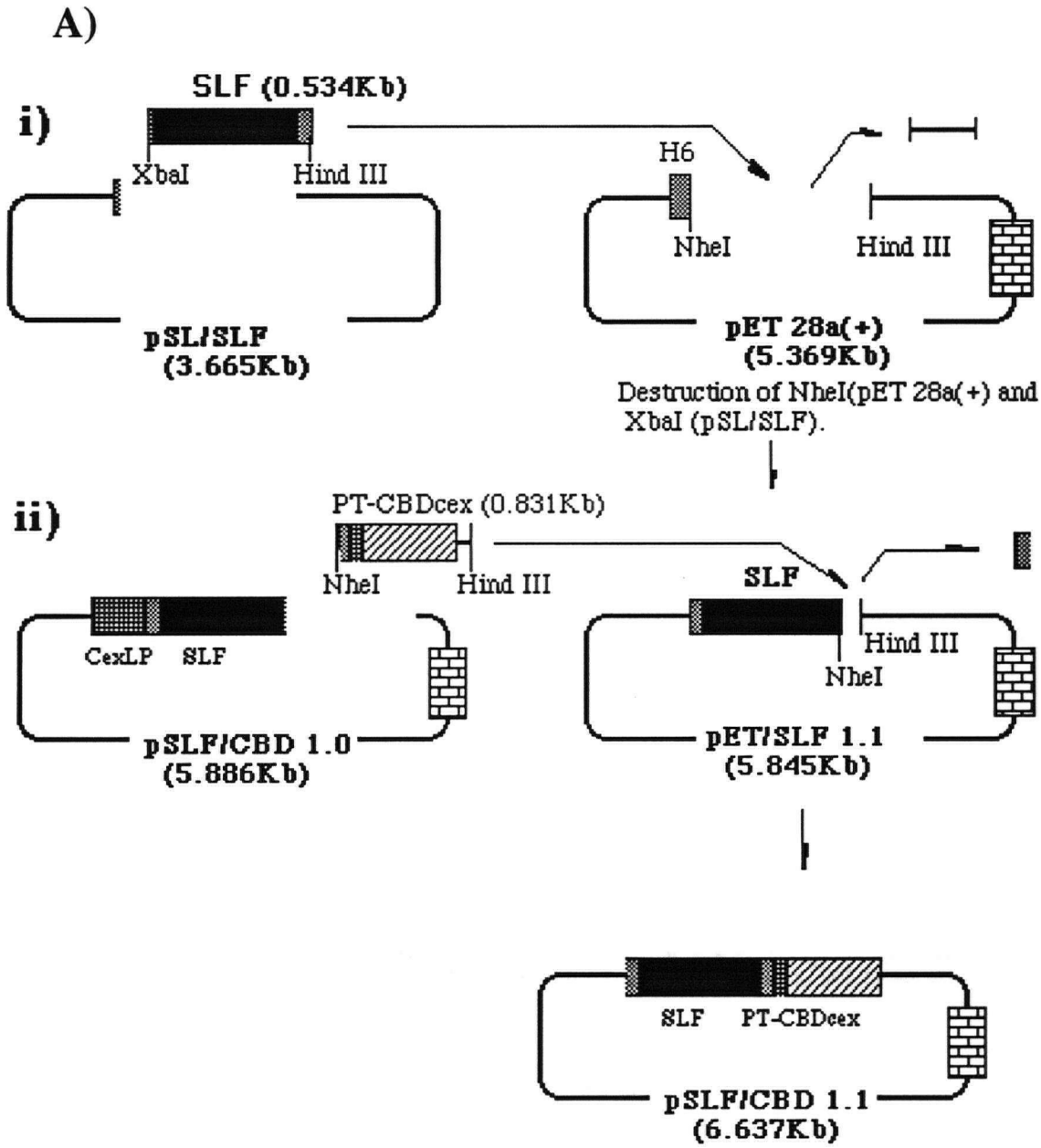



**Figure 3.4 A. Construction of the plasmid pSLF/CBD 1.1.**

**i)** The synthetic gene segment encoding the extracellular domain of SLF was excised from pSL/SLF as an Xba I - Hind III restriction fragment and inserted into the expression plasmid pET 28a(+) to create the intermediate plasmid pET/SLF 1.1. **ii)** $CBD_{Cex}$ was removed from the Cex gene contained within the plasmid pUC12-1.1 (PTIS) as a Stu I - Hind III fragment and inserted into pET/SLF 1.1 to create the plasmid pSLF/CBD 1.1. pSLF/CBD 1.1 was designed for the production of SLF-CBD in the cytoplasm of *E.coli* without export to the periplasm.

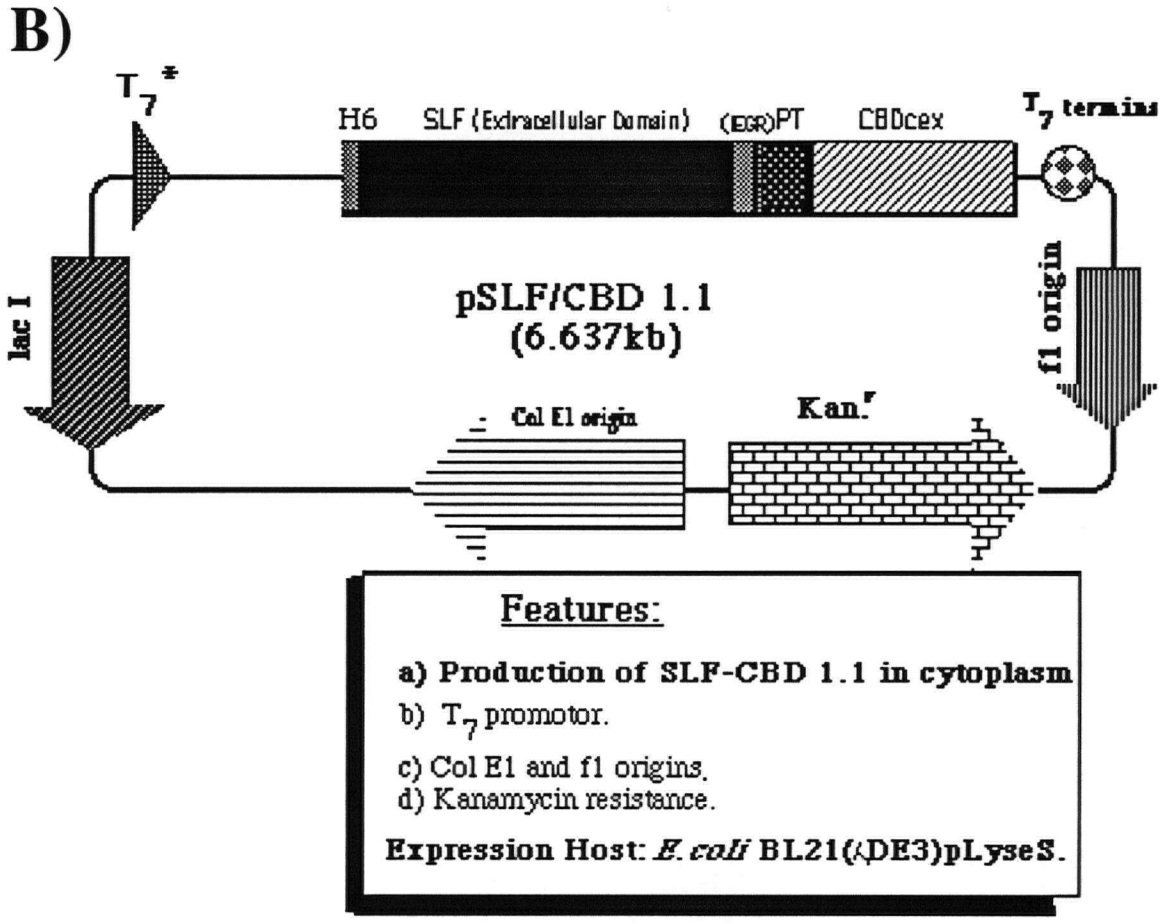



**Figure 3.4 B. The SLF-CBD 1.1 fusion protein expression plasmid pSLF/CBD 1.1 in detail.**

*The plasmid pSLF/CBD 1.1 is based on the plasmid pET 28(a)+ (Studier and Moffatt 1986, and Studier et al. 1990) which maintains transcription under the stringent control of the λT7 RNA polymerase promotor. The λT7 RNA polymerase gene has been integrated into the chromosome of the host cell lysogen BL21 (IDE3)pLysS, the expression of which is induced by 0.1 mM IPTG.

### 3.1.2. Construction of the fusion protein expression plasmid pSLF/CBD 1.1.

A second fusion protein expression plasmid was designed to retain SLF-CBD in the cytoplasm of *E. coli* without export to the periplasm. This plasmid was constructed in case export of the SLF-CBD fusion protein to the periplasm proved to be unsuccessful. The gene segment encoding the modified SLF extracellular domain was excised from pSL/SLF and ligated

into the expression plasmid pET 28a (+) (Novogen Inc., Madison WI.) downstream of a DNA segment encoding a hexahistidine affinity tag (Fig. 3.4 A i). The gene segment encoding the cellulose-binding domain from Cex was then excised from pSLF/CBD 1.0 and inserted downstream of the gene segment encoding the modified SLF extracellular domain (Fig. 3.4 A ii). The resulting plasmid, pSLF/CBD 1.1 (Fig. 3.4 B), was derived from pET 28a (+), which uses a T7 RNA polymerase system (Studier *et al.* 1990). The two expression plasmids (pSLF/CBD 1.0 and pSLF/CBD 1.1), in addition to being designed for different methods of protein production, also encode slightly different amino termini (Fig. 3.5). (The complete DNA coding sequences of the two plasmids are presented in appendix 3. Translated amino acid sequences are presented in appendix 4.)

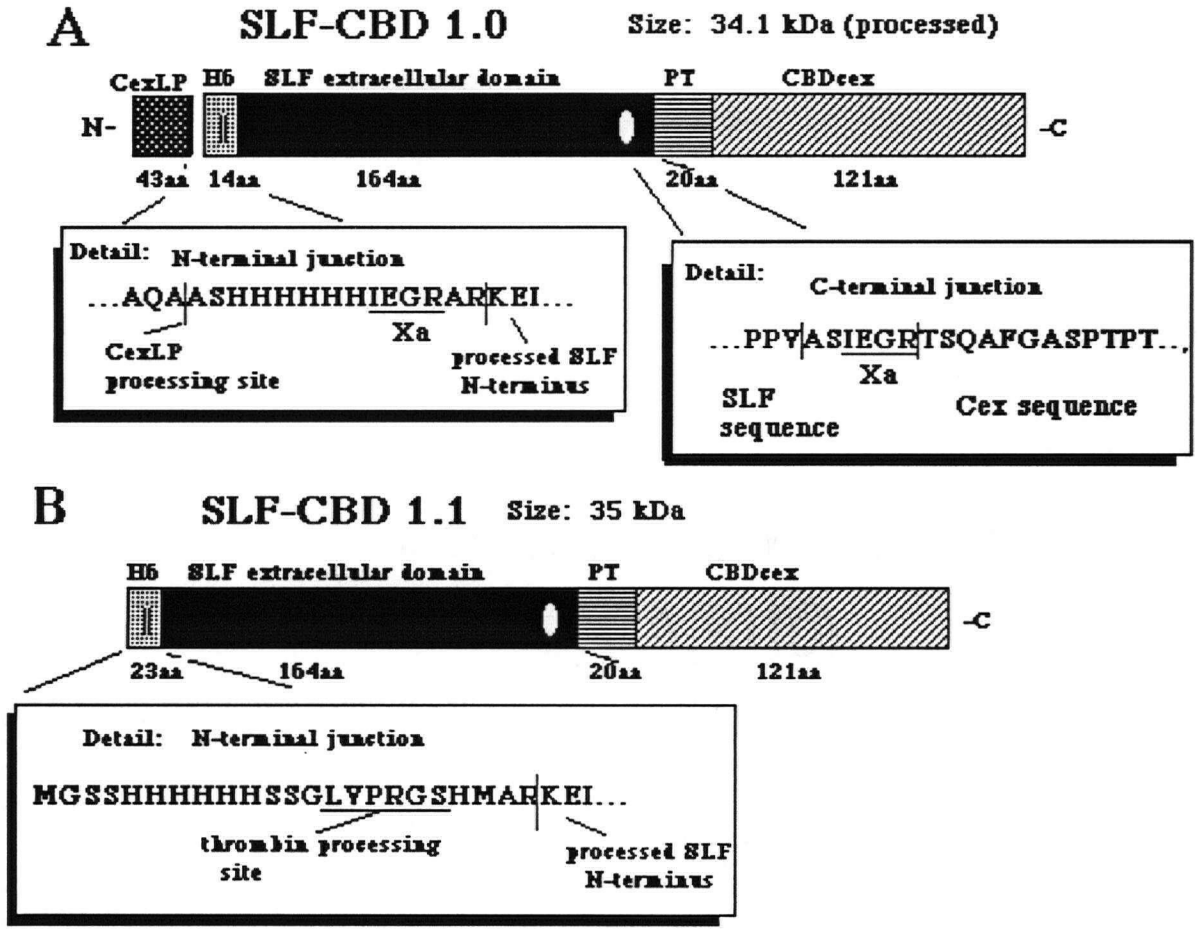



**Figure 3.5. SLF-CBD 1.0 and SLF-CBD 1.1.**

**A)** SLF-CBD 1.0 as encoded by pSLF/CBD 1.0. **B)** SLF-CBD 1.1 as encoded by pSLF/CBD 1.1.

### 3.2. Preliminary expression and binding tests of exported SLF-CBD 1.0.

*E. Coli* cells bearing the plasmid pSLF/CBD 1.0 were then screened for the production of a cellulose-binding protein of the predicted size for the fusion protein. The plasmid pSLF/CBD 1.0 was transformed into *E. coli* JM101 and protein production induced as described in section 2.5. Cells were then separated from the culture supernatant by centrifugation, and periplasmic proteins were extracted by osmotic shock. Cytoplasmic proteins were isolated by rupturing the cells in a French pressure cell. These three samples were assayed individually for the presence of a cellulose-binding protein of the predicted size (34.1 kDa) using the crude Avicel-adsorption assay described in section 2.5. Proteins which had adsorbed to Avicel (microcrystalline cellulose powder) were analyzed by SDS-PAGE (Fig. 3.6). A cellulose-binding protein of the predicted size was found in both the culture supernatant (Fig. 3.6 *lanes 1* and *2*) and the periplasmic extract (Fig. 3.6 *lanes 3* and *4*).

### 3.3. Determination of optimum conditions for SLF-CBD 1.0 production and export to the culture supernatant.

#### 3.3.1. Determination of optimum cell growth temperature.

Three 300 mL cultures of JM101/pSLF/CBD 1.0 were grown at three different temperatures (as described in section 2.6.1) to determine the effects of growth temperature on cell density (without gene expression). Cultures grown at room temperature (about $23^0$ C) reached higher cell densities than cultures grown at either $37^0$ C or $30^0$ C (Fig. 3.7 A).

#### 3.3.2. Effect of gene expression on cell growth kinetics.

To determine the effects of gene expression on cell growth two parallel cultures of JM101/pSLF/CBD 1.0 were grown at $30^0$ C, with induction of one culture at mid-log phase (estimated from the previous experiment). Protein production was not seen to effect cell growth (Fig. 3.7 B).

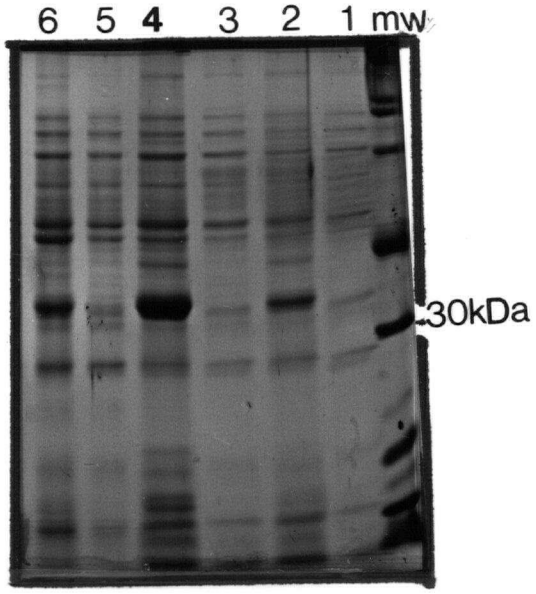



**Figure 3.6. SLF-CBD 1.0 found in various cellular compartments.**

***lane 1***, JM101/pSLF/CBD 1.0 culture supernatant.; ***lane 2***, SLF-CBD 1.0 concentrated from culture supernatant by Avicel; ***lane 3***, JM101/pSLF/CBD 1.0 periplasmic extract; ***lane 4,*** SLF-CBD 1.0 concentrated from periplasmic extract by Avicel; ***lane 5***, JM101/pSLF/CBD 1.0 cytosol; ***lane 6***, SLF-CBD 1.0 concentrated from cytosol by Avicel. (Note: broad range molecular weight (mw) markers are 200 kDa, 116 kDa, 97.4 kDa, 66.0 kDa, 45.0 kDa, 31.0 kDa, 21.5 kDa and 14.5 kDa from top to bottom. The marker 30 kDa marks the approximate position of the 31.0 kDa marker. The fusion protein, with a predicted molecular weight of 34.1 kDa, was expected to run midway between the 31.0 kDa and the 45.0 kDa markers.)

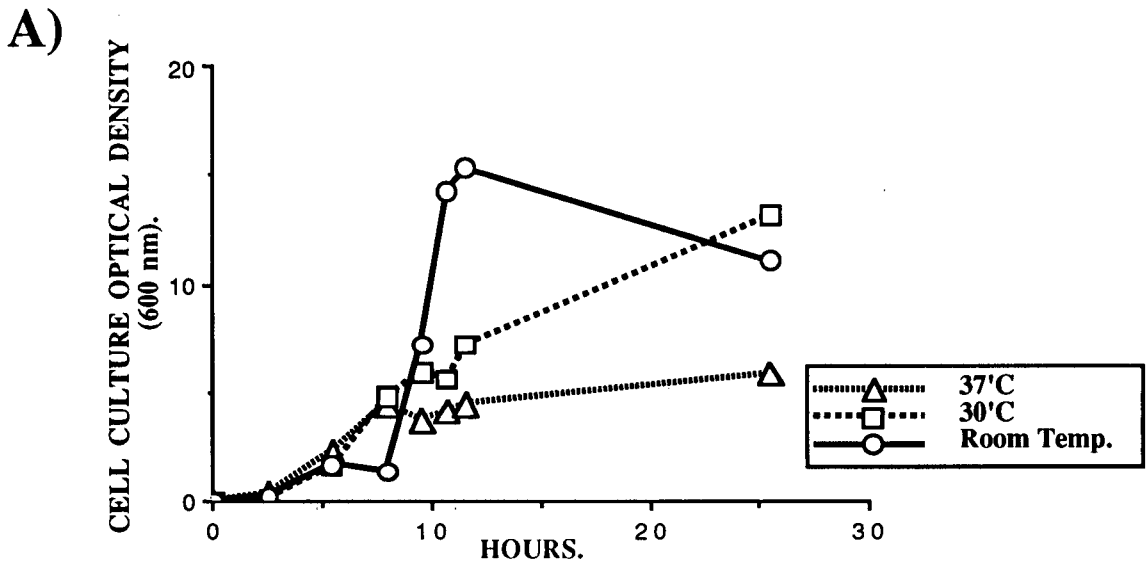



**Figure 3.7 A. Effects of culture growth temperature on cell density.**

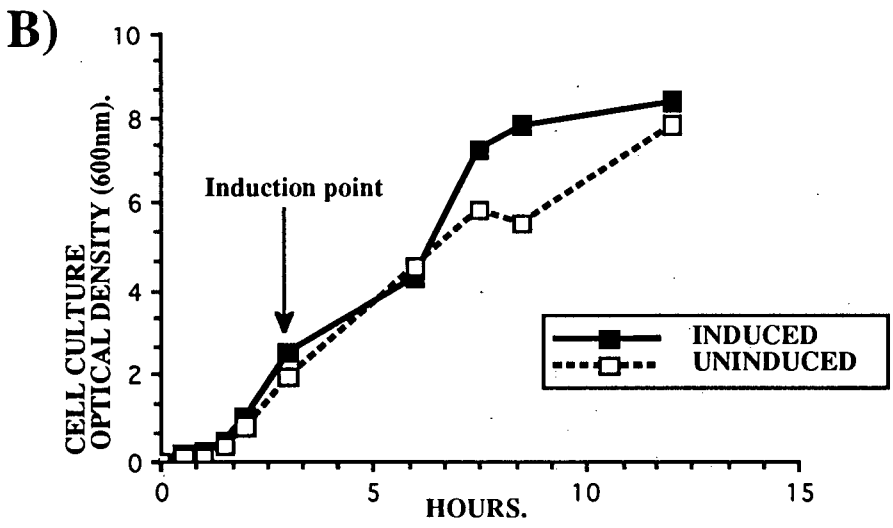



**Figure 3.7 B. Effects of SLF-CBD 1.0 production on cell density.**

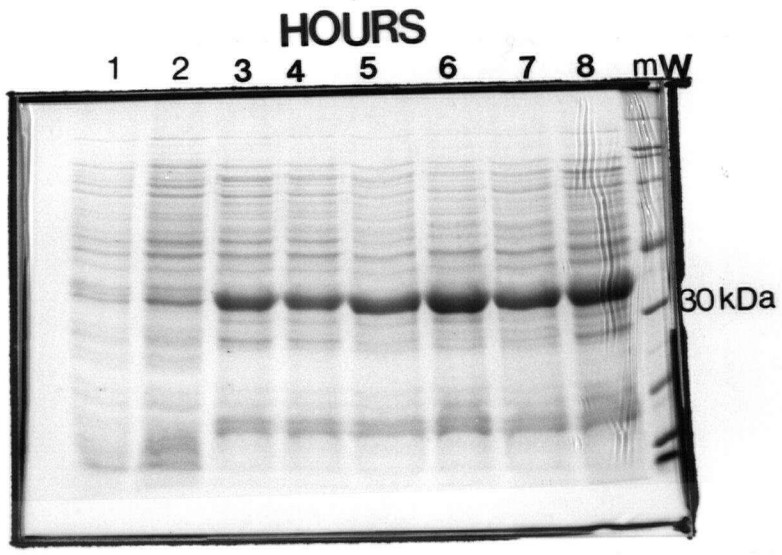



**Figure 3.8. SLF-CBD 1.0 stability in culture supernatant.**

Samples of JM101/pSLF/CBD 1.0 culture supernatant were examined for the presence of SLF-CBD 1.0 during an eight hour induction. (Note: broad range molecular weight (mw) markers are 200 kDa, 116 kDa, 97.4 kDa, 66.0 kDa, 45.0 kDa, 31.0 kDa, 21.5 kDa and 14.5 kDa from top to bottom. The marker 30 kDa marks the approximate position of the 31.0 kDa marker. The fusion protein, with a predicted molecular weight of 34.1 kDa, was expected to run midway between the 31.0 kDa and the 45.0 kDa markers.)

### 3.3.3. Stability of exported SLF-CBD 1.0 in culture supernatant.

To assess the stability of SLF-CBD 1.0 a 300 mL culture of JM101/pSLF/CBD 1.0 was inoculated, grown to mid-log phase at $30^0$ C, and induced with 0.1 mM IPTG as described in section 2.6.2. 1 mL samples of culture were removed every hour, over a period of eight hours, and the culture supernatant analyzed by SDS-PAGE as before. SLF-CBD 1.0 continued to accumulate in the culture supernatant for the duration of the experiment without significant amounts of degradation products being observed (Fig. 3.8).

## 3.4. Affinity chromatography of SLF-CBD 1.0.

A series of experiments was undertaken in order to identify the best method of purifying the fusion protein. Since the fusion protein was designed to bind to cellulose *in situ* it was also possible that the C-terminal cellulose-binding domain could also be used to purify the protein from cell culture supernatants or from cell periplasms. From the previous section it was determined that SLF-CBD 1.0 will adsorb to cellulose. In order to recover a functional protein from cellulose, however, it would also be necessary to have a method of desorbing the protein from cellulose without denaturing it. The protein could be purified, therefore, by sending cell culture supernatants or cell periplasmic extracts through a cellulose column, rinsing other proteins out, and then eluting the desired fusion protein with a suitable reagent.

### 3.4.1. Desorption of SLF-CBD 1.0 from Avicel.

A series of tests was carried out to identify a suitable reagent for desorption of SLF-CBD 1.0 from Avicel without denaturation. 200 mg of Avicel was added to 200 mL of culture supernatant (containing SLF-CBD 1.0), stirred for 10 minutes at $4^0$ C, then recovered by centrifugation and washed in 20 mL of PBS. A small sample of the Avicel was then analyzed by SDS-PAGE to confirm that adsorption of the fusion protein had taken place. The remaining Avicel was divided into 5 mg aliquots, each of which was washed with a different reagent in an attempt to desorb the fusion protein from the cellulose

(Table 3.1). Two different wash volumes (10 mL and 2 mL) were used in each case to allow for the possibility of volume dependent desorption. Each Avicel sample was analyzed by SDS-PAGE (section 2.5) to determine whether or not the fusion protein was still present.

Results of the SDS-PAGE analysis (Table 3.1) indicated that only three of the reagents tested were capable of eluting the fusion protein from its matrix. Carboxymethyl-cellulose (CMC) was the only soluble cellulose tested which mediated desorption of SLF-CBD 1.0 from Avicel. Desorption was not volume dependent, indicating that competitive binding (as discussed in section 1.5) might have been responsible for the elution. Ethylene glycol also caused desorption of the fusion protein from Avicel, with larger volumes being more effective than smaller volumes. The polyethylene glycol solutions tested did not cause desorption of SLF-CBD 1.0 from its matrix. Guanidinium hydrochloride, a chaotrophic agent, was also able to elute SLF-CBD 1.0 from Avicel.

**Table 3.1.**

**Desorption of SLF-CBD 1.0 from Avicel.**

| Reagent. | Desorption (+/-) (2 mL) | (10 mL) |
|---|---|---|
| **ions:** | | |
| 5 M NaCl | - - | - - |
| 1 M NaCl | - - | - - |
| $ddH_2O$ (0 M NaCl) | - - | - - |
| **pH extremes:** | | |
| 1 M Tris-base pH 9.6 | - - | - - |
| 1 M Tris-Cl pH 4.5 | - - | - - |
| **soluble cellulose:** | | |
| hydroxyethyl-cellulose (10 % w/v) | - - | - - |
| cellobiose (10 % w/v) | - - | - - |
| **carboxymethyl-cellulose** | | |
| **(5 % w/v)** | + + | + + |
| (2 % w/v) | - - | - - |
| (1 % w/v) | - - | - - |
| (0.5 % w/v) | - - | - - |
| glucose (20 % w/v) | - - | - - |
| **others:** | | |
| glycerol | - - | - - |
| polyethylene glycol 4000 (12 % w/v) | - - | - - |
| polyethylene glycol 8000 (12 % w/v) | - - | - - |
| **ethylene glycol** | | |
| **(100 %)** | + - | + + |
| **(80 %)** | - - | + - |
| (60 %) | - - | - - |
| (40 %) | - - | - - |
| (20 %) | - - | - - |
| **6M guanidinium hydrochloride** | + + | + + |

**- - presence of SLF-CBD 1.0**
**+ - partial absence of SLF-CBD 1.0 (partial desorption)**
**+ + complete absence of the SLF-CBD 1.0 protein (complete desorption)**

### 3.4.2. Affinity chromatography of SLF-CBD 1.0 on Avicel: desorption with guanidinium hydrochloride.

Guanidinium hydrochloride mediated desorption of SLF-CBD 1.0 from Avicel was examined in greater detail using a guanidinium hydrochloride gradient in an FPLC system. SLF-CBD 1.0 was bound to Avicel by the addition of 5 g of Avicel to 500 mL of culture supernatant. The Avicel was recovered by centrifugation, washed with PBS, and packed into an FPLC column as described in section 2.7.2. A 0 to 6 M guanidinium hydrochloride gradient was then passed through the column, with elution of the fusion protein at 6 M guanidinium hydrochloride (Fig. 3.9). SLF-CBD 1.0 was not seen to elute from the column at lower concentrations of guanidinium hydrochloride.

### 3.4.3. Affinity chromatography of SLF-CBD 1.0 on Avicel: desorption with ethylene glycol.

The above experiment was repeated with an ethylene glycol gradient (as described in section 2.7.3). Although some elution of the protein was seen at ethylene glycol concentrations of less than 100 % (Fig. 3.10) the majority of the protein eluted from the column with 100 % ethylene glycol. This protein sample was re-natured (as described in section 2.7.3), filter sterilized, and stored at $-70^0$ C for biological activity testing (section 3.8).

### 3.4.4. Affinity chromatography of SLF-CBD 1.0 on nickel-sepharose: desorption with imidazole.

The N-terminal hexahistidine tag could also be used to purify the fusion protein by using nickel-sepharose mediated affinity chromatography. This method of purification was also examined in detail, and the activity and purity of the final product was compared to the protein preparations described above.

**i)** SLF-CBD 1.0 was purified using MCAC on nickel-Sepharose, as described in section 2.8.1. Periplasmic proteins were extracted from an

induced culture of JM101/pSLF/CBD 1.0 and purified by MCAC in an FPLC system using a 0 to 500 mM imidazole gradient. The SLF-CBD 1.0 fusion protein eluted from the column at an imidazole concentration between 100 mM and 200 mM (Fig. 3.11 A). Peak fractions were pooled, and the elution buffer exchanged for PBS using ultrafiltration. This sample was filter sterilized and retained for biological activity testing (section 3.8).

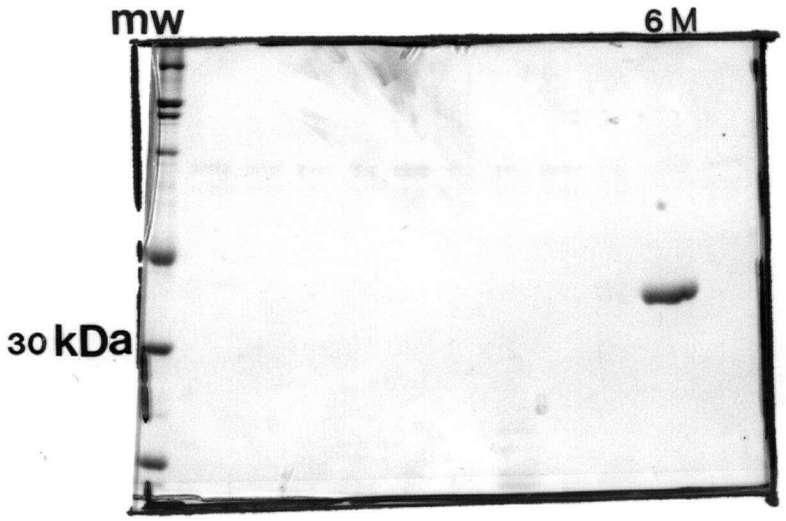



**Figure 3.9. Affinity chromatography of SLF-CBD 1.0 on Avicel: desorption with guanidinium hydrochloride.**

Elution of SLF-CBD 1.0 from Avicel by 6 M guanidinium hydrochloride.
fig 10 ethylene glycol. (Note: broad range molecular weight (mw) markers are 200 kDa, 116 kDa, 97.4 kDa, 66.0 kDa, 45.0 kDa, 31.0 kDa, 21.5 kDa and 14.5 kDa from top to bottom. The marker 30 kDa marks the approximate position of the 31.0 kDa marker. The fusion protein, with a predicted molecular weight of 34.1 kDa, was expected to run midway between the 31.0 kDa and the 45.0 kDa markers.)

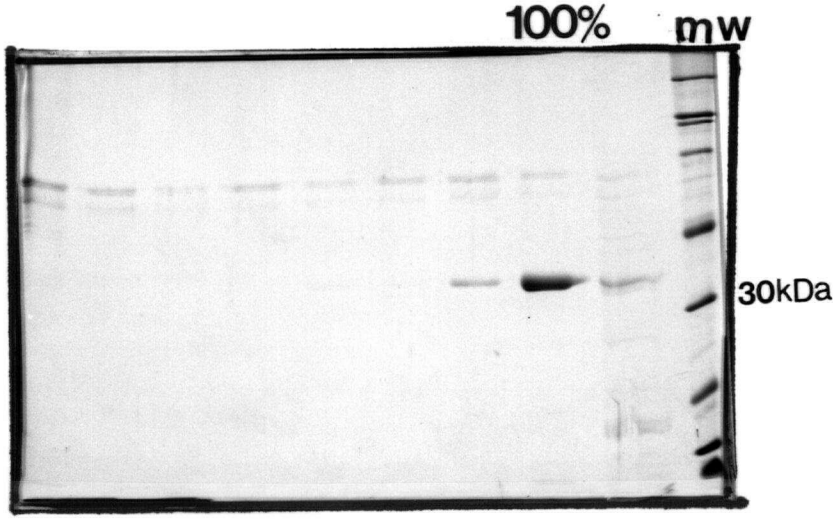



**Figure 3.10. Affinity chromatography of SLF-CBD 1.0 on Avicel: desorption with ethylene glycol.**

Elution of SLF-CBD 1.0 from Avicel by ethylene glycol. (Note: broad range molecular weight (mw) markers are 200 kDa, 116 kDa, 97.4 kDa, 66.0 kDa, 45.0 kDa, 31.0 kDa, 21.5 kDa and 14.5 kDa from top to bottom. The marker 30 kDa marks the approximate position of the 31.0 kDa marker. The fusion protein, with a predicted molecular weight of 34.1 kDa, was expected to run midway between the 31.0 kDa and the 45.0 kDa markers.)

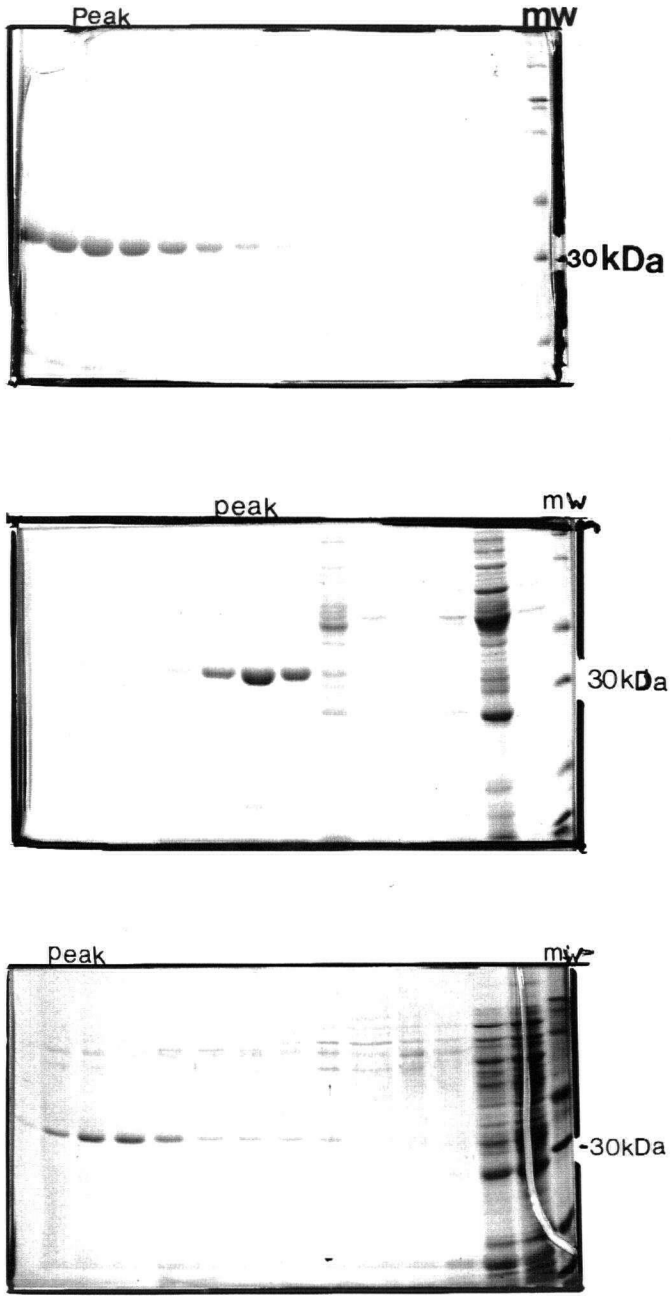



**Figure 3.11. Affinity chromatography of SLF-CBD 1.0 on nickel-Sepharose: desorption with imidazole.**

**A)** Purification of SLF-CBD 1.0 from a periplasmic extract. **B)** Purification of SLF-CBD 1.0 from concentrated culture supernatant. **C)** Purification of SLF-CBD 1.1 as cytoplasmic inclusion bodies. (Note: broad range molecular weight (mw) markers are 200 kDa, 116 kDa, 97.4 kDa, 66.0 kDa, 45.0 kDa, 31.0 kDa, 21.5 kDa and 14.5 kDa from top to bottom. The marker 30 kDa marks the approximate position of the 31.0 kDa marker. The fusion protein, with a predicted molecular weight of 34.1 kDa, was expected to run midway between the 31.0 kDa and the 45.0 kDa markers.)

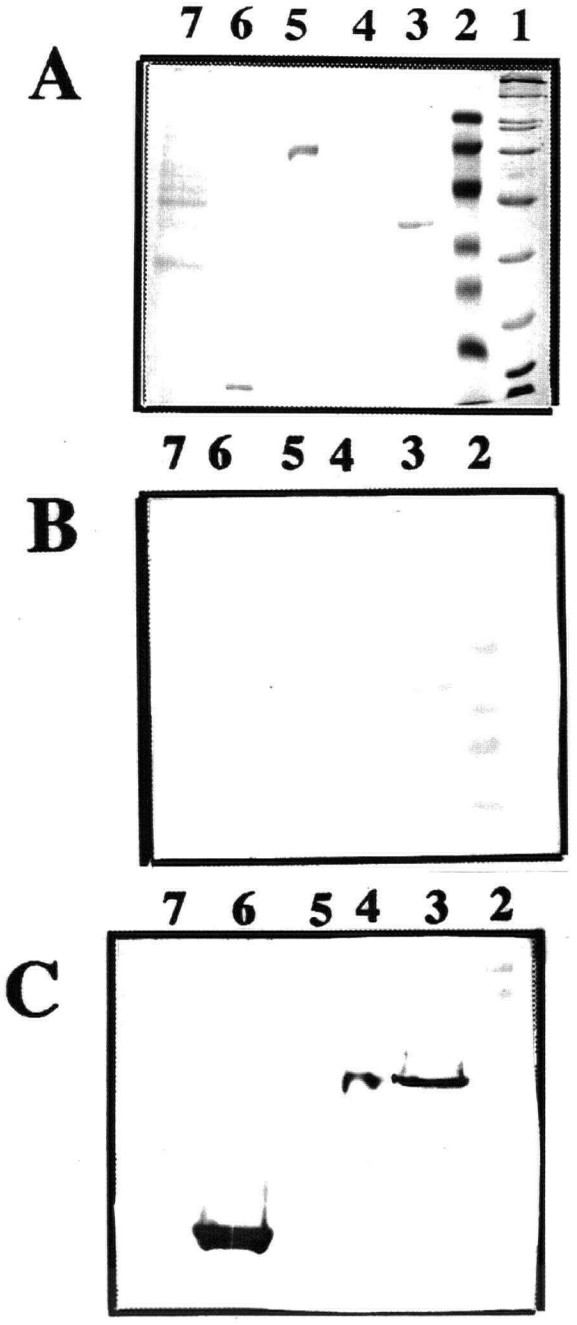



**Figure 3.12. Western blotting analysis of purified SLF-CBD 1.0.**

**A)** SDS-PAGE analysis of proteins (stained with Coomassie blue stain). ***lane 1,*** broad range markers; ***lane 2***, prestained markers; ***lane 3***, 1 ug purified SLF-CBD 1.0; ***lane 4,*** 20 ng SLF-CBD 1.0 cut by factor Xa (not visible on gel); ***lane 5,*** 10 ng recombinant SLF (not visible on gel, dark band is BSA); ***lane 6***, 500 ng $CBD_{Cex}$; ***lane 7***, vector only cell extract. **B)** western blot with anti-SLF polyclonal antibodies (lane designations as for **A**). **C)** western blot with anti-CBD polyclonal antibodies (lane designations as for **A**).

**ii)** SLF-CBD 1.0 was also purified from the culture supernatant (as described in section 2.8.2) and purified using MCAC (Fig. 3.11 B). This sample was also filter sterilized and retained for biological activity testing (section 3.8).

## 3.5. Purification and solid phase re-naturation of SLF-CBD 1.1 inclusion bodies using MCAC.

The plasmid pSLF/CBD 1.1 was designed for the production of an SLF-CBD fusion protein in the cytoplasm of *E.coli* without subsequent export to the periplasm. Inclusion bodies would, however, have to be subsequently renatured. Since this variation of the fusion protein also had an amino-terminal hexahistidine tag it was possible to send inclusion bodies which had been denatured by guanidinium hydrochloride into a nickel-sepharose affinity column, and then renature them through gradual removal of the guanidinium hydrochloride while they remained bound to this matrix.

SLF-CBD 1.1 was produced in the cytoplasm of the *E. coli* host cell strain BL21(λDE3)pLysS as insoluble inclusion bodies. Inclusion bodies were recovered by centrifugation, dissolved in MCAC loading buffer containing 6 M guanidinium hydrochloride, and loaded onto a metal chelate affinity column (as described in section 2.9). SLF-CBD 1.1 was re-natured, while immobilized in the column, by stepwise dilution of the guanidinium hydrochloride. Re-natured SLF-CBD 1.1 was then eluted from the column by an imidazole gradient as above (Fig. 3.11 C). Peak fractions where pooled and the imidazole elution buffer exchanged for PBS. The sample was filter sterilized and retained for subsequent biological activity testing (section 3.8).

## 3.6. Western blotting analysis of purified SLF-CBD 1.0.

Western blotting analysis of SLF-CBD 1.0 (as described in section 2.10.1) indicated that the fusion protein reacted with rabbit antiserum raised to murine SLF (Fig. 3.12 B) as well as with rabbit antiserum raised to recombinant $CBD_{Cex}$ (Fig. 3.12 C) . Factor Xa cleavage of the fusion protein resulted in the separation of the two domains (Fig. 3.12 A lane 4, B lane 4, and C lane 4),

indicating that the factor Xa proteolytic cleavage site was accessible to factor Xa.

### 3.7. N-terminal amino acid sequence analysis of SLF-CBD 1.0.

A small sample of SLF-CBD 1.0 was obtained directly from culture supernatant (as described in sections 2.5 and 3.2) and subjected to an N-terminal amino acid sequence analysis. The first 11 amino acids of the N-terminus of SLF-CBD 1.0 were:

**A S H H H H H H I E G,**

confirming the removal of the CexLP after export to the periplasm.

### 3.8. SLF-CBD stimulated cell proliferation assay in the absence of cellulose.

The ability of SLF-CBD 1.0 and SLF-CBD 1.1 to stimulate proliferation of the steel factor dependent bone marrow cell line B6SUtA was quantified by the MTT cell proliferation assay, as well as by direct cell counts with a hemocytometer (section 2.11.) Recombinant $CBD_{Cex}$, produced in *E. coli* , was used as a negative control while recombinant murine SLF, also produced in *E. coli*, was used as a positive control. Murine SLF produced in *Saccharomyces cerevisiae* was also tested to examine the effects of protein glycosylation on biological activity. As described in section 2.11, protein samples were serially diluted in hybridoma serum-free medium (H-SFM) and added to B6SUtA cells in a standard 96 well tissue culture plate. Protein samples and cells were then incubated for a further 48 hours and the proliferative response measured.

The biological activity of (periplasmic) SLF-CBD 1.0 purified by MCAC was similar to that of the positive controls (Fig. 3.13A), while the biological activity of SLF-CBD 1.0 purified on Avicel with ethylene glycol was significantly lower than that of the positive controls (Fig. 3.13 B). The biological activity of SLF-CBD 1.1 purified from inclusion bodies was also found to be significantly lower than that of the controls (Fig. 3.13 C).

Since the test proteins and the control proteins were of different molecular weights the results were reported as pM protein concentrations. The biological activity of SLF-CBD 1.0 recovered from cell periplasms was similar to that of SLF-CBD 1.0 recovered from cell culture supernatants (data not shown), and subsequent tests were carried out using only the periplasmic isolate. Recombinant $CBD_{Cex}$ was neither stimulatory nor toxic to B6SUtA cells (Fig. 3.13 D).

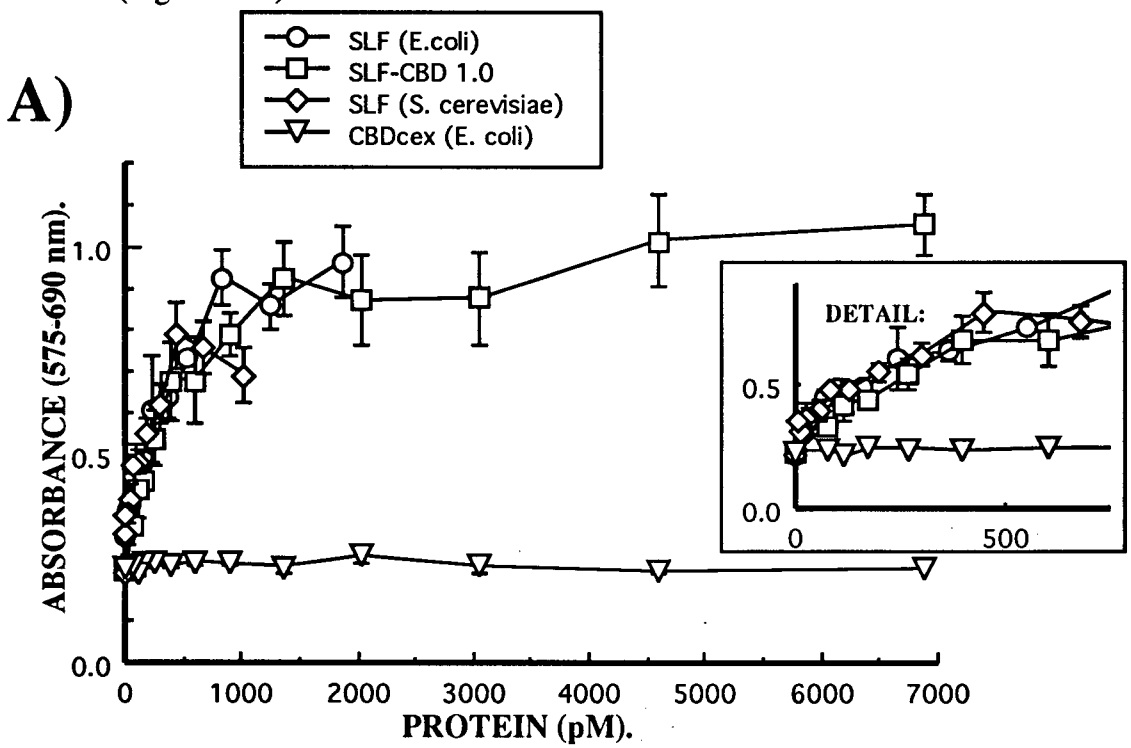



**Figure 3.13 A. Biological activity of SLF-CBD 1.0 and other forms of SLF in the absence of cellulose.**

Cell proliferative response to SLF-CBD 1.0 and controls was measured by MTT.

### 3.9. Neutralization of SLF-CBD 1.0 biological activity by polyclonal antibodies:

The biological activity of SLF-CBD 1.0 was neutralized (as described in section 2.12) by goat α-SLF polyclonal neutralizing antibodies (Fig. 3.14). This indicated that the biological activity of the SLF-CBD 1.0 fusion protein

was due to the specific amino acid sequence of the protein, and not to some coincidental response by the target cells.

## 3.10. Determination of optimal BMCC concentrations for adsorption of SLF-CBD 1.0.

The effect of the surface density of the adsorbed growth factor on cell proliferation was examined by adsorbing a fixed concentration of SLF-CBD 1.0 to a variable amount of BMCC. Adsorption of a fixed amount of SLF-CBD 1.0 to a larger amount of BMCC would result in a lower surface density of immobilized growth factor. Adsorption of the same amount of SLF-CBD 1.0 to a higher concentration of BMCC would result in a higher surface density of immobilized growth factor. The possibility that certain surface densities of adsorbed growth factor may generate greater proliferative responses than others was examined in the studies outlined below.

### 3.10.1. Variation of BMCC concentration with a fixed concentration of SLF-CBD 1.0.

The effect of SLF-CBD 1.0 immobilization on biological activity was examined by adsorbing a fixed amount of fusion protein to a variable amount of BMCC (as described in section 2.14.1). The experiment was repeated for three different concentrations of SLF-CBD 1.0 (Fig. 3.15 A, B and C). In each case an optimal activity peak was observed, the location of which was a function of both SLF-CBD 1.0 and BMCC concentration. In all cases the optimal activity of the protein when immobilized was greater than the activity of the protein when free in solution. A variable concentration of BMCC was not seen to effect the biological activity of the SLF control protein (Fig. 3.15 D).

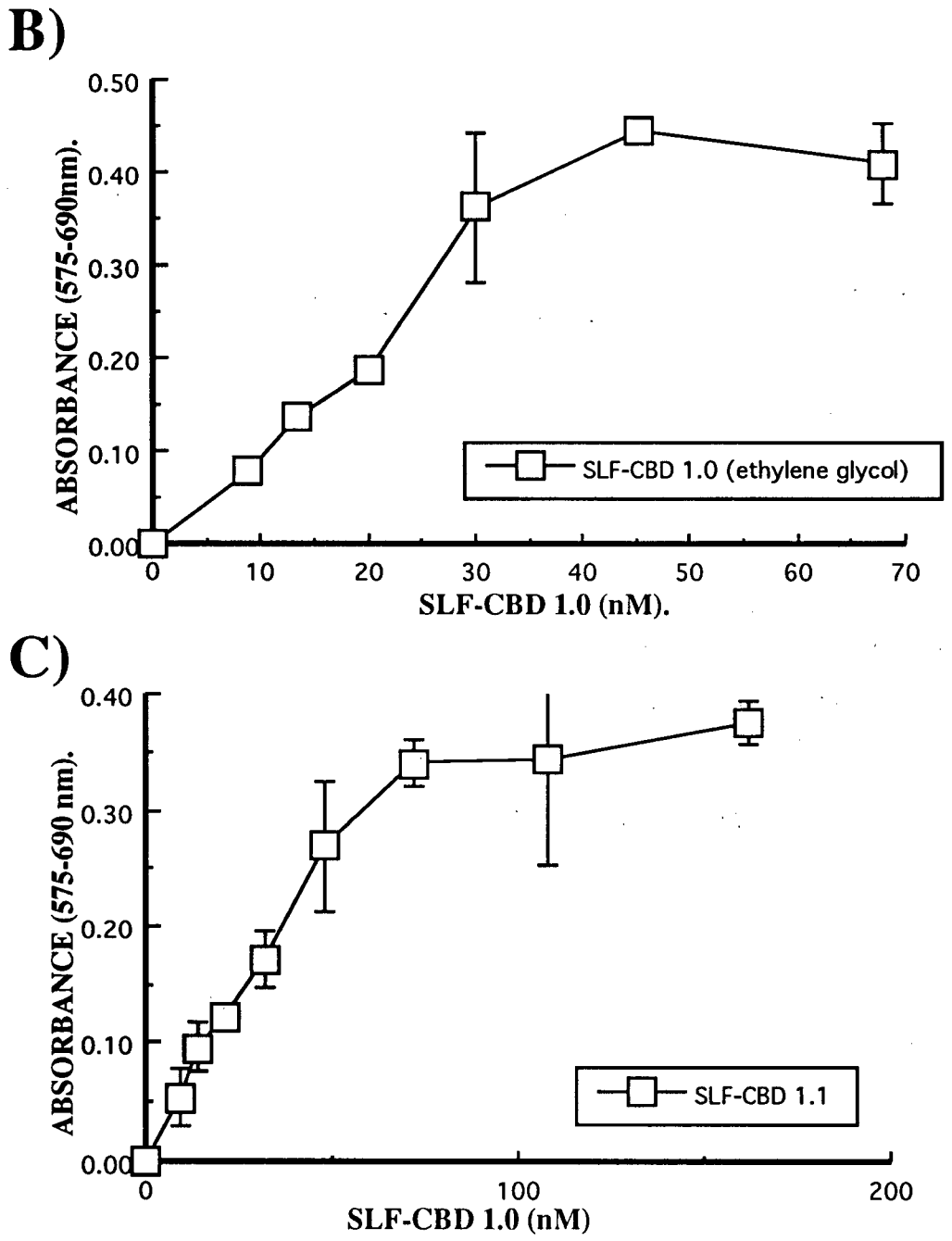



**Figure 3.13 B and C. Biological activity of SLF-CBD 1.0 desorbed from Avicel by ethylene glycol, and of SLF-CBD 1.1 renatured from inclusion bodies.**

**A)** SLF-CBD 1.0 recovered from Avicel by desorption with ethylene glycol. **B)** SLF-CBD 1.1 renatured from inclusion bodies. (baseline activity subtracted).

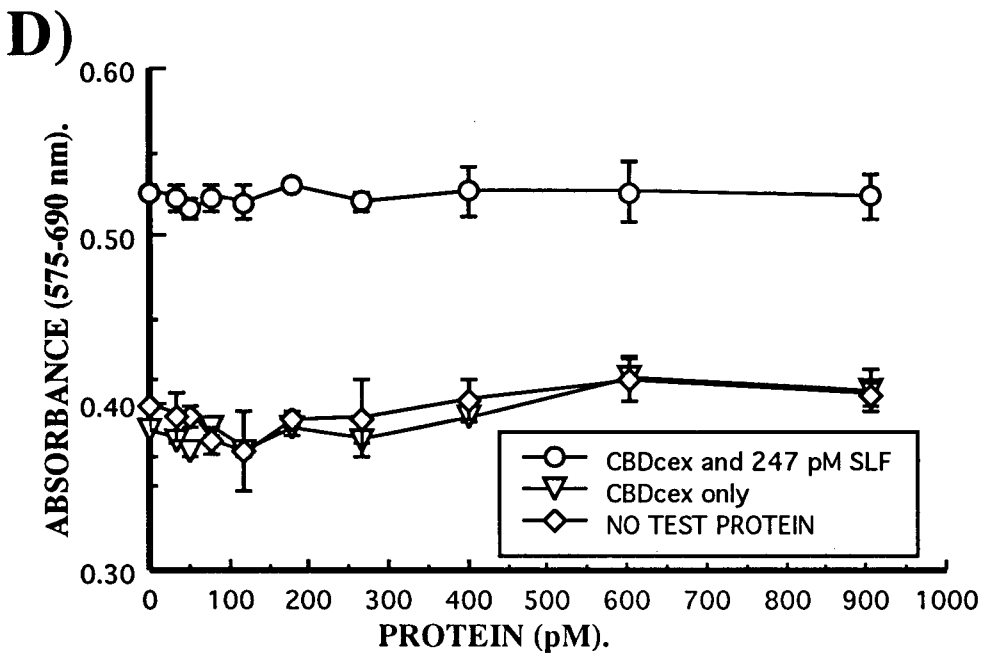



**Figure 3.13 D. Test of $CBD_{Cex}$ for biological activity and toxicity.**

CBDcex was found to be neither stimulatory nor toxic. CBDcex alone did not stimulate proliferation of B6SUtA cells. The addition of CBDcex to SLF did not reduce or enhance its biological activity. (Baseline activity not subtracted.)

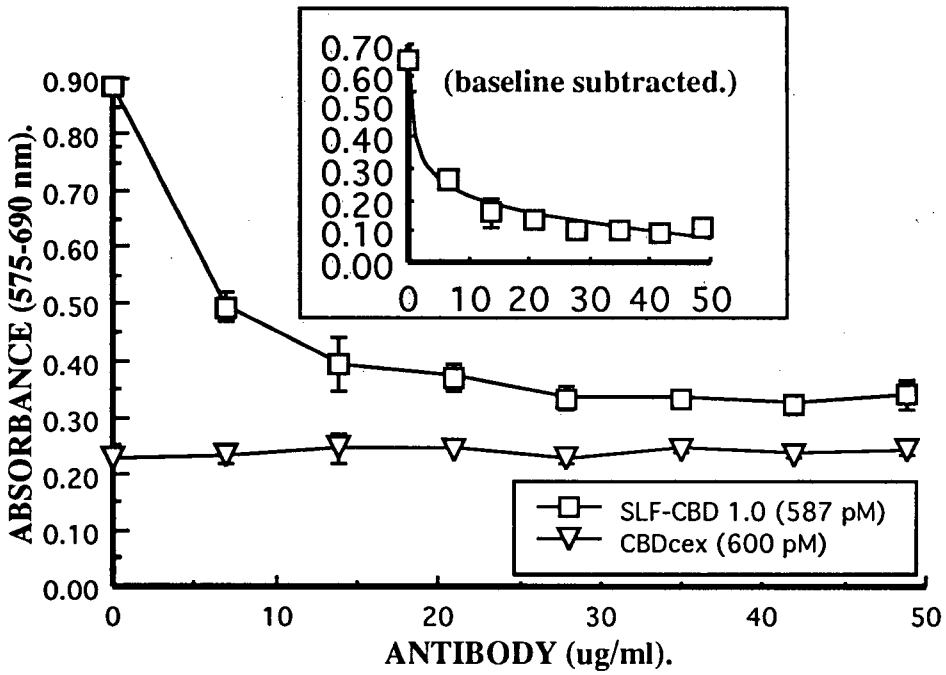



**Figure 3.14. Neutralization of SLF-CBD 1.0 biological activity by polyclonal antibodies.**

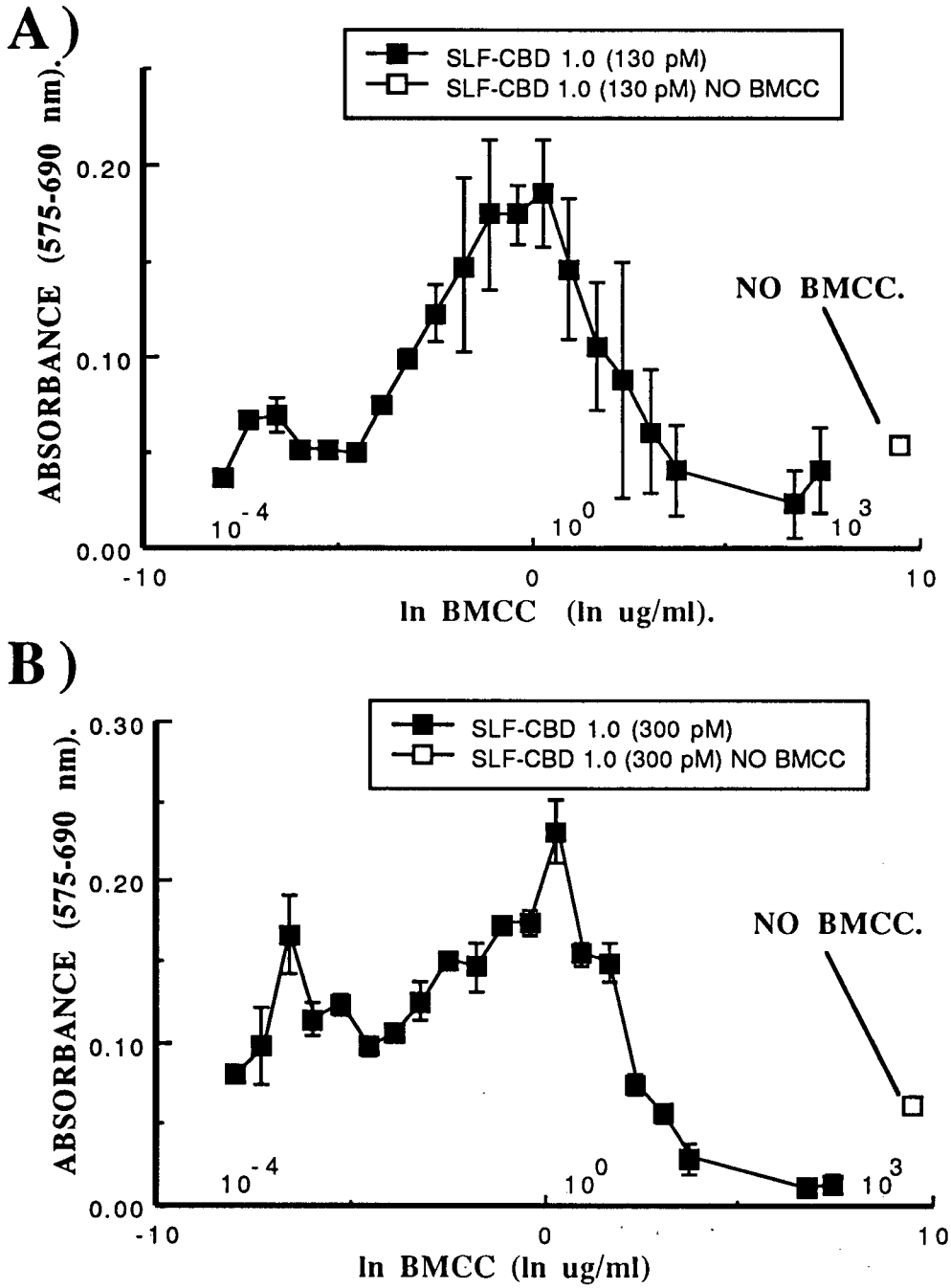



**Figure 3.15 A and B. Variation of BMCC concentration in the presence of a fixed amount of SLF-CBD 1.0.**

**A)** Variation of BMCC concentration in the presence of 130 pM SLF-CBD 1.0. **B)** Variation of BMCC concentration in the presence of 300 pM SLF-CBD 1.0. (baseline activity subtracted.) (note: actual BMCC concentrations (in $\mu g\ mL^{-1}$) are superimposed above the X-axis. Numbers below X-axis are the natural logarithm of the BMCC concentration in $\mu g\ mL^{-1}$).

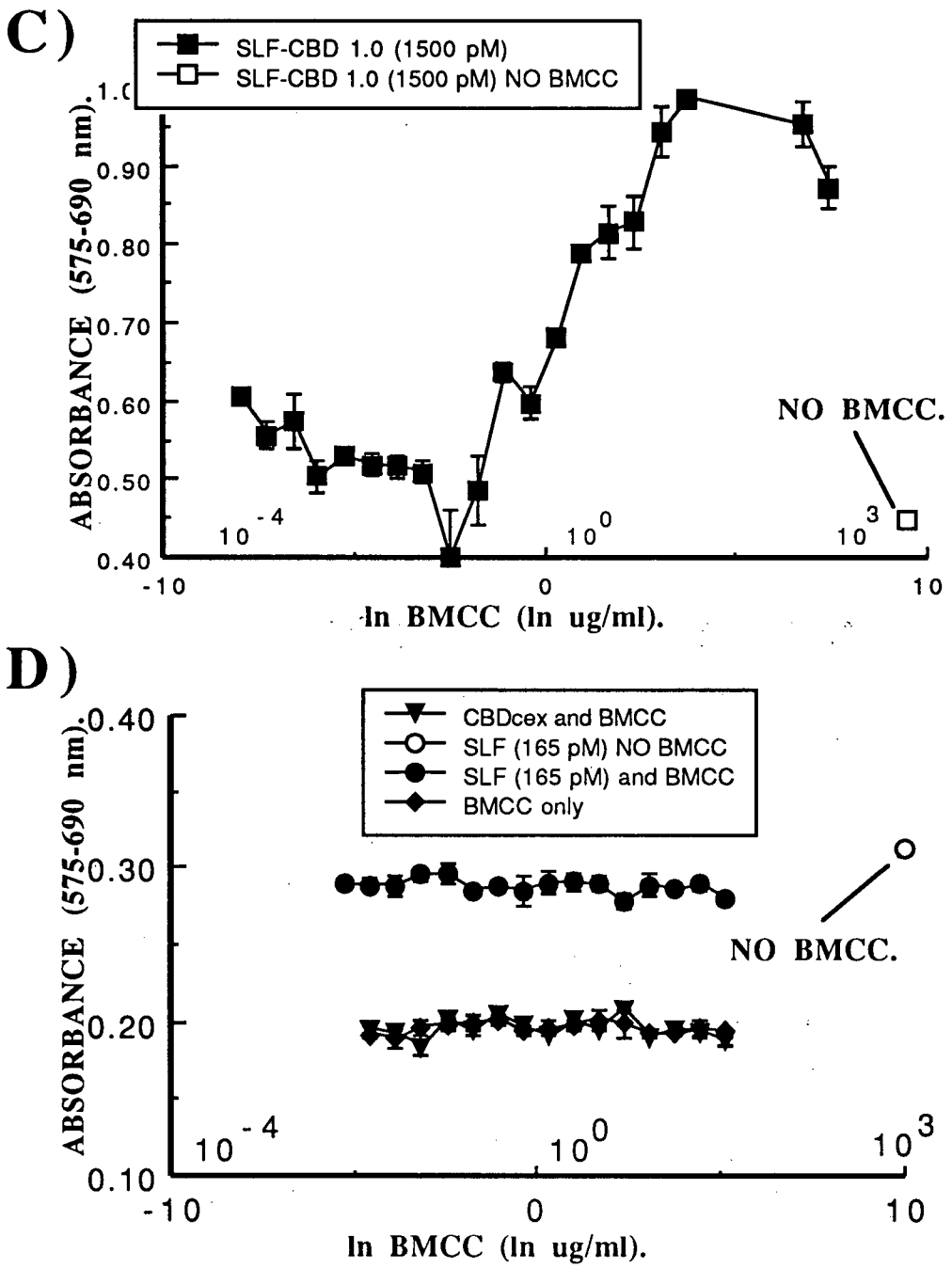



**Figure 3.15 C and D. Effect of varying the BMCC concentration in the presence of a fixed amount of SLF-CBD 1.0.**

C) Variation of BMCC concentration in the presence of 1500 pM SLF-CBD 1.0. (baseline activity subtracted.) D) Test of BMCC and CBDcex alone. (Baseline *not* subtracted.) (note: actual BMCC concentrations (in $\mu g\ mL^{-1}$) are superimposed above the X-axis.)

### 3.10.2. Separation of SLF-CBD 1.0 activity into free and adsorbed components: (variable BMCC concentration).

The completeness of SLF-CBD 1.0 binding to BMCC was examined by separating bound material from free material. Absence of free material in solution would indicate complete adsorption of the fusion protein. Using this method it would also be possible to determine the point at which the BMCC surface becomes saturated by SLF-CBD 1.0. This would allow for the determination of the maximum loading capacity of BMCC for this fusion protein.

SLF-CBD 1.0 was added to a suspension of BMCC as before. BMCC and supernatant were then separated by centrifugation and tested for biological activity separately. The majority of the SLF-CBD 1.0 biological activity was found to be associated with the BMCC (Fig. 3.16 A), while the majority of SLF biological activity was found to be associated with the supernatant (Fig. 3.16 B). Only at very low concentrations of BMCC did free SLF-CBD 1.0 activity exceed immobilized SLF-CBD 1.0 activity.

## 3.11. Variation of SLF-CBD 1.0 concentration with a fixed concentration of BMCC.

### 3.11.1. Influence of immobilization on SLF-CBD 1.0 biological activity.

The biological activities of SLF-CBD 1.0 and SLF were measured in the presence of a 1 $\mu g\ mL^{-1}$ concentration of BMCC. The proliferative response to each was measured by both cell count (Fig. 3.17 A) and MTT (Fig. 3.17 B). In both cases, the biological activity of SLF was not significantly effected by the presence of BMCC. For SLF-CBD 1.0, however, addition of BMCC was accompanied by an increase in biological activity (Table 4.1 of the discussion section).

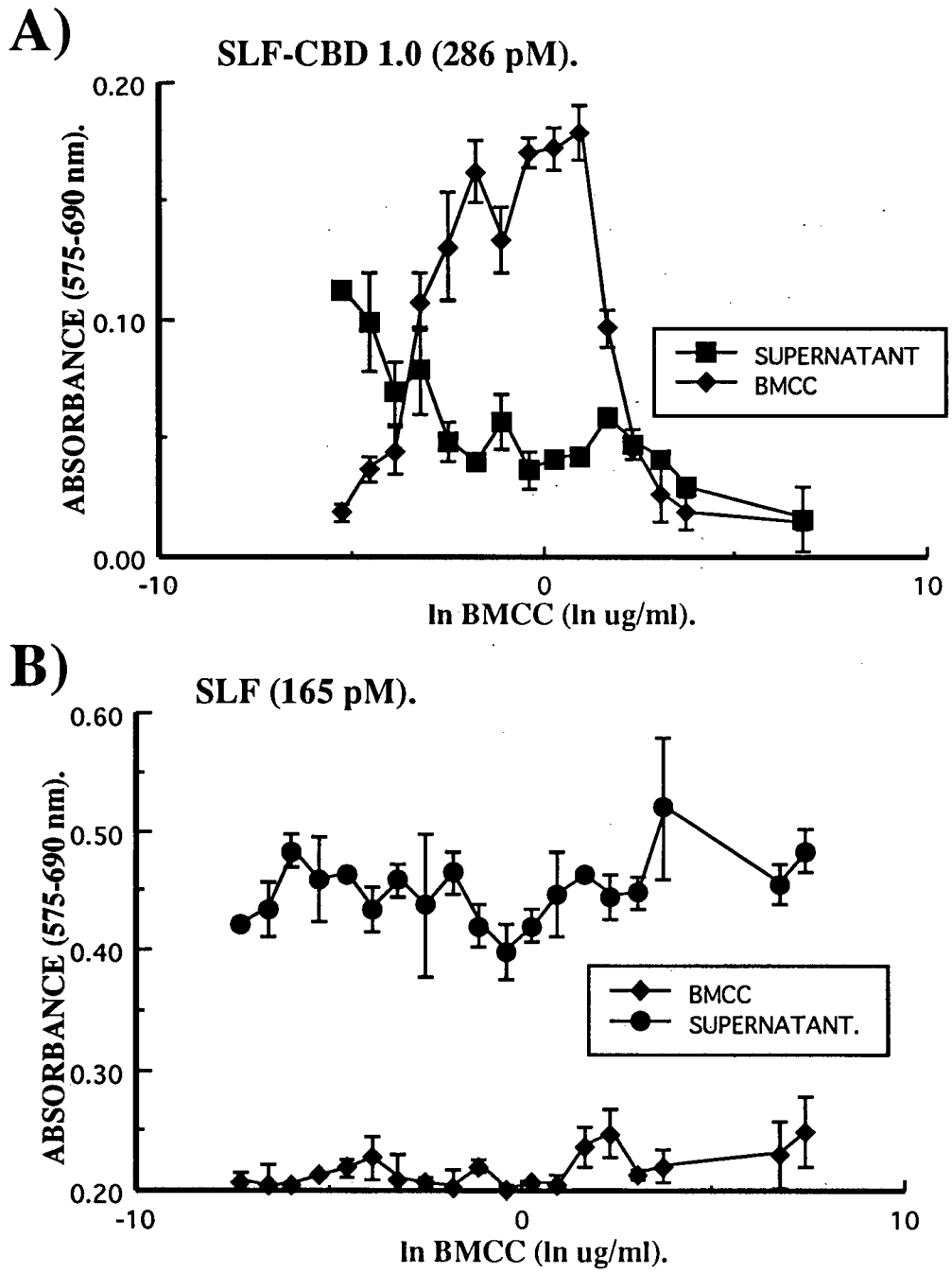



**Figure 3.16. Separation of SLF-CBD 1.0 activity into adsorbed and non-adsorbed components (variable BMCC concentration).**

**A)** SLF-CBD 1.0 was adsorbed to BMCC. BMCC was then removed from suspension by centrifugation and the biological activity associated with the BMCC was tested separately from the biological activity of the supernatant. **B)** As a control the experiment was repeated using SLF which lacked a cellulose binding domain.(baseline subtracted.)

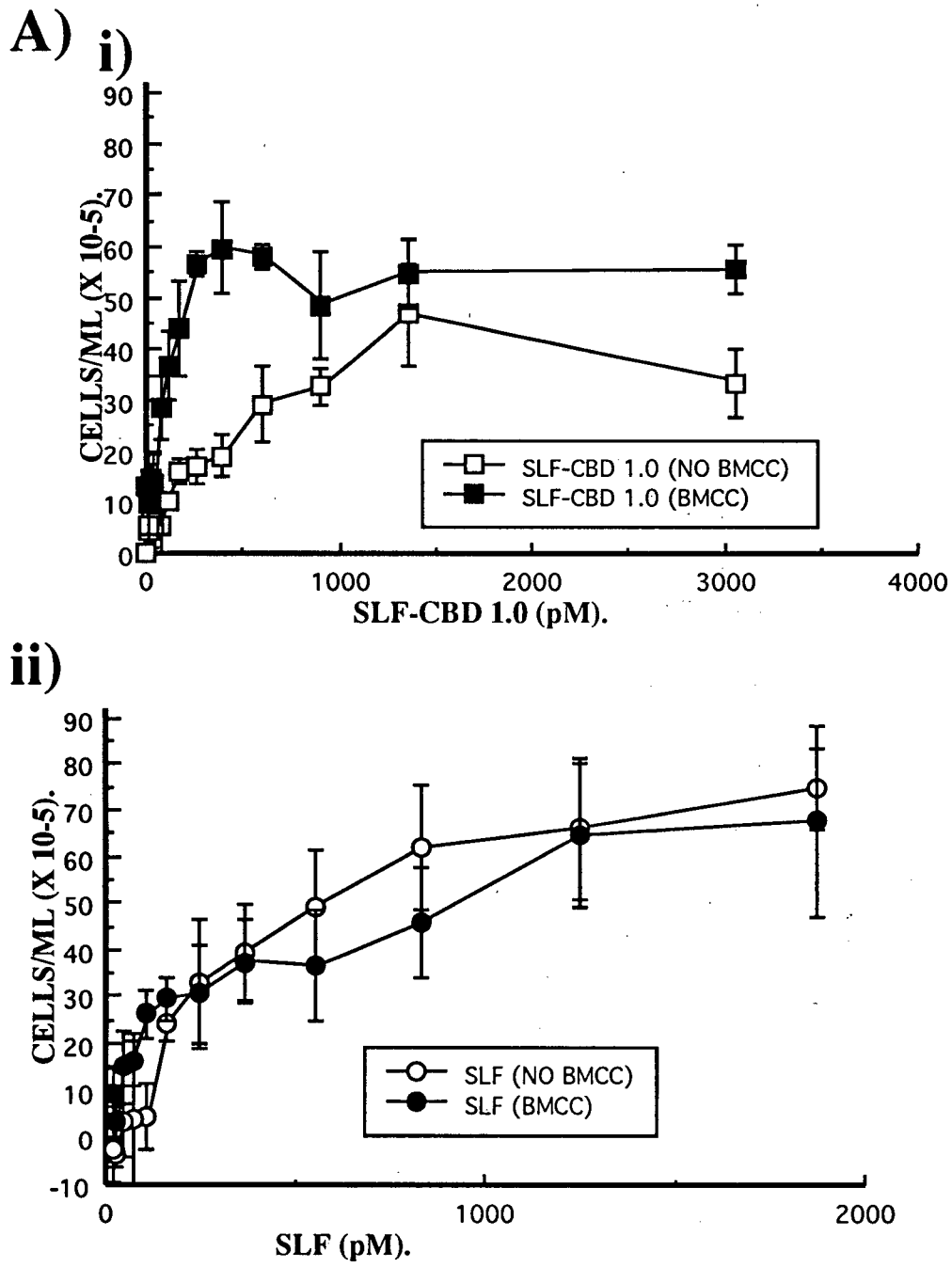



**Figure 3.17 A. Effect of varying the concentration of SLF-CBD 1.0 in the presence of a fixed amount of BMCC (direct cell count).**

**i)** Effects of 1 $\mu g\ mL^{-1}$ BMCC on the biological activity generated by SLF-CBD 1.0.
**ii)** Effects of 1 $\mu g\ mL^{-1}$ BMCC on the biological activity generated by SLF. (Cell starting concentrantion was $2 \times 10^5$ cells $mL^{-1}$)

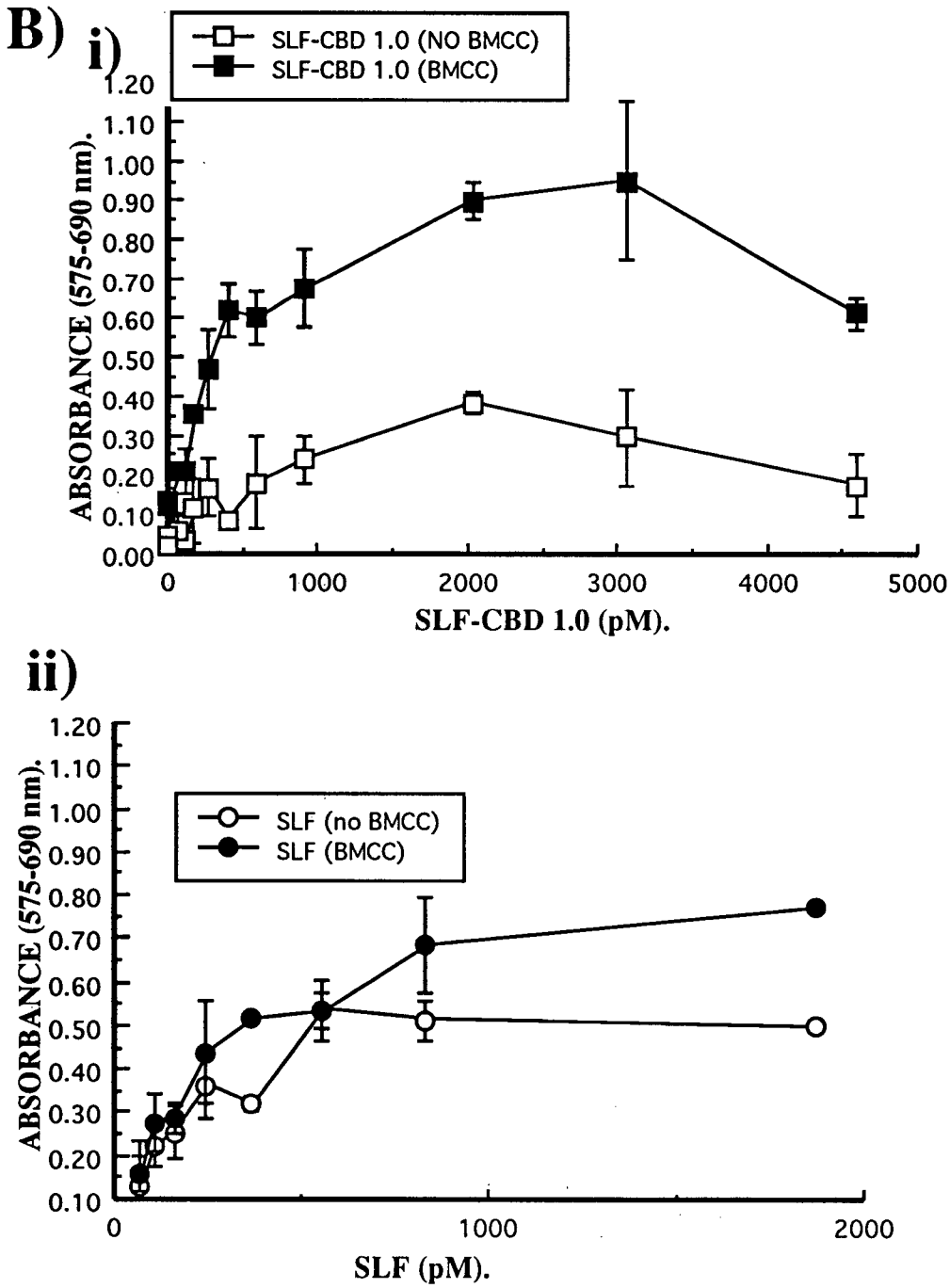



**Figure 3.17 B. Effect of varying the concentration of SLF-CBD 1.0 in the presence of a fixed amount of BMCC (MTT assay).**

**i)** Effects of 1 $\mu g\ mL^{-1}$ BMCC on the biological activity generated by SLF-CBD 1.0.

**ii)** Effects of 1 $\mu g\ mL^{-1}$ BMCC on the biological activity generated by SLF.

### 3.11.2. Adsorption to three different concentrations of BMCC.

The experiment converse to that of section 3.10.1 was carried out in which a variable concentration of SLF-CBD 1.0 was adsorbed to a fixed concentration of BMCC. SLF-CBD 1.0 activity was measured in the presence of three different concentrations of BMCC (Fig. 3.18 ). The results were consistent with those of section 3.10.1, suggesting that higher concentrations of SLF-CBD 1.0 were more effective when adsorbed to higher concentrations of BMCC, while lower concentrations of SLF-CBD 1.0 were more effective when adsorbed to lower concentrations of BMCC.

### 3.11.3. Separation of SLF-CBD 1.0 activity into free and adsorbed components: (variable SLF-CBD 1.0 concentration).

An experiment converse to that of section 3.10.2 was carried out in which adsorbed biological activity and free biological activity were tested separately (as described in section 2.16). As before, the majority of SLF-CBD 1.0 activity was found to be associated with the BMCC matrix (Fig. 3.19 A), while the majority of the SLF activity was found in the supernatant (Fig. 3.19 B).

### 3.11.4. *In situ* cleavage of SLF-CBD 1.0 by factor Xa:

In order to confirm that the cellulose-binding domain was responsible for binding, and that binding and adsorption to cellulose was responsible for an enhancement of the fusion proteins activity the cellulose-binding domain was cleaved by factor Xa *in situ*. The above experiment was repeated with the addition of factor Xa to one set of test samples (as described in section 2.17). Proteolytic cleavage of the cellulose-binding domain was seen to release the fusion protein from its BMCC matrix (Fig. 3.20 A), with the majority of SLF-CBD 1.0 biological activity now being found in the supernatant. The *in situ* removal of the binding domain was accompanied by a concomitant drop in biological activity (Fig. 3.20 B), despite the presence of BMCC. No similar

drop in activity was observed when factor Xa was added to SLF-CBD 1.0 in the absence of BMCC (Fig. 3.20 C).

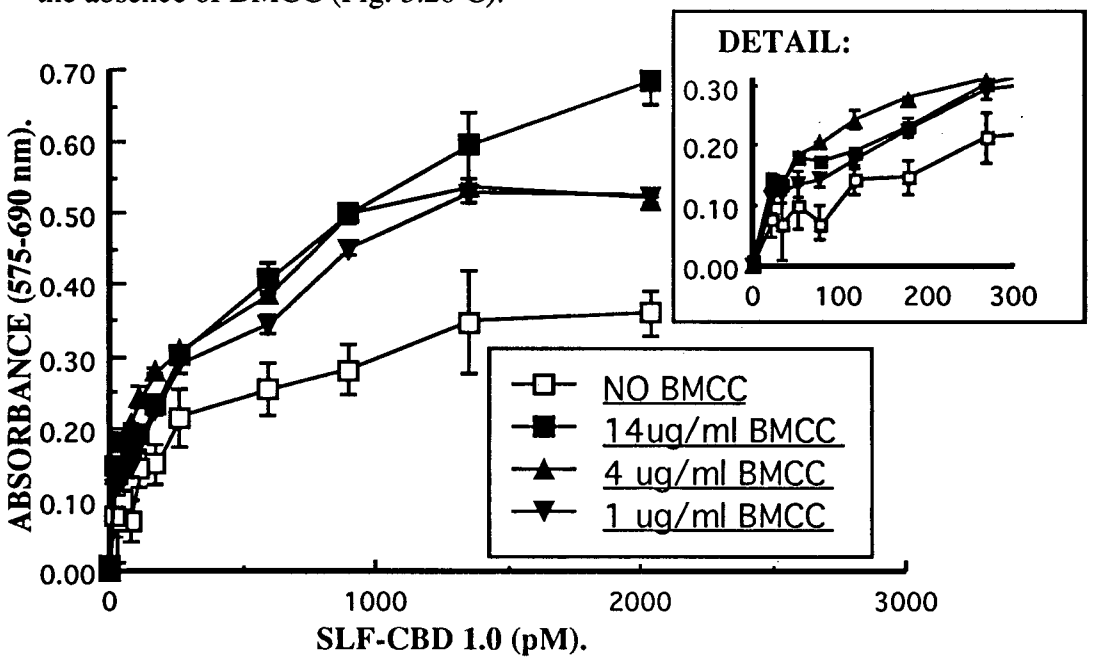
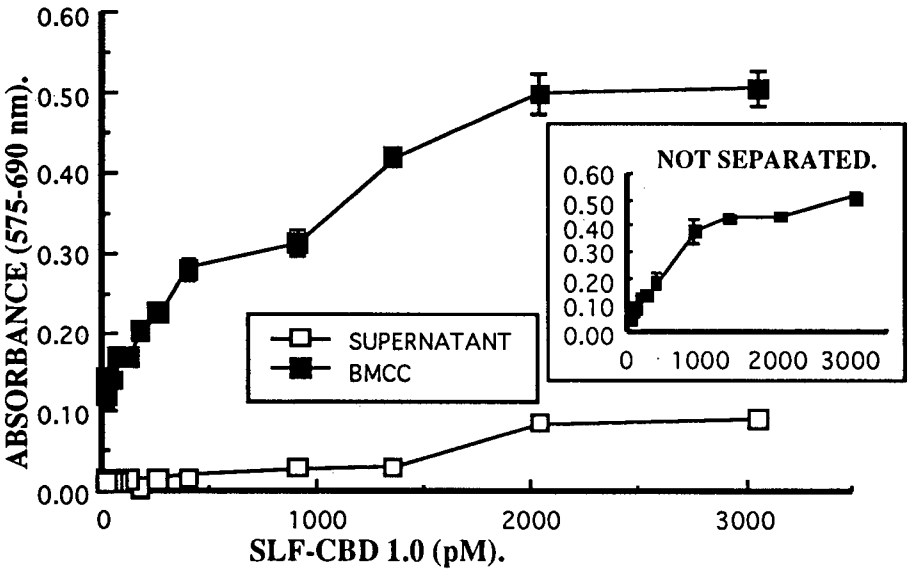



**Figure 3.18. Effects of three different fixed concentrations of BMCC on the biological activity of SLF-CBD 1.0.**

A)



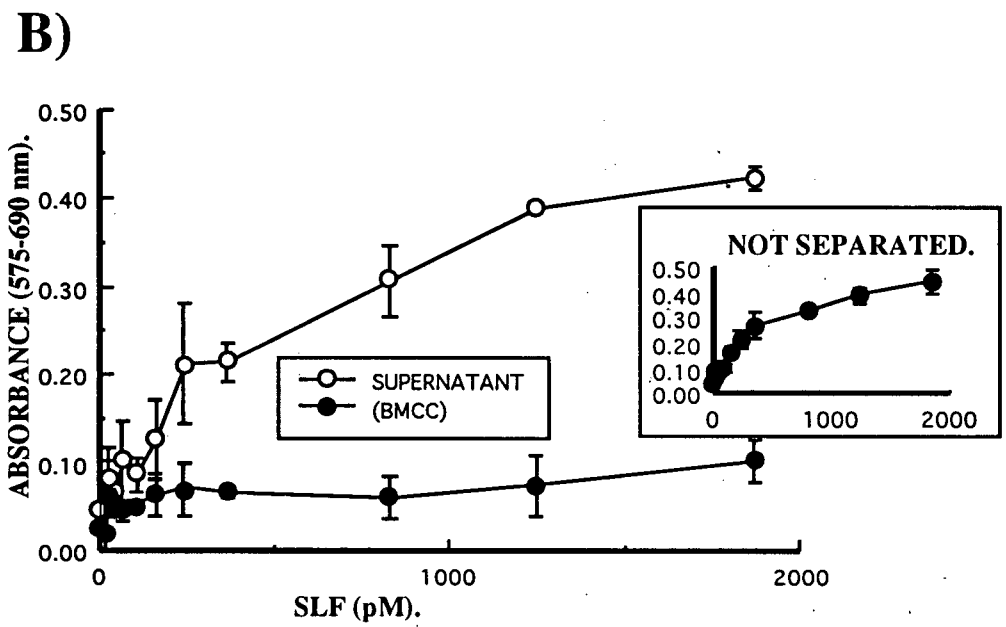
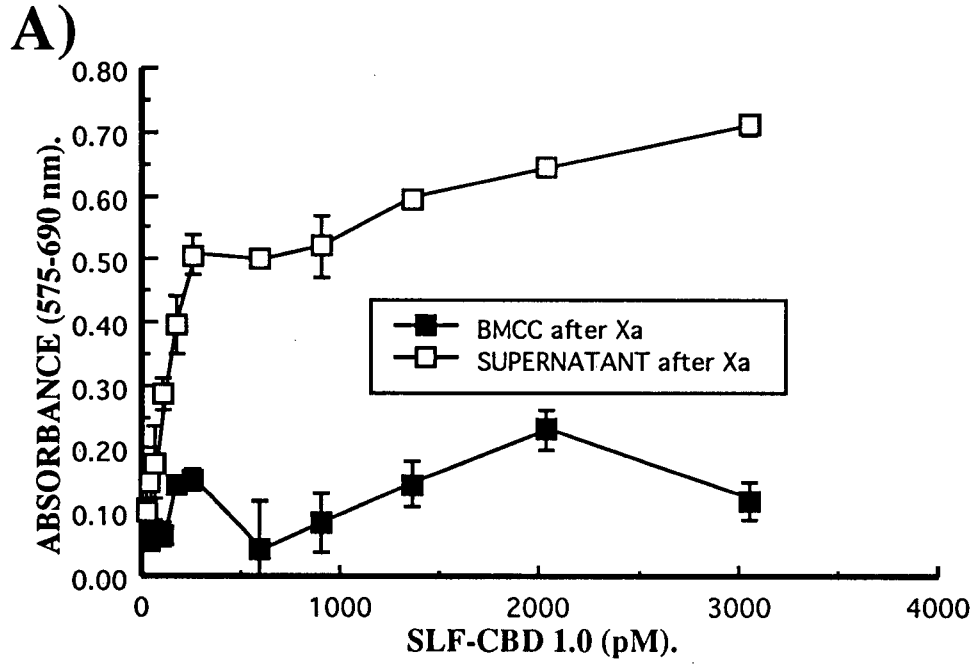



**Figure 3.19. Separation of SLF-CBD 1.0 biological activity into adsorbed and non-adsorbed components (variable SLF-CBD 1.0 concentration).**

**A)** Variable concentrations of SLF-CBD 1.0 were adsorbed to a fixed amount of BMCC ( 1 $\mu g\ mL^{-1}$). BMCC was then removed from suspension by centrifugation and the biological activity associated with the BMCC was assayed separately from the biological activity associated with the supernatant. **B)** Control experiment using SLF lacking the cellulose binding domain. (Baseline subtracted.)



B)

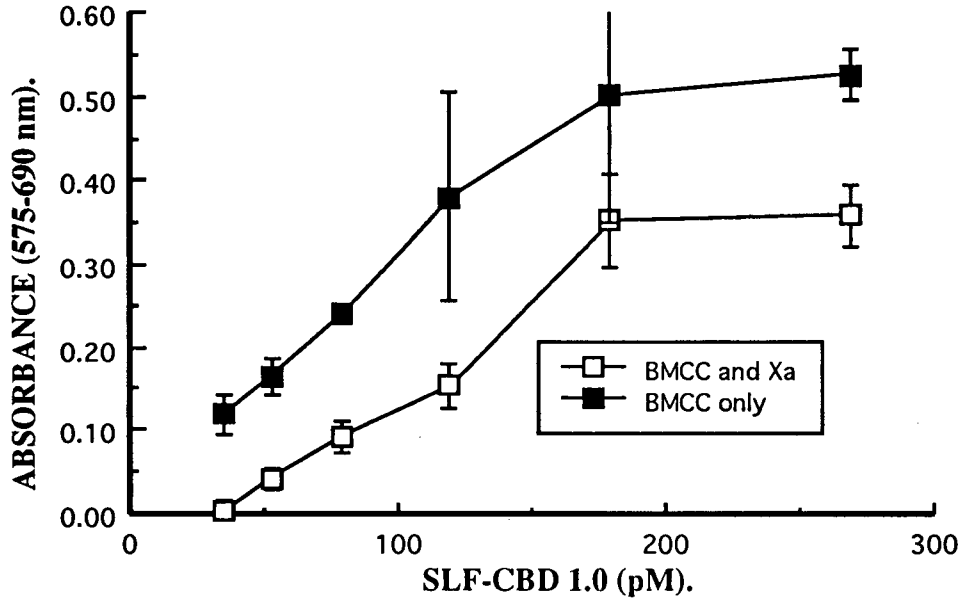



**Figure 3.20 A and B. Cleavage of the SLF-CBD 1.0 fusion protein by factor Xa.**

**A)** SLF-CBD 1.0 was adsorbed to BMCC (1 $\mu g\ mL^{-1}$) in the presence of protease factor Xa (a cleavage site for which was placed between the SLF domain and the cellulose binding domain of SLF-CBD 1.0). BMCC and supernatant were then separated by centrifugation and tested for biological activity separately. **B)** A drop in biological activity was observed concomitant with cleavage of the binding domain. (Baseline subtracted.)

C)

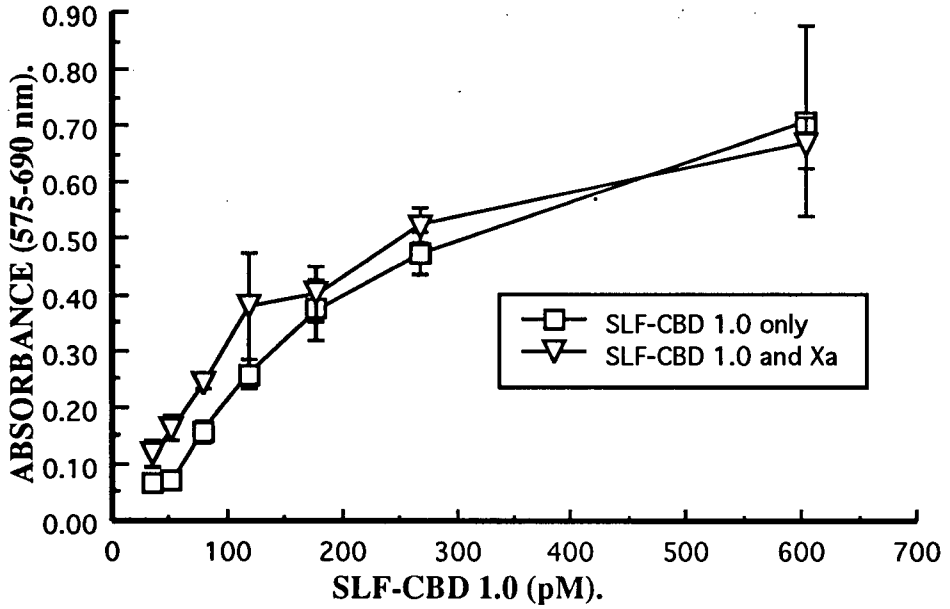



**Figure 3.20 C. Effects of adding factor Xa to SLF-CBD 1.0 in the absence of BMCC.**

### 3.12. SLF-CBD 1.0 adsorption to a cellulose surface regenerated from cellulose-acetate:

BMCC, being difficult and time consuming to make, would not be the ideal matrix for the immobilization of SLF-CBD 1.0. Several types of cellulose would be more convenient, including cellulose acetate which is commonly used in the photography industry. The effective binding of SLF-CBD 1.0 to a cellulose surface regenerated from cellulose acetate was examined in the following manner. Tissue culture plates were coated with cellulose, as described in sections 2.18 and 2.19, and the adsorption of SLF-CBD 1.0 to these surfaces was analyzed by a method analogous to that described in sections 2.16 and 3.11.3. In this case SLF-CBD 1.0 and SLF were allowed to adsorb to the cellulose surface for 24 hours, and the supernatant was then transferred to a tissue culture plate which had not been coated with cellulose. The biological activity associated with the cellulose surface was then assayed separately from the biological activity associated with the supernatant.

The results indicated that the majority of the biological activity generated by SLF-CBD 1.0 was associated with the cellulose surface rather than with the supernatant (Fig. 3.21 A), while the majority of the biological activity generated by SLF was associated with the supernatant rather than with the cellulose surface (Fig. 3.21 B). The activity curve generated by the cellulose-adsorbed SLF-CBD 1.0 was not seen to increase, however, when SLF-CBD 1.0 concentrations of approximately 80 pM or greater were used, suggesting that the binding capacity of the cellulose surface for SLF-CBD 1.0 had been exceeded at these concentrations. This effect was not observed with BMCC (Fig. 3.19 A).

Direct cell count comparisons revealed that cells cultured in regular plates reached higher densities than cells cultured in cellulose-coated plates when both cultures were stimulated by equivalent amounts of soluble SLF (data not shown), indicating that this type of cellulose surface might have had a detrimental effect on the proliferation of B6SUtA cells *in situ*. Hence a direct comparison of soluble SLF-CBD 1.0 and cellulose-adsorbed SLF-CBD 1.0 was not possible in this case. In order to minimize the effect of this situation on the outcome of the experiments, cells cultured in cellulose coated plates were transferred to regular plates prior to incubation with MTT.

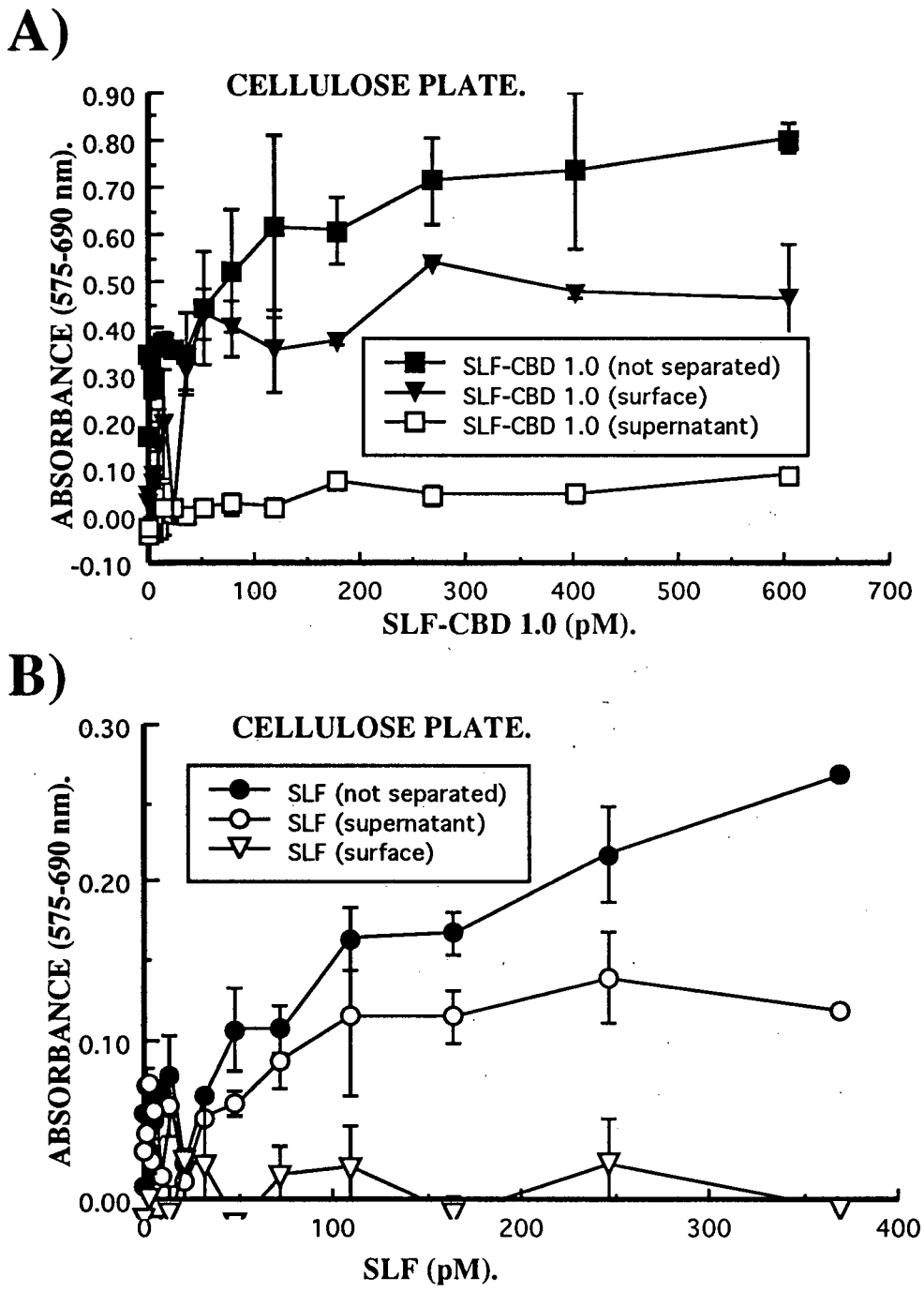



**Figure 3.21. Adsorption of SLF-CBD 1.0 to cellulose coated tissue culture plates.**

**A)** SLF-CBD 1.0 was adsorbed to a cellulose surface generated from the saponification of cellulose acetate. After adsorption, sample supernatants were transferred to a separate plate and the SLF-CBD 1.0 biological activity associated with the cellulose surface was assayed separately from that associated with the supernatant. **B)** The experiment was repeated using SLF which lacked a cellulose binding domain.

### 3.13. Re-use of SLF-CBD 1.0 adsorbed to a regenerated cellulose surface:

An experiment was carried out to determine if a cellulose surface bearing immobilized SLF-CBD 1.0 could be used to support several cycles of cell cultivation, without need for replacement of the immobilized growth factor. A regenerated cellulose surface, bearing SLF-CBD 1.0, was used to support four consecutive rounds of cell culturing. 50 pM of SLF-CBD 1.0 was first adsorbed to the wells of a tissue culture plate and the supernatant was then removed, leaving only bound growth factor in the wells. B6SUtA cells were then added to the wells and allowed to proliverate for 48 hours as before. The cells were then transferred to a new plate, and their proliferative response to the immobilized growth factor was measured by MTT. Fresh B6SUtA cells were then added to the wells of the original plate, and the cell proliferation test was repeated. The surface was used to support four cycles of cell cultivation with the proliferative response being measured each time. The results of the previous section indicated that this type of cellulose surface could be saturated by SLF-CBD 1.0 concentrations greater than 100 pM. The experiment was conducted therefore at an immobilized growth factor concentration of 50 pM.

As a control a 50 pM solution of SLF was added to the wells of a cellulose coated tissue culture plate, and this surface was aslo used to support four consecutive rounds of cell culturing. Since SLF, lacking a cellulose-binding affinity tag, was not expected to bind to the cellulose surface, the SLF containing conditioned culture medium was also recycled to support four rounds of cell culturing. The proliferative response generated by both a cellulose surface exposed to SLF, and recycled culture media containing SLF were compared to the proliferative response generated by a cellulose surface bearing immobilized SLF-CBD 1.0 (Fig. 3.22 A).

The cellulose surface bearing the immobilized SLF-CBD 1.0 fusion protein was capable of supporting cell proliferation over an extended period of re-use, as seen in figure 3.22 A. Both the cellulose surface which had been exposed initially to non-tagged SLF, and the recycled culture medium containing SLF were seen to lose biological activity with each consecutive use. Note, however, that the cellulose surface bearing the immobilized growth factor was showing signs of exhaustion by the fourth cycle of cell cultivation. The biological activity of the SLF surface and the SLF conditioned media are also

shown as a percentage of the biological activity generated by the SLF-CBD 1.0 surface during each cycle of cell cultivation (Fig. 3.22 B).

As an additional control experiment SLF-CBD 1.0 was adsorbed to a cellulose-coated plate, as before, and the supernatant was replaced three times without the addition of cells. Cells were subsequently added to the plate for the third cycle only, and the activity curve generated from this plate was compared to the activity curve generated by the plates to which fresh cells had been added for all three cycles (as illustrated in Figures 3.23 and 3.24). This control experiment was also carried out for the SLF protein. SLF-CBD 1.0 was also adsorbed to a non-cellulose plate, and the supernatant was replaced three times without the addition of cells. Cells were added only at the third cycle. The results indicate that the immobilized SLF-CBD 1.0 fusion protein (unlike the non-tagged SLF) had not been removed from the surface as a result of repeated replacement of the overlying supernatant. Furthermore, the activity generated by the unused surface was similar to that of the used surface, again indicating that the majority of the immobilized growth factor had remained bound to the cellulose surface dispite replacement of the overlying supernatant.

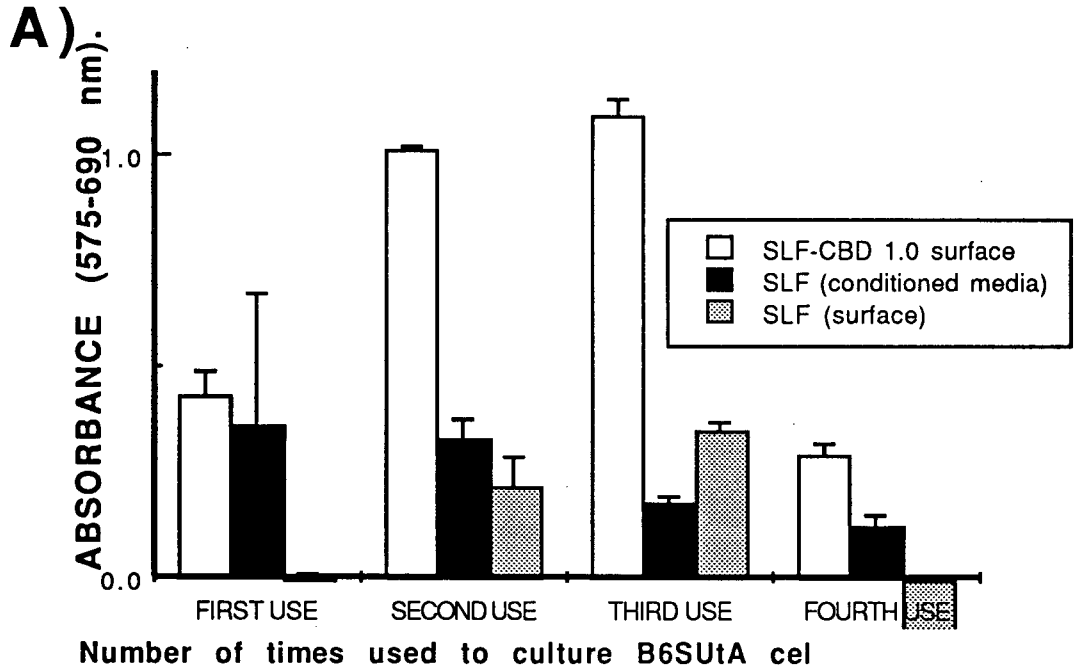



**Figure 3.22 A. Re-use of a regenerated cellulose surface bearing SLF-CBD 1.0.**

A cellulose surface bearing immobilized SLF-CBD 1.0 was used to support four consecutive rounds of cell cultivation. The proliferative response to this surface was compared to that of a cellulose surface which had been exposed to non-tagged SLF. Recycled SLF containing culture media was also used to support four consecutive cell cultivation cycles.

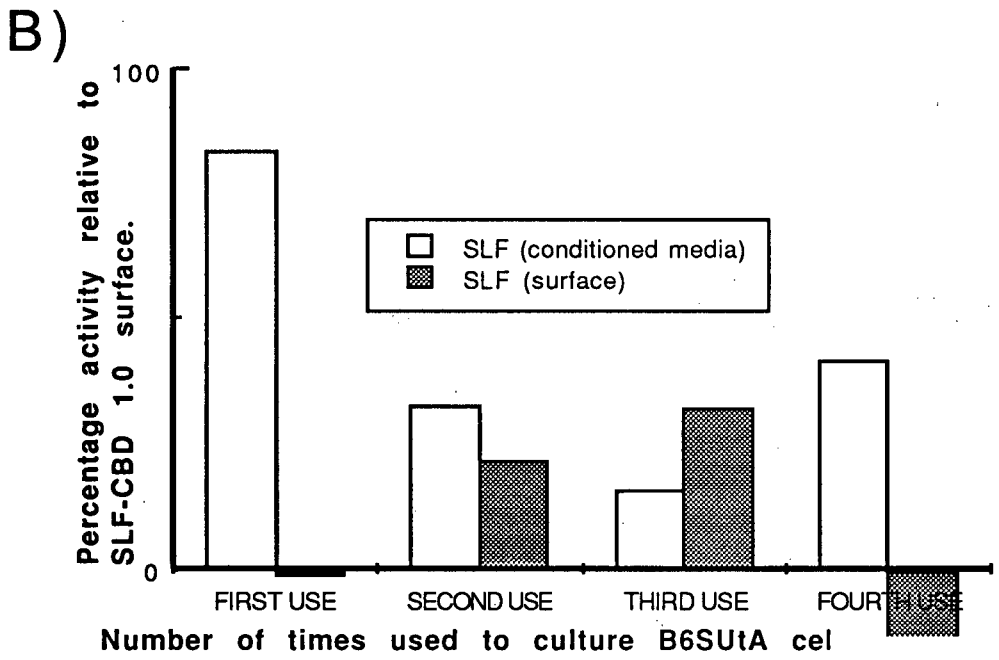



**Figure 3.22 B. Proliferative response to SLF containing conditioned media, and an SLF surface expressed as a percentage of that generated by a SLF-CBD 1.0 surface.**

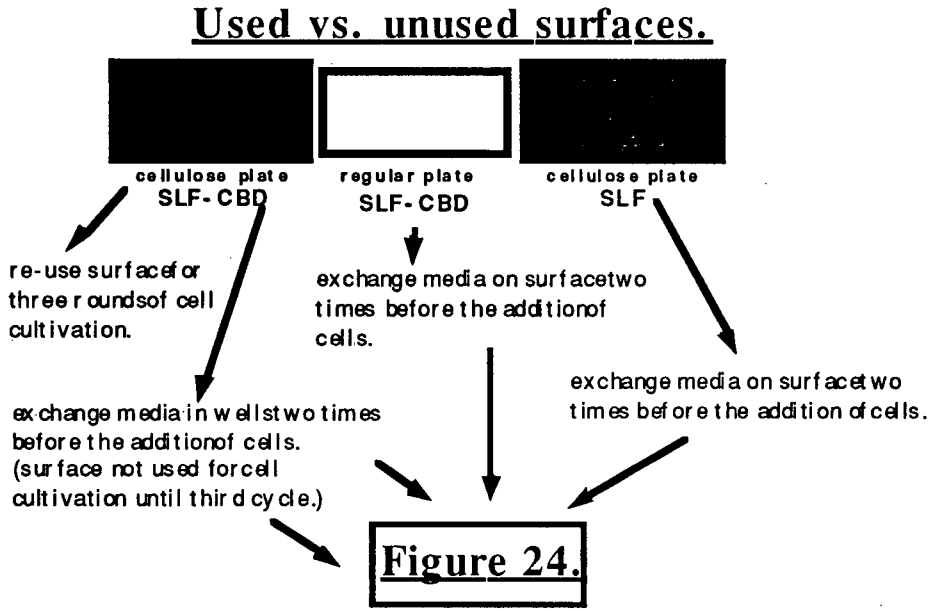



**Figure 3.23. Comparison of used and unused growth factor-bearing cellulose surfaces.**

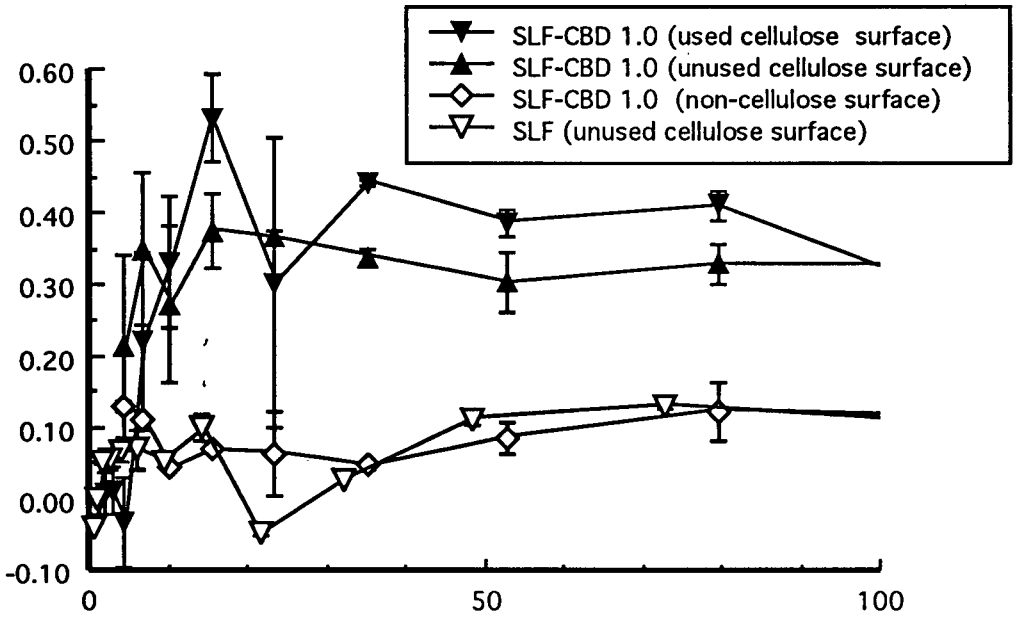



**Figure 3.24. Comparison of used and unused cellulose surfaces.**

SLF-CBD 1.0 was first adsorbed to a regenerated surface. This surface was then used for three consecutive rounds of cell culturing, with cell proliferation being measured for the third use. Activity was compared to that of a surface (on the same plate) which had not been used for cell culturing. These results were compared to a non-cellulose surface, and a non-affinity tagged growth factor.

# 4. DISCUSSION:

## 4.1. Design of the fusion protein.

In the present study protein domain swapping was used to create a chimerical protein in which the extracellular domain from murine steel factor(SLF) (Fig. 4.1 A) was fused to the cellulose binding domain from the cellulase Cex ($CBD_{Cex}$) (Fig. 4.1 B). The resulting hybrid was designated SLF-CBD 1.0 (Fig. 4.1 C), with the two domains being loosely tethered together by the proline-threonine spacer arm from Cex. Omission of this spacer arm resulted in fusion proteins which had only marginal biological activity, and only a marginal affinity for cellulose. Murine rather than human steel factor was selected because of its ability to stimulate both murine and human steel factor dependent cells, while the reverse relationship is not true (Martin *et al.* 1990). A murine based fusion protein could therefore be used on a broader range of cell types.

SLF-CBD 1.0 was designed to be model of naturally occurring steel factor; with its ability to bind to cellulose surfaces being analogous to the native proteins ability to bind to stromal surfaces. To create a model which was both functionally and structurally similar to the original, the cellulose-binding domain was placed at the location previously occupied by the transmembrane anchor, and a factor Xa site was substituted for the native cleavage site. SLF-CBD 1.0 could thus be immobilized in the same orientation as SLF, with cleavage of the CBD by factor Xa generating a free protein similar to the one generated by cleavage of the SLF transmembrane anchor *in vivo*. Because of the possibility that the hematopoietic process may be partially regulated by the temporal cleavage of native SLF from stromal cells *in vivo*, the factor Xa site was also included to facilitate the study of hematopoietic stem cell regulation *in vitro*. $CBD_{Cex}$ was chosen as the binding domain because it is naturally positioned at the carboxyl terminus of its parent enzyme, and would be more likely to retain its functional properties at the C-terminus of a fusion protein.

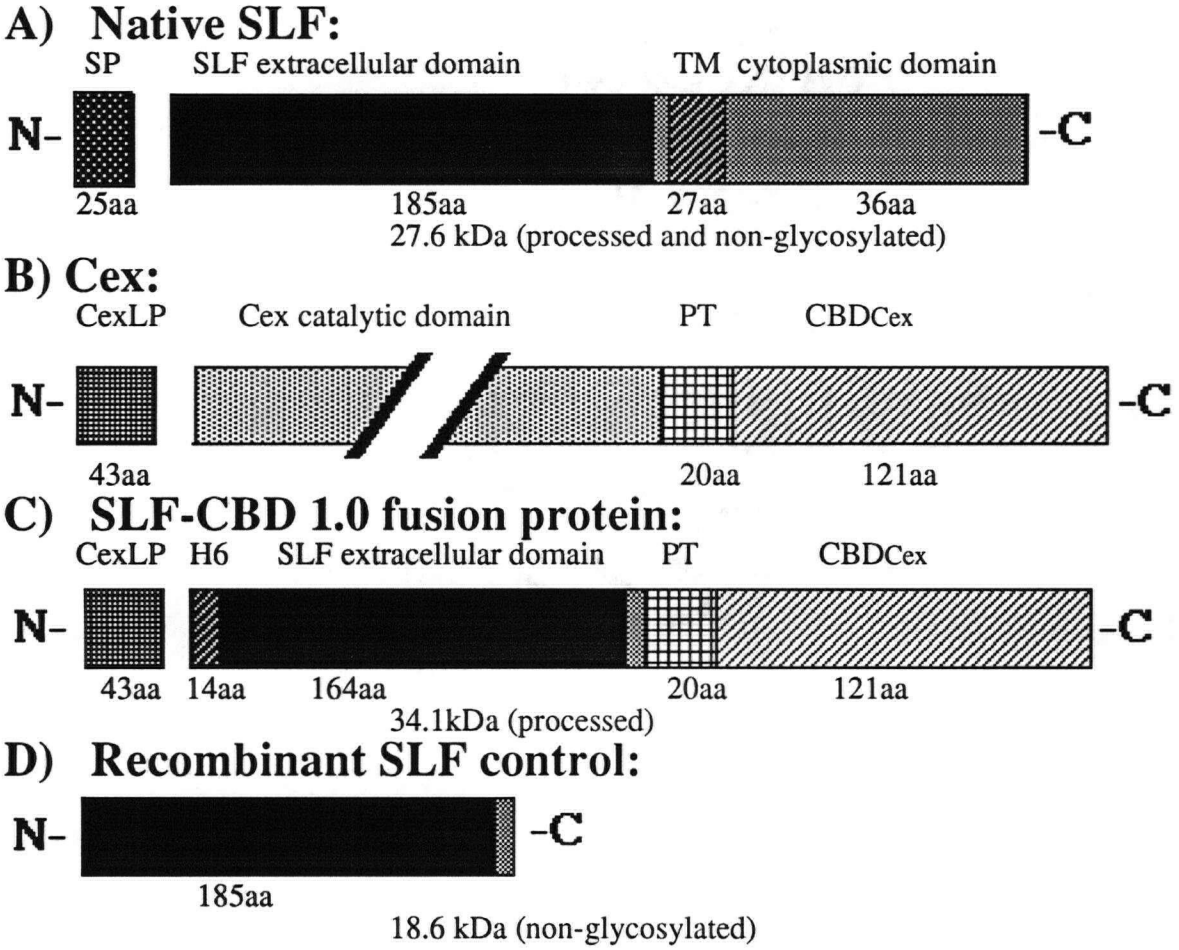



**Figure 4.1. SLF-CBD 1.0 and its components.**

**A)** Murine steel factor. **B)** Cex. **C)** SLF-CBD 1.0 fusion protein. **D)** Recombinant SLF control protein.

### 4.2. Fusion protein production and purification.

SLF-CBD 1.0 was designed to be a secretion-dual affinity tag fusion protein, of the sort illustrated in Fig. 1.2 vii. The Cex signal peptide was used to direct export of the protein to the periplasm of the *E. coli* expression host, with subsequent isolation of periplasmic proteins by osmotic shock. The SLF-CBD 1.0 was then purified using the amino-terminal polyhistidine tag and metal chelate affinity chromatography (MCAC). The carboxy-terminal cellulose

binding domain was later used to adsorb SLF-CBD 1.0 to cellulose for *in situ* experimentation.

Mammalian cytokines are frequently difficult to express in *E. coli*, and usually form insoluble inclusion bodies (Marston and Hartley 1990). In the present study this problem was circumvented by using a bacterial signal peptide to direct export of the protein to the periplasm, where mammalian proteins are more likely to retain their proper conformation (Hsiung *et al.* 1988, Pollitt and Zalkin 1983). Furthermore, since the mammalian peptide was bracketed on both ends by bacterial peptides, the host cell may have been more likely to regard it as a native bacterial protein, and not subject it to the same degree of proteolytic degradation as other mammalian proteins. Since both the Cex signal peptide and the Cex binding domain were derived from a highly soluble bacterial protein it is also possible that fusion of these two peptides to steel factor allowed it to remain soluble, and not form inclusion bodies.

A second fusion protein (SLF-CBD 1.1) was designed for intracellular expression, without export to the periplasm. This protein was recovered from *E. coli* cytoplasms as inclusion bodies, purified, and subsequently re-natured. A comparison of the biological activities of the two proteins revealed that the secreted protein was the more potent of the two, with a specific activity similar to that of commercial recombinant SLF (Fig. 13). The secretion method was therefore adopted as the preferred production method.

Use of the CBD as an affinity tag for purification was also explored (sections 3.4.1 and 3.4.3). CBDs had been used previously for the purification of fusion proteins (Greenwood *et al.* 1992), and the use of the C-terminal cellulose-binding domain for protein purification in this case would have eliminated the need for an N-terminal polyhistidine tag. Elution of the fusion protein from cellulose was not found to be possible without the use of chaotrophic agents, however, and MCAC purification was adopted as the preferred method of purification.

### 4.2.1. Protocol for rapid production and purification of SLF-CBD 1.0.

Of the three temperatures tested, culture growth at room temperature resulted in the highest cell density (Fig. 3.7 A), and was therefore adopted as the standard growth temperature. Induction of protein production with 0.1 mM IPTG was found to be sufficient for most purposes. Although slightly higher

yields of SLF-CBD 1.0 were elicited by higher concentrations of IPTG, the high cost of this reagent does not make this an economical means of increasing protein production.

For small scale production of SLF-CBD 1.0 therefore it is recommended that freshly transformed cultures of JM101/pSLF/CBD 1.0 be grown in shake flasks at room temperature to an optical density ($OD_{600}$) of 5 to 10 Absorbance units, induced with 0.1 mM IPTG, and harvested eight hours after induction (the longest induction period tested in this study). The fusion protein can then be isolated from the harvested cells by osmotic shock. SLF-CBD 1.0 can subsequently be separated from other periplasmic proteins using MCAC as described in section 2.8.1. In most cases approximately two thirds of the fusion protein will have been retained in the periplasmic compartment of the cell, with the remaining third being found in the cell culture supernatant. Fusion protein can also be purified from the culture supernatant, if desired, using the method described in section 2.8.2.

### 4.3. Biological activity of SLF-CBD 1.0.

The biological activity of free SLF-CBD 1.0 was found to be similar to that of commercial rmSLF (section 3.8, Fig. 3.13 A). The cellulose-binding domain, alone, was neither stimulatory nor toxic to the target cells (Fig. 3.13 D).

### 4.4. Effects of SLF-CBD immobilization on biological activity.

The results presented in sections 3.10 and 3.11 indicated that the biological activity of SLF-CBD 1.0 could be enhanced as a result of immobilization. This was likely due to an increase in the local concentration of the growth factor at the point of contact between the cell surface and the BMCC matrix to which the SLF-CBD 1.0 was adsorbed. By first adsorbing the fusion protein to its matrix and then removing the matrix from suspension, with subsequent testing of the resulting supernatant and matrix separately, it was possible confirm binding of the fusion protein to BMCC (Figures 3.16 A and 3.19 A). Cleavage of the cellulose-binding domain by factor Xa (Fig. 3.20 A) confirmed that the CBD was responsible for binding. The resulting drop in biological activity

associated with cleavage of the binding domain confirmed that the original enhancement of activity was a result of immobilization (Fig. 3.20 B).

Figure 3.16 A indicates that the binding capacity of BMCC had only been exceeded at very low matrix concentrations. Only at these lower BMCC concentrations was the activity associated with the supernatant seen to exceed the activity associated with the matrix. The first point (in Fig. 3.16 A) at which the free activity exceeds the adsorbed activity corresponds to a BMCC concentration of about 3 ng $mL^{-1}$. By multiplying protein concentration by Avogadro's number it was possible to estimate the number SLF-CBD 1.0 molecules present. Assuming that most of the SLF-CBD 1.0 molecules had bound to the matrix it was then possible to calculate the approximate growth factor surface density for each point by dividing the number of molecules present by the available surface area for BMCC ($1.22 \times 10^6$ $cm^2$ $g^{-1}$, Gilkes *et al. 1992*). Saturation of the BMCC surface occurred therefore at an SLF-CBD 1.0 surface density of approximately $5 \times 10^{13}$ molecules $cm^{-2}$.

### 4.4.1. Effect of immobilization on cell proliferation.

By adsorbing a fixed concentration of SLF-CBD 1.0 to a variable concentration of BMCC (Figures 3.15 and 3.16) it was possible to make two key observations. Firstly, an optimal activity peak was always observed, for which the activity of the adsorbed growth factor was greater than that of the free growth factor. Secondly, the location on this peak appeared to be a function of both the BMCC concentration and the SLF-CBD 1.0 concentration. Since the net effect of holding the SLF-CBD 1.0 concentration constant while varying the BMCC concentration was to vary the surface density of the immobilized growth factor, this second observation would tend to suggest that the target cells were optimally stimulated by a specific surface density of SLF-CBD 1.0.

Adsorption of SLF-CBD 1.0 to BMCC only enhanced biological activity when the fusion protein was adsorbed to a specific amount of BMCC. This optimal concentration of BMCC was found to be different for each concentration of SLF-CBD 1.0 tested (Table 4.1), with higher concentrations of BMCC being optimal for higher concentrations of SLF-CBD 1.0, and lower concentrations of BMCC being optimal for lower concentrations of SLF-CBD 1.0. This observation is consistent with the surface density hypothesis. When fixed concentrations of SLF-CBD 1.0 were adsorbed to excessive amounts of

BMCC the surface density of immobilized growth factor would have been too low to elicit an optimum proliferative response. Increasing the amount of immobilized growth factor, however, would counteract this effect. The surface density of adsorbed growth factor could also be increased by reducing the amount of BMCC present, but this would have the effect of reducing the number of BMCC particles available to stimulate cells. The proliferative response elicited would therefore be limited on the one hand by the surface density of immobilized growth factor, and on the other hand by the available surface area of the BMCC (and the number of BMCC particles present).

**Table 4.1**
**Optimal BMCC concentrations observed with fixed concentrations of SLF-CBD 1.0.**

| SLF-CBD 1.0 (pM) | BMCC* ($\mu g\ mL^{-1}$) | adsorbed activity† (abs. units) | free activity‡ (abs.units) |
|---|---|---|---|
| **130** | **1.412** | 0.187 | 0.055 |
| **300** | **1.412** | 0.230 | 0.063 |
| 1500 | 44.100 | 0.986 | 0.450 |

* Optimal BMCC concentration was defined as the point at which the highest activity was measured.
† Biological activity in the presence of BMCC.
‡ Biological activity in the absence of BMCC.

These observations were reinforced when variable amounts of SLF-CBD 1.0 were adsorbed to fixed amounts of BMCC (section 3.11). Again, higher concentrations of BMCC were found to be optimal for higher concentrations of fusion protein, while lower concentrations of BMCC were found to be optimal for lower concentrations of fusion protein. BMCC concentrations of 14 $\mu g\ mL^{-1}$ appeared to be optimal for SLF-CBD 1.0 concentrations greater than 1000 pM (Fig. 3.18), while BMCC concentrations of 1 $\mu g\ mL^{-1}$ were found to be optimal for lower concentrations of SLF-CBD 1.0 in some cases (Fig. 3.18 (inset)).

One further observation can be made from the results presented sections 3.10 and 3.11. In most experiments the proliferative response was measured by both the MTT test and direct cell count. Both methods confirmed that immobilization of SLF-CBD 1.0 resulted in an enhancement of biological activity, but both methods were not always in agreement as to the magnitude of

the enhancement. Activity increases measured by the MTT test were usually larger than increases measured by direct cell count. While direct cell count measures only the net number of live cells the MTT test is a measure of both cell number and cell respiration. A comparison of figures 3.17 A and 3.18 A reveals that the increases in cell respiration elicited by immobilized growth factor concentrations of (approximately) 300 pM or less were accompanied by equivalent increases in cell number. The increases in cell respiration elicited by immobilized growth factor concentrations greater than 300 pM, however, were not accompanied by equivalent increases in cell number. A direct cell count was used therefore to quantify the enhancement of SLF-CBD 1.0 activity as a result of immobilization (below), since this was believed to be a more accurate measurement of the proliferative response.

The fact that increases in cell respiration are not accompanied by increases in cell division at higher concentrations of growth factor has important implications for the study of cell regulation. It is possible that a feedback mechanism exhists which stops cells from dividing when overstimulated by too much steel factor. A similar abundance of steel factor may be found on certain stromal layers in the bone marrow microenvironment. The body may therefore maintain a viable source of bone marrow stem cells by immobilizing them through their c-kit receptors to a stromal layer of feeder cells. An over abundant supply of surface presented SLF may maintain these cells in a viable, but non-mitotic state. Subsequent release of the cells, through the cleavage of the membrane bound SLF, would result in an entrance into mitosis, and a replenishment of the bone marrow. It is possible that this mechanism of cell regulation is important to the hematopoietic system for maintaining and regulating the supply of hematopoietic stem cells.

### 4.4.2. Effects of immobilization on SLF-CBD 1.0 specific activity.

Adsorption of SLF-CBD 1.0 to cellulose was also accompanied by a drop in the half-maximal effective dose (the $ED_{50}$) of the protein, meaning that less of the fusion protein was required to elicit the maximum response when immobilized. In this case, as before, the two methods of measurement were not in total agreement. Manual cell counts were believed to be a more genuine

measure of cell proliferation, and were used to assess the impact of growth factor immobilization on target cell proliferation (Table 4.3).

The results, in this case, indicated that the adsorption of SLF-CBD 1.0 to 1μg $mL^{-1}$ of BMCC was accompanied by a 35% increase in the final cell number, and a 73% drop in the $ED_{50}$. No equivalent trends were observed for the SLF control protein which could not be adsorbed to the BMCC.

### 4.5. Effects of ligand surface density on cell proliferation.

By adsorbing a fixed concentration of SLF-CBD 1.0 to a variable concentration of BMCC (section 3.10) it was possible to examine the effects of growth factor surface density on cell proliferation. The results illustrated in Figure 3.15 indicated that each concentration of growth factor was able to elicit a maximum response when adsorbed to an optimum amount of BMCC, suggesting that the magnitude of the response was actually a function of the

**Table 4.2.**

**Increased SLF-CBD 1.0 activity as a result of adsorption to BMCC.***

**A) SLF-CBD 1.0 (test protein).**

| | Adsorbed to BMCC. | Free in solution. |
|---|---|---|
| $ED_{50}$ (pM): | **65 ± 17** | **250 ± 83** |
| Maximum response (cells X $10^5$): | **67 ± 4** | **50 ± 6** |

**B) SLF (control protein).**

| | Adsorbed to BMCC. | Free in solution. |
|---|---|---|
| $ED_{50}$ (pM): | **280 ± 76** | **360 ± 72** |
| Maximum response (cells X $10^5$): | **70 ± 8** | **82 ± 8** |

*Adsorption to 1 μg mL-1 BMCC.
(Confidence intervals represent the 95% confidence interval for the standard error of the estimate as calculated by the GraFit program.)

growth factor surface density. By again multiplying the SLF-CBD 1.0 concentrations listed in Table 4.1 by Avogadro's number, and dividing by the available BMCC surface area present, the approximate optimal growth factor surface density for each experiment was calculated (Table 4.3). In all three cases the optimal growth factor surface densities were found to be within the same order of magnitude. Alternatively, the optimal SLF-CBD 1.0 surface density could be calculated from experiments where the BMCC concentration was held constant while the growth factor concentration was varied (section 3.11). The SLF-CBD 1.0 surface concentration which elicited maximum cell proliferation when adsorbed to 1 $\mu g\ mL^{-1}$ (Fig. 3.17 A) was calculated using a growth factor concentration of approximately 130 pM (twice the $ED_{50}$) as the optimum value. This optimal SLF-CBD 1.0 concentration corresponds to a surface density of approximately $6 \times 10^{10}$ molecules $cm^{-2}$, and is again within the same order of magnitude as the other values.

**Table 4.3.**
**SLF-CBD 1.0 surface densities which elicited maximum cell proliferation.**

| SLF-CBD 1.0 (pM). | reference figure. | density (molecules/$cm^2$) |
|---|---|---|
| 130 | 3.15 A | $5 \times 10^{10}$ |
| 300 | 3.15 B | $1 \times 10^{10}$ |
| 1500 | 3.15 C | $2 \times 10^{10}$ |
| variable (1 $\mu g\ mL^{-1}$) | 3.17 A | $6 \times 10^{10}$ |

Although the surface density of the c-kit receptor on B6SUtA cells is not known, the approximate receptor density of two similar cell types (MO7e and OCIM1) can be calculated. For a cell diameter of approximately 10 μm, and 35 000 to 80 000 receptors per cell (Turner *et al.* 1995) these values would be approximately $1 \times 10^{10}$ to $3 \times 10^{10}$ receptors $cm^{-2}$ respectively. If B6SUtA cells have a similar receptor density then an optimal proliferative response is elicited when the surface density of the ligand is approximately equal to the surface density of the receptor. It is possible that the enhanced biological activity generated by this density of ligand was the result of surface compression of the receptors. An increased local density of ligand could have brought the

surface receptors into closer proximity and allowed them to dimerize and cluster more easily, thus setting off the signal transduction cascade.

### 4.6. Adsorption of SLF-CBD 1.0 to a regenerated cellulose surface.

SLF-CBD 1.0 immobilization experiments, previously carried out using BMCC, were repeated using a flat cellulose surface regenerated from a solution of cellulose acetate. Manual cell counts of B6SUtA cells grown on cellulose coated tissue culture plates in the presence of SLF indicated that these surfaces might have an adverse effect on cell growth. For unknown reasons, cells cultured in regular wells generally reached densities which were 20% to 25% greater than cells cultured in cellulose coated wells. As a result of this a direct comparison between immobilized SLF-CBD 1.0 and free SLF-CBD 1.0 was not possible when this kind of a surface was used. Comparisons between SLF-CBD 1.0 and SLF were comparable, however, when both were cultured in cellulose coated plates (Fig. 3.21).

The affinity of SLF-CBD 1.0 for a regenerated cellulose surface (Fig. 3.21 A) was found to be lower than the affinity of SLF-CBD 1.0 for a BMCC surface (Fig. 3.19 A). As before, SLF-CBD 1.0 was first adsorbed to the cellulose, then supernatant was transferred to a fresh plate for separate testing. When adsorbed activity and soluble activity were tested separately it became apparent that the addition of SLF-CBD 1.0 concentrations greater than 75 pM did not result in a higher adsorbed activity being observed. Presumably the surface had become saturated at this point.

The low binding capacity of this surface cannot be explained by a lack of surface area relative to BMCC. The cellulose surface area present in a tissue culture well with a diameter of 5 mm (the kind used here) was about 0.6 $cm^2$, compared to the 0.2 $cm^2$ cellulose surface present in each well during the BMCC experiments described above. Figure 3.19 A indicates that the binding capacity of this smaller BMCC surface had not been exceeded even by SLF-CBD 1.0 concentrations of 3000 pM.

There are two possible explanations for this apparent difference in binding capacity. Firstly, since the regenerated cellulose surface was flat, while the BMCC surface was crystalline, it is possible that the cellulose binding domain ($CBD_{Cex}$) had a specific binding preference for crystalline, rather than flat

surfaces. Binding studies, in which labeled $CBD_{Cex}$ was tested on different cellulose surfaces, showed marked binding preferences for different kinds of surfaces. When labeled $CBD_{Cex}$ was adsorbed to cellulose acetate cover slip label was evident only on the edges (Eric Jervis, personal communication). Very little $CBD_{Cex}$ was seen to adsorb to the flat of the surface. This is in contrast to BMCC, which bound labeled protein over its entire surface. Furthermore, when labeled $CBD_{Cex}$ was adsorbed to cellulose fibers with rectangular cross sections, the protein again displayed a marked preference for the edges of the fibers (Neena Din, personal communication).

If $CBD_{Cex}$ actually has a preference for edges, rather than flat surfaces, then the comparison between BMCC binding capacity and flat surface binding capacity becomes one of available edge area, rather than one of available surface area. A crystalline matrix would most likely have more edges available for binding, per unit of surface area, than would a flat surface, generated from cellulose acetate. Indeed, when the solution of cellulose acetate was added to the tissue culture wells, to regenerate the cellulose surface used in these experiments, the solution evaporated in such a way as to create a series of concentric rings. It is possible that the edges of these rings were responsible for the majority of the SLF-CBD 1.0 binding, with very little protein binding to the flat of the surface.

A second possible explanation for these results is that this kind of functional binding assay is not a valid measure of binding when used with this kind of surface. The method of separating adsorbed activity from free activity was developed as an alternative to measuring SLF-CBD 1.0 binding by ELISA, since ELISA was not sensitive enough to measure the minute quantities of protein used in these experiments. This method, although sensitive, has other limitations which the ELISA method does not share. It was critical, for example, that all of the cells present had equal access to the bound growth factor in order to give a faithful estimate of how much protein was actually present. A microscopic examination of B6SUtA cultures showed that these cells usually formed spherical clusters when grown to high density. It was possible, therefore, that not all of the cells in a cluster had equal access to the growth factor when it was immobilized on a flat surface. Only the cells at the bottom of the cluster would have been in contact with the surface. BMCC, by contrast, surrounded the cell clusters, so that more of the cells in the cluster had access to the surface. It should be noted that, in Figure 3.21 A, the observed plateau in

the immobilized activity was not accompanied by an equivalent rise in the observed soluble activity. The fact that these two curves are not additive suggests that the second explanation is, at least partially, correct.

### 4.7. Re-use of a regenerated cellulose surface bearing SLF-CBD 1.0.

The results presented in section 3.14 indicated that it was possible to use a regenerated cellulose surface, bearing immobilized SLF-CBD 1.0, to support several generations of cell growth. This technique could be used to cultuvate several generations of cell culture in the same flask by simply removing the previouse generation of cells, and the spent culture media, and replacing it with fresh media. Immobilization of growth factors on the interior of the culture flask would eliminate the need to constantly supply fresh cytokines to the culture. The interior surfaces of rollerbottles, for example, could be coated with regenerated cellulose, and this cellulose saturated with cellulose-binding growth factors. When these rollerbottles were subsequently used to cultivate cells, the same bottles could be used to bring several generations of cells to confluence without the addition of fresh growth factors. One culture, after having reached its maximum density, could simply be poured out of the bottle (leaving only a few cells to seed the next culture), and the media replaced.

Although growth factor receptors are generally internalized and degraded after stimulation, the fact that the growth factor is internalized and degraded along with the receptor may be merely coincidental, and not necessary. Immobilization of the growth factor may prevent co-internalization with the receptor, provided that binding to the matrix is stronger than binding to the receptor. The use of this technique could potentially prevent the consumption of many crowth factors from cell cultures, thus conserving these growth factors and lowering the cost of *ex vivo* cell culturing.

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# Appendix 1.

**PCR primers used to create the synthetic SLF gene:**

**Primer 1:**
(G50) N-terminal primer to introduce Nco I and Xba I restriction sites into the DNA encoding the amino-terminus of SLF.

**5'-ATGGCATCTAGAAAGGAGATCTGCGGGAATCCTGTGACTG-3'**

**Primer 2:**
(G51) C-terminal primer to introduce Hind III, Stu I, Nhe I and Spe I sites into the DNA encoding the carboxy-terminus of the SLF extracellular domain.

**5'-AGTGCCAAGCTTCGCGCCGAAGGCCTGACTAGTCCT GCCCTCGATGCTAGCAACAGGGGGTAACATAAATGG-3'**

# Appendix 2.

**Calculation of the theoretical extinction coefficient for SLF-CBD:**

$\varepsilon_{280}$= **(5700 X nW) + (1300 X nY)/ mol.weight of protein (Da).**

Where nW= number of tryptophans
nY=number of tyrosines.

$\varepsilon_{280}$= **(5700 X 6) + (1300 X 3) / 34100 = 1.11 mg/ml.**

# Appendix 3.

**Coding sequences of pSLF/CBD 1.0 and pSLF/CBD 1.1.**

**A) pSLF/CBD 1.0.**

ATGgctCCTAGGACCACGCCCGCACCCGGCCACCCGGCCCGCGGCGCCC
GCACCGCTCTGCGCACGACGCTCGCCGCCGCGGCGGCGACGCTCGTCG
TCGGCGCCACGGTCGTGCTGCCCGCCCAGGCCGCTAGtcatcaccatcaccatcac
atcgaaggtagggCTAGAaaggagatctgcgggaatcctgtgactgataatgtaaaagacattacaaaactggtg
gcaaatcttccaaatgactatatgataaccctcaactatgtcgccgggatggatgttttgcctagtcattgttggctacgag
atatggtaatacaattatcactcagcttgactactcttctggacaagttctcaaatatttctgaaggcttgagtaattactcca
tcatagacaaacttgggaaaatagtggatgacctcgtgttatgcatggaagaaaacgcaccgaagaatataaaagaat
ctccgaagaggccagaaactagatcctttactcctgaagaattctttagtattttcaatagatccattgatgcctttaaggac
tttatggtggcatctgacactagtgactgtgtgctctcttcaacattaggtcccgagaaagattccagagtcagtgtcaca
aaaccatttatgttacccccctgttGCTAGCATCGAGGGCAGGACTAGTCAGGCCTTCGG
CGCGAGCCCGACGCCGACGCCCACCACGCCGACCCCGACGCCCACGAC
GCCGACGCCGACCCCGACGTCCGGTCCGGCCGGGTGCCAGGTGCTGTG
GGGCGTCAACCAGTGGAACACCGGCTTCACCGCGAACGTCACCGTGAA
GAACACGTCCTCCGCTCCGGTCGACGGCTGGACGCTCACGTTCAGCTTC
CCGTCCGGCCAGCAGGTCACCCAGGCGTGGAGCTCGACGGTCACGCAG
TCCGGCTCGGCCGTGACGGTCCGCAACGCCCCGTGGAACGGCTCGATC
CCGGCGGGCGGCACCGCGCAGTTCGGCTTCAACGGCTCGCACACGGGC
ACCAACGCCGCGCCGACGGCGTTCTCGCTCAACGGCACGCCCTGCACG
GTCGGCTGA

**B) pSLF/CBD 1.1.**

atggGCAGCAGCCATCATCATCATCACAGCAGCGGCCTGGTGCCGCG
CGGCAGCCATATGGCTAGAaaggagatctgcgggaatcctgtgactgataatgtaaaagacattaca
aaactggtggcaaatcttccaaatgactatatgataaccctcaactatgtcgccgggatggatgttttgcctagtcattgtt
ggctacgagatatggtaatacaattatcactcagcttgactactcttctggacaagttctcaaatatttctgaaggcttgagt
aattactccatcatagacaaacttgggaaaatagtggatgacctcgtgttatgcatggaagaaaacgcaccgaagaata
taaaagaatctccgaagaggccagaaactagatcctttactcctgaagaattctttagtattttcaatagatccattgatgc
ctttaaggactttatggtggcatctgacactagtgactgtgtgctctcttcaacattaggtcccgagaaagattccagagtc
agtgtcacaaaaccatttatgttacccccctgttGCTAGCATCGAGGGCAGGACTAGTCAGGC
CTTCGGCGCGAGCCCGACGCCGACGCCCACCACGCCGACCCCGACGCC
CACGACGCCGACGCCGACCCCGACGTCCGGTCCGGCCGGGTGCCAGGT
GCTGTGGGGCGTCAACCAGTGGAACACCGGCTTCACCGCGAACGTCAC
CGTGAAGAACACGTCCTCCGCTCCGGTCGACGGCTGGACGCTCACGTTC
AGCTTCCCGTCCGGCCAGCAGGTCACCCAGGCGTGGAGCTCGACGGTC
ACGCAGTCCGGCTCGGCCGTGACGGTCCGCAACGCCCCGTGGAACGGC
TCGATCCCGGCGGGCGGCACCGCGCAGTTCGGCTTCAACGGCTCGCAC
ACGGGCACCAACGCCGCGCCGACGGCGTTCTCGCTCAACGGCACGCCC
TGCACGGTCGGCTGA

# Appendix 4.

**Amino acid sequences of SLF-CBD 1.0 and SLF-CBD 1.1.**

**A)i SLF-CBD 1.0** (unprocessed. Export of fusion protein to periplasm of E. coli.)

**MAPRTTPAPGHPARGARTALRTTLAAAAATLVVGATVVLPAQAASHH
HHHHIEGRARKEICGNPVTDNVKDITKLVANLPNDYMITLNYVAGMD
VLPSHCWLRDMVIQLSLSLTTLLDKFSNISEGLSNYSIIDKLGKIVDDLV
LCMEENAPKNIKESPKRPETRSFTPEEFFSIFNRSIDAFKDFMVASDTSD
CVLSSTLGPEKDSRVSVTKPFMLPPVASIEGRTSQAFGASPTPTPTTPTP
TPTTPTPTPTSGPAGCQVLWGVNQWNTGFTANVTVKNTSSAPVDGWT
LTFSFPSGQQVTQAWSSTVTQSGSAVTVRNAPWNGSIPAGGTAQFGFN
GSHTGTNAAPTAFSLNGTPCTVGZ**

363 Amino Acids MW : 38280 Dalton (unprocessed)

| | n | n(%) | MW | MW(%) |
|---|---|---|---|---|
| A ala alanine | 34 | 9.4 | 2415 | 6.3 |
| C cys cysteine | 6 | 1.7 | 618 | 1.6 |
| D asp aspartic acid | 15 | 4.1 | 1725 | 4.5 |
| E glu glutamic acid | 11 | 3.0 | 1419 | 3.7 |
| F phe phenylalanine | 15 | 4.1 | 2206 | 5.8 |
| G gly glycine | 26 | 7.2 | 1482 | 3.9 |
| H his histidine | 9 | 2.5 | 1233 | 3.2 |
| I ile isoleucine | 14 | 3.9 | 1583 | 4.1 |
| K lys lysine | 13 | 3.6 | 1665 | 4.4 |
| L leu leucine | 24 | 6.6 | 2714 | 7.1 |
| M met methionine | 7 | 1.9 | 917 | 2.4 |
| N asn asparagine | 19 | 5.2 | 2166 | 5.7 |
| P pro proline | 32 | 8.8 | 3105 | 8.1 |
| Q gln glutamine | 10 | 2.8 | 1280 | 3.3 |
| R arg arginine | 13 | 3.6 | 2029 | 5.3 |
| S ser serine | 33 | 9.1 | 2872 | 7.5 |
| T thr threonine | 45 | 12.4 | 4547 | 11.9 |
| V val valine | 27 | 7.4 | 2674 | 7.0 |
| W trp tryptophan | 6 | 1.7 | 1116 | 2.9 |
| X ukw unknown | - | - | | |
| Y tyr tyrosine | 3 | 0.8 | 489 | 1.3 |
| Z --- STOP | 1 | 0.3 | | |

**ii SLF-CBD 1.0** (signal peptide removed):

**ASHHHHHHIEGRARKEICGNPVTDNVKDITKLVANLPNDYMITLNYVA
GMDVLPSHCWLRDMVIQLSLSLTTLLDKFSNISEGLSNYSIIDKLGKIV
DDLVLCMEENAPKNIKESPKRPETRSFTPEEFFSIFNRSIDAFKDFMVAS
DTSDCVLSSTLGP**

**EKDSRVSVTKPFMLPPVASIEGRTSQAFGASPTPTPTTPTPTPTTPTPTP TSGPAGCQVLWGVNQWNTGFTANVTVKNTSSAPVDGWTLTFSFPSGQ QVTQAWSSTVTQSGSAVTVRNAPWNGSIPAGGTAQFGFNGSHTGTNA APTAFSLNGTPCTVGZ**

320 Amino Acids MW : 34123 Dalton

| | n | n(%) | MW | MW(%) |
|---|---|---|---|---|
| A ala alanine | 21 | 6.6 | 1491 | 4.4 |
| C cys cysteine | 6 | 1.9 | 618 | 1.8 |
| D asp aspartic acid | 15 | 4.7 | 1725 | 5.1 |
| E glu glutamic acid | 11 | 3.4 | 1419 | 4.2 |
| F phe phenylalanine | 15 | 4.7 | 2206 | 6.5 |
| G gly glycine | 23 | 7.2 | 1311 | 3.8 |
| H his histidine | 8 | 2.5 | 1096 | 3.2 |
| I ile isoleucine | 14 | 4.4 | 1583 | 4.6 |
| K lys lysine | 13 | 4.1 | 1665 | 4.9 |
| L leu leucine | 20 | 6.2 | 2261 | 6.6 |
| M met methionine | 6 | 1.9 | 786 | 2.3 |
| N asn asparagine | 19 | 5.9 | 2166 | 6.3 |
| P pro proline | 27 | 8.4 | 2620 | 7.7 |
| Q gln glutamine | 9 | 2.8 | 1152 | 3.4 |
| R arg arginine | 9 | 2.8 | 1404 | 4.1 |
| S ser serine | 33 | 10.3 | 2872 | 8.4 |
| T thr threonine | 38 | 11.9 | 3839 | 11.3 |
| V val valine | 23 | 7.2 | 2278 | 6.7 |
| W trp tryptophan | 6 | 1.9 | 1116 | 3.3 |
| X ukw unknown | - | - | | |
| Y tyr tyrosine | 3 | 0.9 | 489 | 1.4 |
| Z --- STOP | 1 | 0.3 | | |

**B) SLF-CBD 1.1** (production of SLF-CBD as inclusion bodies in E. coli.)

**MGSSHHHHHHSSGLVPRGSHMARKEICGNPVTDNVKDITKLVANLPN DYMITLNYVAGMDVLPSHCWLRDMVIQLSLSLTTLLDKFSNISEGLSN YSIIDKLGKIVDDLVLCMEENAPKNIKESPKRPETRSFTPEEFFSIFNRSI DAFKDFMVASDTSDCVLSSTLGPEKDSRVSVTKPFMLPPVASIEGRTSQ AFGASPTPTPTTPTPTPTTPTPTPTSGPAGCQVLWGVNQWNTGFTANV TVKNTSSAPVDGWTLTFSFPSGQQVTQAWSSTVTQSGSAVTVRNAPW NGSIPAGGTAQFGFNGSHTGTNAAPTAFSLNGTPCTVGZ**

329 Amino Acids MW : 34981 Dalton

| | n | n(%) | MW | MW(%) |
|---|---|---|---|---|
| A ala alanine | 20 | 6.1 | 1420 | 4.1 |
| C cys cysteine | 6 | 1.8 | 618 | 1.8 |
| D asp aspartic acid | 15 | 4.6 | 1725 | 4.9 |
| E glu glutamic acid | 10 | 3.0 | 1290 | 3.7 |
| F phe phenylalanine | 15 | 4.6 | 2206 | 6.3 |
| G gly glycine | 25 | 7.6 | 1425 | 4.1 |
| H his histidine | 9 | 2.7 | 1233 | 3.5 |
| I ile isoleucine | 13 | 4.0 | 1470 | 4.2 |
| K lys lysine | 13 | 4.0 | 1665 | 4.8 |
| L leu leucine | 21 | 6.4 | 2374 | 6.8 |
| M met methionine | 8 | 2.4 | 1048 | 3.0 |
| N asn asparagine | 19 | 5.8 | 2166 | 6.2 |
| P pro proline | 28 | 8.5 | 2717 | 7.8 |
| Q gln glutamine | 9 | 2.7 | 1152 | 3.3 |
| R arg arginine | 9 | 2.7 | 1404 | 4.0 |
| S ser serine | 37 | 11.2 | 3220 | 9.2 |
| T thr threonine | 38 | 11.6 | 3839 | 11.0 |
| V val valine | 24 | 7.3 | 2377 | 6.8 |
| W trp tryptophan | 6 | 1.8 | 1116 | 3.2 |
| X ukw unknown | - | - | | |
| Y tyr tyrosine | 3 | 0.9 | 489 | 1.4 |
| Z --- STOP | 1 | 0.3 | | |

## Appendix 5.

## MTT cell proliferation assay for the B6SUtA cell line:

**The following protocol was developed to measure B6SUtA (Greenberger *et al.* 1983) cell proliferation in response to the hybrid cytokine fusion protein SLF-CBD (Doheny *et al* 1996), using MTT rather than $^3$H-thymidine. It was adapted from the MTT test (Denizot and Lang 1986, Mosmann 1984) and a standard $^3$H-thymidine uptake test protocol provided by Ms. Vivian Lam (Terry Fox Laboratory).**

**Principle:** Cell proliferation is measured indirectly through measurement of net cell respiration (assuming the net amount of cell respiration is proportional to the number of cells present). The tetrazolium salt MTT (3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is cleaved by the mitochondrial enzyme succinate-dehydrogenase to form a blue colored product (formazan), the concentration of which can be measured by a spectrophotometer (Denizot and Lang 1986, Mosmann 1983).

**Cell proliferation assay reagents:**
B6SUt cells
Spleen cell conditioned medium PWM-SCCM (STEMCELL TECHNOLOGIES INC. TERRY FOX LABORATORY. cat. no. HCC-2100)
Hybridoma Serum Free Medium (H-SFM, Gibco BRL cat. no. 12045-019)†
MTT ((3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Sigma cat. no. M-2128)‡
FCS (Fetal Bovine Serum, Gibco BRL cat. no. 26140-038)‡
PBS (phosphate buffered saline)
Insulin (Bovine Pancreas) (Sigma cat. no. I-5500)‡
holo-Transferrin (Human) (Sigma cat. no. T-4778)‡
BSA (Albumin Fraction V) (Boehringer Mannheim cat.no. 735 094)‡
$NaHCO_3$ (BDH Chemicals cat. no. K92564)‡
100X Antibiotics (pen./strep.) (Gibco BRL cat. no. 15145-014)
rmSLF (positive control) (R&D systems cat. no. 455-MC)‡

**1) Grow B6SUtA cells to one day past maximum density.** Approximately 5 X $10^6$ cells $mL^{-1}$. Cells should be split to a ratio of approximately 1/100 every two days. If a 5 mL tissue culture flask is used, therefore, 0.5 mL of cells at maximum density can be used to seed a 5 mL flask every other day. For use in the cell proliferation assay a freshly split flask can be left for approximately three days before being used in the cell proliferation assay.

**2) Stain a sample of cells with trypan blue dye** (0.25% wt/vol)**, and count in a hemocytometer.** Cells should be at a density of approximately 5 X $10^6$ cells $mL^{-1}$, with at least 90% of the cells being viable.

**3) Wash the cells once with H-SFM.** Remove cells from suspension by spinning at approximately 400g (approximately 1200 rpm in a standard bench top centrifuge used for mammalian cell work) and re-suspend cells in H-SFM.

**4) Re-suspend cells in supplemented H-SFM (see below) at a density of approximately 1-2 X $10^5$ cells $mL^{-1}$.**

**5) Add 20 uL of test protein sample (either SLF-CBD, SLF, or $CBD_{cex}$) to the wells of a standard 96 well tissue culture plate.** [a] (Commercial rmSLF can be used as a positive control. $CBD_{cex}$ or BSA can be used as a negative control. For less accurate work B6SUt cells can be added to wells containing no test protein as a negative control.)

**6) Add 100 ul of B6SUt cells in supplemented H-SFM to the wells containing the protein test samples.**

**7) Incubate at 37°C/5% $CO_2$ for 24-48 hours.** (48 hours is recommended, but 24 hours is sufficient for less accurate work.)

**8) Quickly add 20 uL MTT solution (see below) to each well** (mixing with the tip) **and continue to incubate at 37°C/5% $CO_2$ for an additional 3 hours.** ***(Note: MTT is toxic: handle with care. See MTT Safety sheets for proper disposal protocol..)*** (The sensitivity of the assay can be increased by adding 5 uL of FCS to each well. This will have the effect of increasing the respiration level of any cells present, resulting in a greater color change. The addition of 5 uL of FCS was not seen to alter the $ED_{50}$ of the activity curve. To save time the FCS can be added to the MTT solution immediately before use. FCS should not be stored in MTT.)

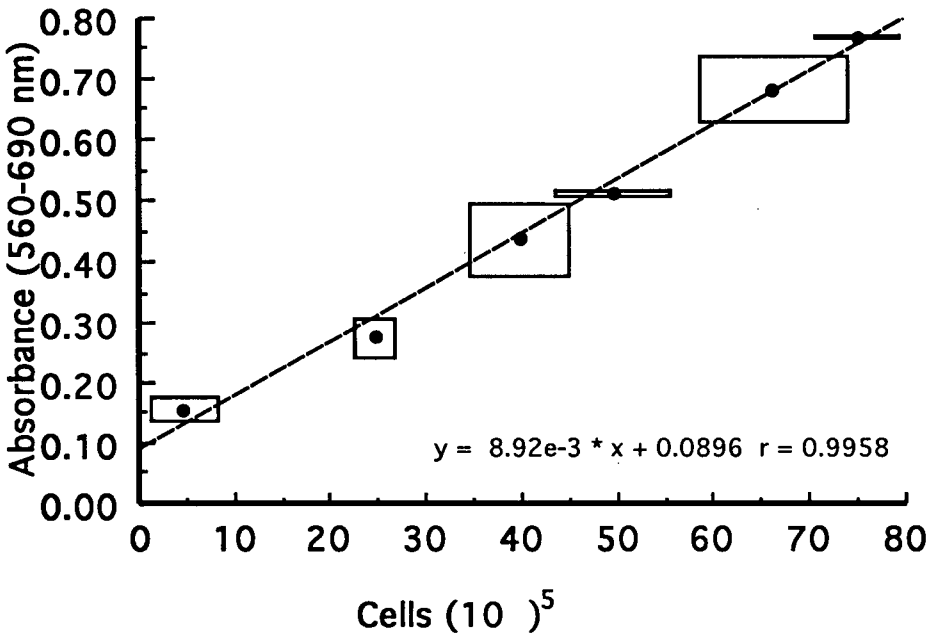
**9) Remove plate from incubator, place in a fume hood, and quickly add 120 uL of MTT solvent (see below). Mix vigorously by pipetting up and down several times** (a multichannel pipetteman is sufficient for this, provided that no solution is left in the tips when moving from one row to the next). **Leave plate for an additional 3 hours to allow MTT (formazan) to dissolve.** (For better results the mixing step can be repeated occasionally during this period.)

**10) Read Absorbance (560-690 nm) in a standard plate reader.** (560 nm is the wavelength of maximum absorbance for formazan. 690 nm was found to be the most efficient reference wavelength for use with tissue culture plates (Denizot and Lang 1986).

**Assay accuracy range:**

i) Comparisons of the MTT test with direct cell counts indicated a linear relationship between cell number and Absorbance (560-690 nm) for cell densities between $1 \times 10^5$ cells mL-1 and $5 \times 10^6$ cells mL-1 (see below).

ii) Half-maximal cell proliferative responses were found to be elicited by growth factor concentrations of approximately 100-400 pM. The assay is only recommended, therefore, for growth factor concentrations of approximately 5 to 3000 pM.




**Linear relationship between cell number and absorbance (560-690 nm) produced by MTT cleavage in the B6SUtA cell line.**

**Conversion factors:**

Molecular masses:

SLF-CBD: 34.1 kDa

SLF (R&D control cat. no. 455-MC): 18.6 kDa

SLF-CBD:

1 pM is equal to an approximate concentration of 3.4 X $10^{-5}$ ug $mL^{-1}$

2000 pM is equal to an approximate concentration of 6.8 X $10^{-2}$ ug $mL^{-1}$

5 ug $mL^{-1}$ is equal to an approximate molarity of 1.5 X $10^{5}$ pM

SLF (R&D control):

1 pM is equal to an approximate concentration of 1.9 X 10-5 ug $mL^{-1}$

2000 pM is equal to an approximate concentration of 3.8 X 10-2 ug $mL^{-1}$

5 ug $mL^{-1}$ is equal to an approximate molarity of 2.7 X $10^{5}$ pM

**Troubleshooting:**

**a)** The accuracy of the assay was found to be extremely sensitive to variations in volume. It is recommended therefore that the most accurate pipettemen be used to add samples, cells, and MTT reagents to the wells. Only p20 pipettemen should be used for small samples (less than 30 uL), and only p200 pipettemen should be used for larger samples (30-200 uL).

The use of multichannel pipettemen (8 or 12 channel) will result in less accurate results. Comblitip™ and Rainin™ devices designed to deliver

repeated amounts of a set volume were also found to introduce significant errors to the measurements. These devices, when set to deliver repeated amounts of a fixed volume, were found to deliver more than the set volume on the first delivery, and progressively smaller volumes for each subsequent delivery. This pattern of delivery (where sequentially smaller amounts of either cells, growth factors, or MTT reagents are delivered to each well) can easily be mistaken for a dose-response curve upon development. It is for this reason that the use of such devices is not recommended. Where the use of these devices cannot be avoided however (i.e.- in complicated tests, requiring the use of several plates), a strategy should be developed to counterbalance this delivery pattern. For example, cells could be added to the wells from left to right, and MTT could be added to the wells from right to left. Subsequent results should be viewed, however, as being less reliable than those for which more reliable delivery devices were used.

## Recipes:

**B6SUtA maintenance medium:**
H-SFM supplemented with 10% FCS and 5% spleen cell conditioned medium.

**Supplemented H-SFM: (500 mL) (100X stock).**
mix.
50 mL H-SFM
0.004 g Insulin
0.5 mL 100X antibiotics
0.1 g Transferrin
4 g BSA
0.2 g $NaHCO_3$

filter through 0.22 uM filter for sterilization. Dispense 5 mL aliquots into sterile 55 mL Falcon™ tubes. Store at -70°C until needed. For use, add 50 mL H-SFM. (For less accurate work 5 mL of this stock can be used to make up to 200 mL of supplemented H-SFM.)

**MTT solution:**
Make as 10 mg $mL^{-1}$ in PBS. Filter through a 0.45 uM filter to remove any insoluble MTT. (Store refrigerated, in a dark container, for no more than two weeks.)

**MTT (formazan) solvent: (200 mL).**
mix.
1.7 mL HCl
20 mL Triton X-100
Isopropanol to 200 mL.

† H-SFM was found to give better results than the following: RPMI 1640 (Gibco BRL), DMEM (Gibco BRL), ISCOVES media (with or without phenol red pH indicator), and Macrophage Serum Free Media (without phenol red pH indicator) (Gibco BRL). At the time of printing H-

SFM without phenol red pH indicator was unavailable, but media lacking phenol red is recommended for use in the MTT assay (Denizot and Lang 1986).

‡ Other sources of these reagents are also available. It may be possible to obtain more accurate results by using murine Transferrin and murine Insulin, but these were not tested in the present study.

J.G. Doheny

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