THE REGULATION OF CD44-MEDIATED ADHESION TO HYALURONAN

by

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ABSTRACT

Although the widely distributed cell surface glycoprotein CD44 has been shown to function as a receptor for the glycosaminoglycan hyaluronan (Aruffo *et al.*, 1990; Miyake *et al.*, 1990b), not all CD44 expressing cell lines are able to bind this particular ligand. This observation suggests that the hyaluronan adhesive capacity of the CD44 molecule is not determined simply by its expression on the cell surface. At present, however, the mechanisms regulating CD44-mediated adhesion to hyaluronan remain largely undefined.

Using expression cloning, a cDNA clone designated B6F1.3, was isolated that appears to "activate" the hyaluronan-binding capacity of CD44 upon transfection into the murine fibroblastoid cell line MOP8. The putative regulatory molecule encoded by this clone was found to be the murine interleukin-2 receptor γ chain (mIL-2Rγ; Cao *et al.*, 1993). Mutation within this molecule is the mechanism causing X-linked severe combined immunodeficiency (XSCID) in humans (Noguchi *et al.*, 1993). The human interleukin-2 receptor γ chain (hIL-2Rγ) was originally isolated as a component of functional IL-2 receptor complexes (Takeshita et al., 1992). More recent studies have demonstrated a link between this molecule and the cytokine receptors for IL-4 (Kondo *et al.*, 1993; Russell *et al.*, 1993) and IL-7 (Noguchi *et al.*, 1993; Kondo *et al.*, 1994). Furthermore, the receptors for the cytokines IL-9 and IL-13 also are suspected to use the IL-2Rγ chain as a functional component.

The expression of B6F1.3 was determined by Northern blot analysis. B6F1.3 mRNA was found at high levels on all tested murine hemopoietic cell lines and tissue types with the exception of the bone marrow cells in which little message was observed. Upregulation of CD44 does not seem to be the mechanism by which the "activation" of adhesion occurs as CD44 levels were apparently unaffected upon transfection of B6F1.3. Moreover, a cytoplasmic deletion mutant of B6F1.3 was not able to induce this adhesive event. These findings suggest a functional link between the IL-2 receptor complex and CD44, which may play a role in contributing to the XSCID phenotype in humans.

We further demonstrated that stable expression of the mIL-2Rγ in MOP8 cells by retroviral-mediated gene transfer not only enhanced binding of these cells to hyaluronan but also upregulated c-jun expression without affecting cell surface levels of CD44. Transient expression of the human c-jun cDNA in MOP8 cells by a plasmid-based system using pCDM8 (Seed and Aruffo, 1987) also induced adhesion to hyaluronan, again with no alteration of CD44 expression levels. These data suggest a functional link between the proto-oncogene c-jun and the adhesion protein CD44 in the signal transduction pathways regulating cellular adhesion.

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CHAPTER I

INTRODUCTION

CD44 was originally defined by Dalchau and colleagues (1980) as the broadly-distributed human cell surface glycoprotein reactive with the monoclonal antibody (mAb) F10-44-2. Many other independently characterized mAbs are now known to recognize CD44 and these have been instrumental in demonstrating an important role for this molecule in various adhesion-dependent cellular processes including lymphocyte and progenitor cell homing, tumour metastasis, lymphocyte and macrophage activation, and hemopoiesis (Haynes *et al.*, 1989; Herrlich *et al.*, 1993).

CD44 is very polymorphic, and species ranging in size from 80 to 250 kD have been detected on various normal and transformed cell types (Dalchau *et al.*, 1980; reviewed in Lesley *et al.*, 1993). CD44H, the most prevalent form expressed on the majority of resting hemopoietic cells, migrates at 80 to 90 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). cDNA clones encoding this molecule have been isolated by two independent groups (Stamenkovic *et al.*, 1989; Goldstein *et al.*, 1989). Stamenkovic and colleagues (1989) utilized a eukaryotic expression cloning strategy in which COS cells transfected with libraries prepared from the histiocytic lymphoma cell line U937, the B lymphoblastoid line JY, the Burkitt's lymphoma line Raji, and the myeloid leukemia line KG-1 were screened for reactivity with the anti-CD44 mAb J173 (Pesando *et al.*, 1986). A 1354 nucleotide cDNA terminating in a short poly(A) tail was isolated. The predicted sequence contained a single long open reading frame of 341 amino acids corresponding to a typical type I integral membrane protein (Figure 1). The extracellular domain of 248 residues is followed by 21 mostly hydrophobic amino acids, corresponding to the predicted membrane-spanning domain. The intracellular domain consisted of 72 predominantly hydrophilic residues.

Goldstein and coworkers (1989) also reported the isolation of the CD44 cDNA. In these studies, the human B lymphoblastoid cell line KOA was used as the source of poly(A)-selected RNA for the production of a cDNA library in the λgt11 expression vector. Recombinant plaques were screened using an anti-gp90^{Hermes} serum (Jalkanen *et al.*, 1987). The predicted amino acid sequence for this clone contained an open reading frame of 293 residues. This clone was identical to the CD44 clone isolated by Stamenkovic and colleagues (1989) except that it contained a truncated cytoplasmic domain consisting of only 3 amino acids.

1.1 Structure of CD44

The predicted core protein of CD44 is expected to be approximately 37 kD and can be subdivided into several domains (Zhou *et al.*, 1989; Figure 1). The transmembrane region shows greater than 80% sequence identity among different species. The CD44 protein has been demonstrated to be acidic, with estimates of isoelectric point ranging from 4.2 to 5.8 (Jalkanen *et al.*, 1988; Kalomiris and Bourguignon, 1988; Picker *et al.*, 1989a; Culty *et al.*, 1990).

1.1.1 Extracellular domain

The extracellular domain of CD44 contains several salient features including a region that appears to be important for ligand binding specificity. This N-terminal functionally important region is well conserved and will be discussed below. The extracellular domain is extensively modified with O- and N-linked glycosylation. Six potential N-linked glycosylation sites of Asn-X-Ser/Thr motif, are observed. Furthermore, the region proximal to the membrane-spanning domain demonstrates only approximately 50% sequence identity between species. This region contains four serine-glycine dipeptides that are potential sites for chondroitin

sulfate attachment. The location for the insertion of sequences generating higher molecular mass CD44 isoforms is also found proximal to the transmembrane domain and will be discussed below.

1.1.2 Cytoplasmic domain

The 72 amino acid cytoplasmic domain is highly conserved, demonstrating 80 to 90% sequence identity among species (Stamenkovic *et al.*, 1989). Seven serine residues exist in the intracellular domain of human CD44. Five of these residues are conserved in human, mouse, baboon, cow, and hamster (Stamenkovic *et al.*, 1989; Idzerda *et al.*, 1989; Zhou *et al.*, 1989; Aruffo *et al.*, 1990; Bosworth *et al.*, 1991) while four are conserved in the rat (Günthert *et al.*, 1991). The serine residue at position 296 is not phosphorylated in intact epithelial cells (Neame and Isacke, 1992) although it is a potential substrate for cAMP- and cGMP-dependent protein kinases (Wolffe *et al.*, 1990). Neame and Isacke (1992) have demonstrated that both Ser-303 and -305 may be phosphorylated and that mutation of either residue disrupts the ability of CD44 to be phosphorlyated in epithelial cells. Furthermore, Camp and colleagues (1993) have shown that a mutant form of CD44, in which the Ser-305 was changed to an alanine residue, was not phosphorylated when transiently expressed in COS cells. Another mutant form of CD44, in which the Ser-303 was altered, showed reduced phosphorylation. These data suggest that both Ser-303 and Ser-305 may be important for the phosphorylation of the cytoplasmic tail of CD44. The effect of phosphorylation on this molecule, however, is not well defined.

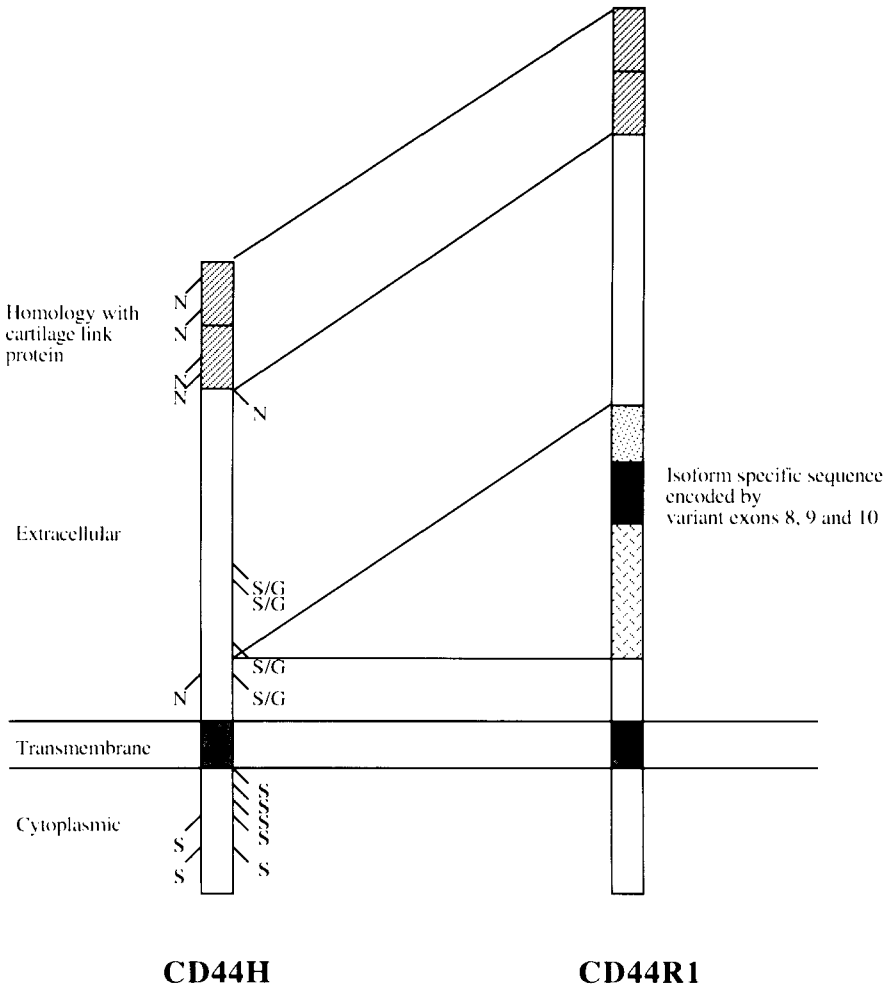

Figure 1: Structure of the CD44 protein. CD44 species range in size from 80-250 kD. The major form expressed by most resting hemopoietic cells (CD44H; left) has an apparent molecular mass of 80-90 kD. Higher molecular mass isoforms are generated by the alternative splicing of at least 10 exons producing additional peptide sequences of varying lengths that are inserted into a single site within the extracellular domain proximal to the membrane spanning domain. One particular isoform is CD44R1 (right) which contains a sequence encoded by the variably expressed exons 8, 9, and 10. The extracellular domain contains 6 potential sites of N-linked glycosylation (N), numerous sites of potential O-linked glycosylation, and 4 serine-glycine (S/G) motifs, that may serve as sites of chondroitin sulphate attachment. The cytoplasmic tail contains 7 serine residues (S) that may be important for cell signalling. The N-terminus contains a tandemly repeated domain implicated in hyaluronan binding.

1.2 Distribution and Heterogeneity of CD44

1.2.1 The Expression of CD44

Although CD44 was originally implicated in lymphocyte homing, its expression is not restricted to T and B lymphocytes. CD44 has been observed in a wide variety of tissues including the astrocytes and glial cells of the central nervous system, lung, epidermis, liver, and pancreas (Picker *et.al.*, 1989) as well as cell types such as ovarian carcinomas (Pals *et al.*, 1989), monocytes, granulocytes, fibroblasts, keratinocytes, erythrocytes and brain tissue (Haynes *et al.*, 1983; Dalchau *et al.*, 1980). Although expression levels are extremely heterogeneous, most hemopoietic cells of mouse and man express CD44 (Kansas *et al.*, 1989, 1990; Trowbridge *et al.*, 1982). In the murine system, CD44 has been demonstrated to be expressed in every hemopoietic lineage (Spangrude *et al.*, 1989; Trowbridge *et al.*, 1982).

The expression of CD44 in hemopoietic lineages, appears to be dependent upon the degree of differentiation. Murine prothymocytes capable of homing to and populating the thymus express CD44 (Spangrude *et al.*, 1989; Trowbridge *et al.*, 1982). CD44 expression is lost during T cell development upon the expression of the α chain of the interleukin-2 receptor complex (IL-2Rα; Lesley *et al.*, 1993). CD44 reappears on more mature CD4 and CD8 single-positive thymocytes (Lynch and Ceredig, 1989). In fact, murine CD44 is expressed on bone marrow prothymocytes, on 80-90% of day 13-14 fetal thymocytes, and on only about 5% of adult thymocytes (Trowbridge *et al.*, 1985). The pattern of CD44 expression in human fetal thymus is similar to that of the murine thymus (Horst *et al.*, 1990). Approximately 60% of immature $CD4^-$ $CD8^-$ $CD3^-$ human thymocytes are strongly CD44-positive (Denning *et al.*, 1989). Furthermore, most lymphocytes in the human peripheral blood express CD44 (de los Toyos *et al.*, 1989; Kansas *et al.*, 1989; Horst *et al.*, 1990).

The expression of CD44 also appears to be elevated in both memory and activated T cells. Budd and colleagues (1987a,b) demonstrated that memory cytotoxic T cell precursors

elicited in response to different antigens were CD44-positive as well as CD8+ve. This study was performed using the C57BL/6 strain of mice which express low numbers of T cells expressing CD44 in the thymus and periphery. Although CD44 appears to be a good marker for memory T cells, the use of this antigen is restricted to mouse strains that express low levels of CD44-positive cells in mature thymus and peripheral T cell populations (Lynch and Ceredig, 1989). In C57BL/6 mice, the expression of CD44 was observed to be elevated in helper memory T cells, defined by markers such as low CD45RB and low L-selectin expression (Butterfield *et al.*, 1989; Swain *et al.*, 1990). Furthermore, the expression of CD44 appeared to be responsive to activation state. Human T cells activated *in vitro* were observed to express more CD44 (de los Toyos *et al.*, 1989; Oppenheimer-Marks *et al.*, 1990; Haegel and Ceredig, 1991). Haegel and Ceredig (1991) demonstrated that stimulating T cells with either mitogens or antigens increased surface expression of CD44. Furthermore, three major mRNA species of approximately 4.5, 3.5, and 1.6 kb are observed (Haegel and Ceredig, 1991).

The expression pattern of CD44 during B cell development is similar to that of maturing T cells. CD44 expression on CD10-positive immature B cells isolated from human bone marrow was observed to be low. Subsequently, the level of CD44 is upregulated on CD20 positive mature B cells (Kansas and Dailey, 1989). Furthermore, the level of CD44 on B cells is influenced by activation state. B cells activated with either LPS or anti-IgD-dextran demonstrated an upregulation of the CD44 expression (Hathcock *et al.*, 1993).

1.2.2 Higher Molecular Mass CD44 Isoforms

Biochemical studies have suggested that multiple isoforms of CD44 exist (Kansas *et al.*, 1989). These higher molecular mass isoforms may potentially arise by posttranslational modification such as chondroitin sulphate attachment or glycosylation of the extracellular domain. A 180 to 200 kD form observed on some lymphocytes was determined to be sensitive to chondoitinase ABC treatment (Jalkanen *et al.*, 1988), therefore implicating chondroitin

sulfate attachment as a mode of generating higher molecular mass isoforms. Furthermore, other forms are generated by extensive glycosylation in which more than half of the apparent molecular mass in accounted for by N- and O-linked carbohydrate addition.

1.2.3 CD44 Isoforms by Alternative Splicing

The production of higher molecular mass CD44 species cannot be attributed solely to posttranslational modifications of a common polypeptide core. Numerous CD44 variants have been reported to be generated by alternative splicing, which results in the insertion of peptide sequences of varying lengths into a single site within the extracellular domain proximal to the membrane spanning domain (Dougherty *et al.*, 1991; Stamenkovic *et al.*, 1991; He *et al.*, 1992; Screaton *et al.*, 1992; Günthert *et al.*, 1991; Figure 1). Furthermore, these inserted amino acid sequences are produced by the alternative splicing of a contiguous series of exons present within a single copy CD44 gene (Figure 2). This finding was facilitated by the determination of the structure of the human CD44 genomic gene (Cooper *et al.*, 1992; Screaton *et al.*, 1992), which is located on the short arm of chromosome 11. To date, 12 of the 20 exons that make up the CD44 gene have been observed to be alternatively spliced. Ten exons within the extracellular domain and 2 exons within the cytoplasmic domain, are alternatively utilized (Screaton *et al.*, 1992; Figure 2). Moreover, one example has been found in which a "constant" exon (exon 16) was deleted (Günthert *et al.*, 1991). Thus it is clear that a great number of different CD44 isoforms could potentially be generated by this alternative splicing mechanism. Whether every possible splice variant is translated into a mature protein is unclear, however, numerous species have been confirmed by immunoprecipitation and/or Western blot analysis (Dougherty *et al.*, 1991; Stamenkovic *et al.*, 1991; He *et al.*, 1992; Screaton *et al.*, 1992; Günthert *et al.*, 1991). Moreover, the alternative splicing mechanism may allow for the production of soluble CD44 by

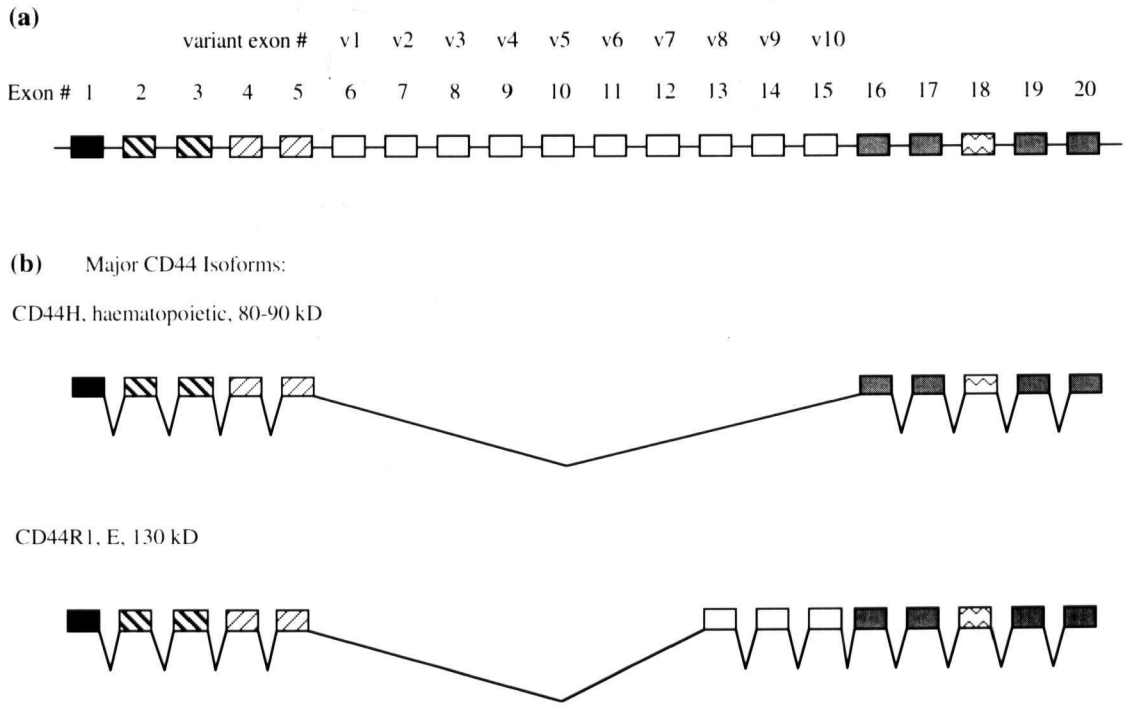

Figure 2: The genomic organization of the CD44 gene. To date, the human genomic CD44 structure is known to consist of 20 exons. Exon 1 encods the leader peptide whereas exon 2 and 3 represent the putative hyaluronan binding domain. The exons 6 through 15 correspond with the variant exons 1 to 10. These exons can be alternatively spliced to generate higher molecular mass CD44 isoforms. The isoform CD44R1 containing variant exons 8, 9, and 10 is also shown. Exons 19 and 20 encode the cytoplasmic domain with exon 18 forming the transmembrane domain. The exons and introns are not drawn to scale.

introducing potential protease cleavage sites within the variant peptide sequences (Dougherty *et al.*, 1991; He *et al.*, 1992) It has been reported that arginine dipeptides are introduced into human CD44 isoforms (Dougherty *et al.*, 1991). In mice, one similar site is located proximal to the transmembrane domain in all known splice variants, and two sites exist in high molecular mass variants (He *et al.*, 1992).

1.3 CD44 and the Extracellular Matrix

1.3.1 General Features of the Extracellular Matrix

Most cells in multicellular organisms are in contact with a complex network of interacting, extracellular macromolecules that constitute the extracellular matrix (ECM). The components of the ECM were originally recognized to function mainly as a relatively inert scaffolding that stabilized the physical structure of tissues. More recently, it has been realized that the ECM plays an active and complex role in regulating the function of cells that come into contact with the ECM structures. The ECM has been implicated in a variety of cellular functions including cell adhesion, migration, proliferation, and differentiation (reviewed in Toole, 1990).

The macromolecules that make up the ECM are secreted by a variety of cells, including fibroblasts and also chondroblasts in cartilage and osteoblasts in bone. Two of the main constituents of the ECM are collagenous proteins and proteoglycans (Lindenhal and Höök, 1978). The latter are usually several polysaccharide glycosaminoglycans generally covalently linked to a protein core.

A major component of the ECM in some tissues is the glycosaminoglycan hyaluronan. Hyaluronan exists as a single, very long carbohydrate chain of sugar residues in a regular, repeating sequence of D-glucuronic acid (1-β-3) and *N*-acetyl-D-glucosamine (1-β-4) (reviewed in Toole, 1990; Laurent and Fraser, 1992; Figure 3). The polymer can have a molecular weight of 400 to 8 million. In solution, hyaluronan behaves as a random coil

(Laurent and Fraser, 1992). A large quantity of solvent is trapped within the coil and the molecule can be considered to be a highly hydrated sphere (Laurent and Fraser, 1992). Hyaluronan is strongly negatively charged due to its content of carboxyl groups. Hyaluronan, however, differs from the typical glycosaminoglycans in three key features. First, the others tend to contain a number of different disaccharide units in a complex arrangement. Furthermore, typical glycosaminoglycans have fewer than 300 sugar residues. Finally, hyaluronan appears not to be covalently linked to a protein core, although it is frequently found attached to the surface of mesenchymal cells via appropriate hyaluronan-binding proteins (Knudson *et al.*, 1993).

Hyaluronan appears to play an important role in regulating cell behavior. Oligosaccharide degradation products of hyaluronan (3 to 16 dissacharides) have been demonstrated to be able to stimulate cell proliferation (West and Kumar, 1989). This observation contrasts with the finding that higher molecular mass hyaluronan polymers have been shown to be inhibitory to cell growth (West and Kumar, 1989).

Lymphoma cells and macrophages can be induced to homotypically aggregate following the addition of hyaluronan even at low concentrations. Divalent cation-independent cellular aggregation can be inhibited by treatment with hyaluronidase or large quantities of soluble hyaluronan. Cell lines that display hyaluronan-dependent homotypic aggregation appear to do so by crosslinking of the hyaluronan receptors on adjacent cells with hyaluronan (Toole, 1990). The aggregation of lymphocytes that is mediated by hyaluronan, can be blocked with antibodies directed against CD44 (Lesley *et al.*, 1990). The work of Lesley and coworkers (1990) suggested that CD44 mediated both hyaluronan-dependent self-aggregation and the binding of soluble hyaluronan to some lymphoid cell lines. Studies by St. John and colleagues (1990) further supported this idea. This latter group demonstrated that a fibroblast cell line transfected with a CD44 cDNA could be induced to homotypically aggregate. These studies support the role for hyaluronan in a variety of cell functions. The role that hyaluronan

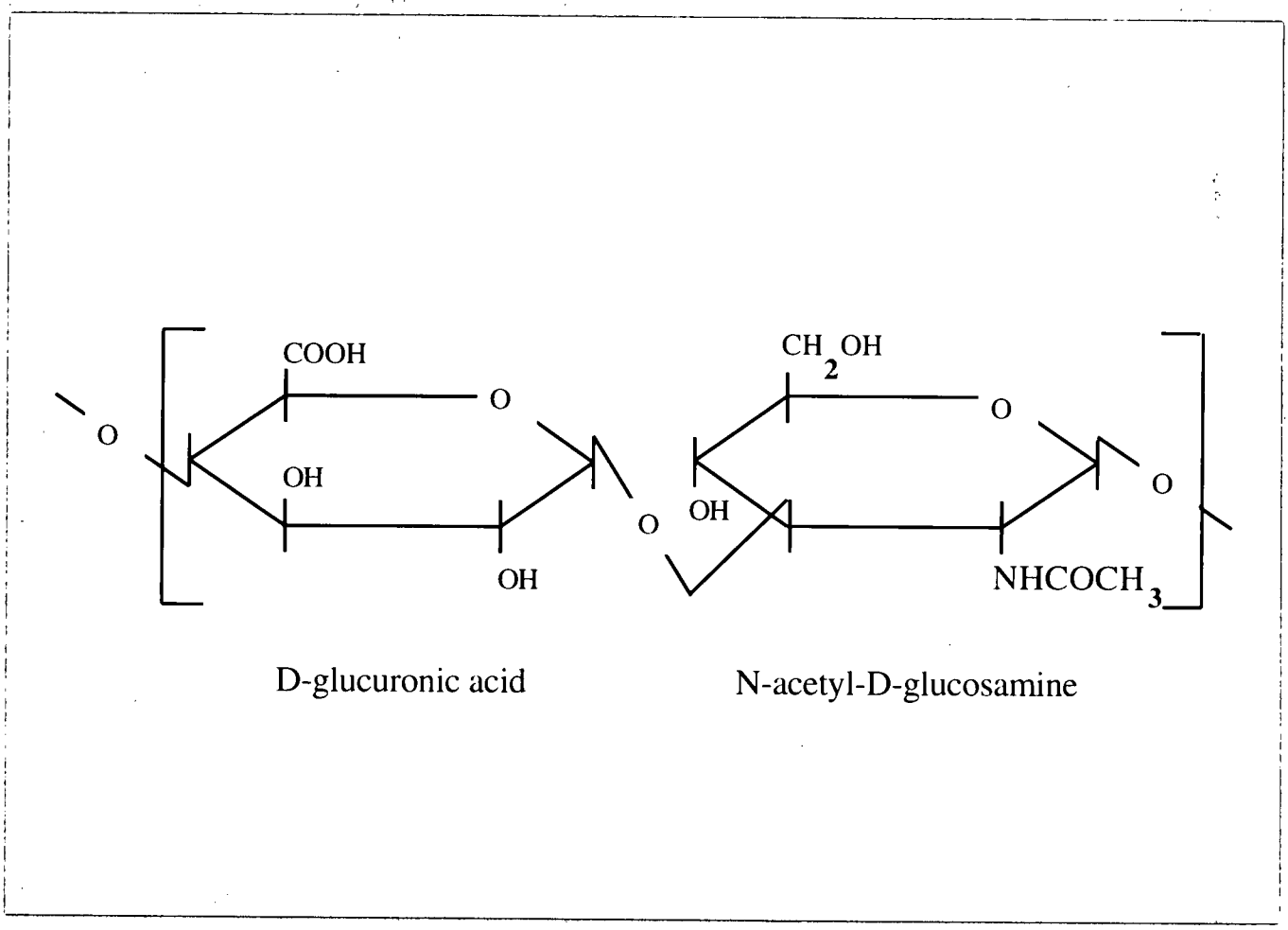

Figure 3: Structure of hyaluronan. Hyaluronan is a simple glycosaminoglycan consisting of alternating residues of D-glucuronic acid and N-acetyl-D-glucosamine. It has a molecular weight of up to several million, and in solution forms an extended random coil that can trap large quantities of solvent.

plays in these functions is, itself, regulated by at least three aspects: the size and concentration of hyaluronan and the affected cell type (reviewed in Toole, 1990).

The synthesis of hyaluronan appears to be regulated by various growth factors (Heldin *et al.*, 1989). Hyaluronan production takes place at the inner face of the plasma membrane. The newly generated hyaluronan remains bound to the hyaluronan synthetase during production and is extruded through the plasma membrane (Ng and Schwartz, 1989). Many cellular growth factors including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β), appear to influence hyaluronan synthesis at least in fibroblasts (Heldin *et al.*, 1989). Furthermore, cells have been observed to be arrested in mitosis if hyaluronan synthesis is inhibited (Toole, 1990). Recently, bacterial hyaluronidase has been cloned from *Streptococcus pneumoniae* (Berry *et al.*, 1994). The clone contained an open reading frame sufficient to encode a polypeptide with an approximate molecular weight of 108 kD. However, an active hyaluronidase was purified with an approximate molecular weight of 89 kD (Berry *et al.*, 1994).

1.4 CD44 Adheres to Hyaluronan

1.4.1 Sequence of CD44 Suggests a Potential Role in Hyaluronan Recognition

A region of the extracellular domain of CD44, residues 12-101 in humans, demonstrates greater than 80% sequence identity between species. Sequence comparisons indicate that this region includes a tandemly repeated domain with low but significant homology (approximately 30% sequence identity) to the second (B) subdomain of cartilage proteoglycan core and link proteins (Goldstein *et al.*, 1989; Stamekovic *et al.* 1989; Idzerda *et al.*, 1989; Wolffe *et al.* 1990; Doege *et al.*, 1991). Further comparisons demonstrated that this region is present within a number of other proteins that recognize and bind HA, including aggrecan, versican, and a

recently described molecule, designated TSG-6, that is induced in fibroblasts by TNF-α or IL-1β, and in peripheral blood mononuclear cells by mitogen stimulation (Stamenkovic *et al.*, 1989; Goldstein *et al.*, 1989; Lee *et al.*, 1993). More limited sequence homology is also observed to RHAMM, a 58 kD surface receptor that plays an important role in hyaluronan-mediated cellular migration events (Hardwick *et al.*, 1992). This domain containing 6 cysteine residues may potentially be linked by disulphide bonds to form a single globular domain (Goldstein *et al.*, 1989).

Recently, Yang and colleagues (1994) determined that only two hyaluronan binding domains exist in the hyaluronan receptor, RHAMM. Furthermore, these two binding domains contributed approximatedly equally to the hyaluronan binding ability of RHAMM. The first binding domain contained two sets of two basic amino acids, each spaced seven residues apart. Mutations of these basic residues decreased the ability of RHAMM to bind to hyaluronan (Yang *et al.*, 1994). Mutational analysis of the second binding domain implicated Lys-423 and Arg-431, spaced seven amino acids apart, as critical for hyaluronan binding (Yang *et al.*, 1994). This group suggested that the minimal requirement for hyaluronan binding activity is two basic residues flanking a seven amino acid stretch, a motif found in all hyaluronon binding proteins to date including CD44 and cartilage link protein. Site-directed mutations of these motifs in CD44 sequences abolished hyaluronan-binding activity (Yang *et al.*, 1994).

1.4.2 Evidence for Hyaluronan as a Ligand for CD44

Many independent research groups have demonstrated by transfection studies that the ligand for CD44 is hyaluronan. One such group showed that the expression of human CD44H in the CD44-negative human Burkitt B cell lymphoma line Namalwa allowed the transfectants to bind to lymph node high endothelial cells (Aruffo *et al.*, 1990). This adhesion event was inhibited by a polyclonal antisera specific for CD44, the addition of excess soluble hyaluronan or pretreatment of the target cells with the enzyme hyaluronidase. Furthermore, hamster CD44

transfected into COS cells reacted with an antibody directed against the hamster fibroblast hyaluronan receptor (Aruffo *et al.*, 1990).

Lesley and coworkers (1992) studied the alteration of adhesion of a murine T cell hybridoma line designated AKR1 after transfection of the cell line with a murine CD44 cDNA. The transfectants acquired the ability to bind immobilized hyaluronan and fluorescein-conjugated hyaluronan from solution. This binding was inhibited by unconjugated hyaluronan or pretreatment with a mAb directed against CD44.

To study the adhesion of CD44 to hyaluronan further, a chimeric protein in which the extracellular domain of human CD44 was fused with the hinge domains (C_H2 and C_H3) of human IgG_1, was generated (Aruffo *et al.*, 1990). This soluble CD44-immunoglobulin fusion protein adhered to lymph node high endothelial cells when expressed in COS cells and this interaction was abolished in the presence of hyaluronan but not other glycosaminoglycans. Furthermore, the binding of the CD44-immunoglobulin fusion protein was inhibited by the pretreatment of the high endothelial cells with hyaluronidase but not enzymes that do not digest hyaluronan.

1.4.3 Features of CD44 Important for HA binding

Recent studies on cartilage link protein have suggested that the interaction of CD44 with hyaluronan is largely ionic in nature. Furthermore this binding is mediated by negatively charged carboxyl groups on hyaluronan and clusters of positively charged basic residues in the protein (Jackson *et al.*, 1991). Using deletion analysis, Peach and colleagues (1993) have localized the hyaluronan-binding domain of CD44 to the first 186 amino acid residues of the molecule. Unlike the remainder of the extracellular domain, this region is highly conserved among different mammalian species (>90% identity between mouse and man) (Wolffe *et al.*, 1990; Nottenburg, *et al.*, 1989). The fact that this amino-terminal region is involved in the binding of hyaluronan has been inferred from the sequence identity this domain shares with

regions contained in other hyaluronan binding proteins. Importantly, the region defined by Peach and coworkers (1993) contains two short stretches of 13 amino acids that include 3 and 4 positively charged arginine or lysine residues, respectively. Although only the first of these basic amino acid clusters appears to be present in other hyaluronan-binding proteins (Stamenkovic *et al.*, 1989; Goldstein *et al.*, 1989; Lee *et al.*, 1993), site-directed mutagenesis studies suggest that both contribute to the hyaluronan-binding ability of CD44, with the arginine residue at position 41 being particularly important (Peach *et al.*, 1993). It has been suggested that only residues 18-30 and 88-112 are likely to be exposed at the surface (Lesley *et al.*, 1993). Furthermore, an antibody generated against the peptide consisting of residues 18-30 was not able to interfere with the binding of CD44 to hyaluronan, thus suggesting that these residues are not critical for hyaluronan interaction (Lesley *et al.*, 1993).

1.5 Regulation of CD44-mediated Adhesion to Hyaluronan

Although CD44 is necessary for hyaluronan binding, there is not a one-to-one correlation between the expression of CD44 on the cell surface and the ability of cells to bind hyaluronan.

1.5.1 Evidence for Regulation of Hyaluronan Binding

The evidence that the interaction of CD44 with hyaluronan was regulated first came from observations of the binding abilities of murine hemopoietic cell lines expressing CD44. Many cell lines such as the CD44-positive T cell lines SAKRTLS 12, EL4 and the B cell lines 70Z/3 and RAW 253 were not able to bind hyaluronan (Lesley *et al.*, 1990). Lesley and coworkers (1990) further demonstrated that the inability to bind hyaluronan in these cell lines was not due to a masking of the hyaluronan receptor activity by endogenous hyaluronan. This conclusion was made following the observation that treatment of the nonbinding cell lines with hyaluronidase or chondroitinase ABC does not recover the hyaluronan binding capacity of the

cell lines (Lesley *et al.*, 1990). These data suggest that the adhesive function of CD44 is not regulated simply by the expression of the molecule, and that other regulatory mechanisms must exist.

Some T cell lines could be induced to bind hyaluronan following treatment with phorbol ester (Lesley *et al.*, 1990). The cell lines used express CD44H and after stimulation, did not demonstrate an isoform shift. Furthermore, the induction required several hours at 37°C. The expression level of CD44 increased upon stimulation, leading to the idea that upregulation of CD44 by phorbol ester is the mechanism that regulates the adhesive function. This notion is discounted by the observations of Hyman and colleagues (1991) by studying two variants of the SAKRTLS 12 cell line. One such variant was selected by fluorescence-activated cell sorting for high CD44 expression whereas the other was selected for hyaluronan binding. Interestingly, the variant expressing high levels of CD44 was unable to bind hyaluronan. Furthermore, the hyaluronan binding variant expressed lower levels of CD44 than the CD44^{hi} variant (Hyman *et al.*, 1991). Thus the adhesive function of CD44 can be induced by phorbol esters and the upregulation of CD44 does not appear to be the mechanism by which the enhancement of binding occurs.

The hyaluronan binding capacity of CD44 can also be induced by treatment with certain mAbs directed against CD44. Lesley and coworkers (1992) demonstrated that mAb IRAWB 14 can "activate" the hyaluronan binding of several T cell lines including SAKRTLS 12 and EL4. Unlike the induction seen with phorbol ester, the mAb treatment was rapid and occurred at 0°C (Lesley and Hyman, 1992). Moreover, Fab fragments of the inducing antibodies were unable to mimic the effects of whole antibodies, suggesting that crosslinking of the CD44 protein on the cell surface is required.

1.5.2 Other Adhesion Molecules that Require Activation

The failure of many cell lines expressing high levels of CD44 to adhere to hyaluronan (Hyman *et al.*, 1991; Lesley *et al.*, 1990) suggests that CD44 is one of many cell adhesion molecules known to require activation to most efficiently bind its ligand (Dustin and Springer, 1991; Hynes, 1992; Altieri *et al.*, 1988; Graham and Brown, 1991; Neugebauer and Reichardt, 1991; Spertini *et al.*, 1991). Examples include L-selectin (Spertini *et al.*, 1991) and many of the integrins such as LFA-1, Mac-1/CR3, and the platelet integrin gpIIb-IIIa (Dustin and Springer, 1991; Hynes, 1991).

L-selectin is an adhesion molecule that belongs to a family of proteins that contain a lectin-like domain. It can recognize and bind to carbohydrate ligands. L-selectin is expressed on the surface of neutrophils and lymphocytes but its binding function can be activated by stimuli such as G-CSF, GM-CSF, and tumor necrosis factor (TNF)-α (Spertini *et al.*, 1991). The corresponding molecule found on lymphocytes can also be stimulated by crosslinking the T cell receptor or CD2. This enhancement of the function of L-selectin occurs in a matter of minutes and does not involve the upregulation of expression of the molecule.

LFA-1 (CD11a/CD18) is expressed on T lymphocytes and is critically important for the interaction between helper T cells and antigen-presenting cells or between cytotoxic T cells and target cells (Dustin and Springer, 1989; van Kooyk *et al.*, 1989). The counter-receptors for this dimeric protein were determined to be intercellular cell adhesion molecule (ICAM)-1, ICAM-2, ICAM-3 and ICAM-4 (Marlin and Springer, 1987; Staunton *et al.*, 1989; de Fougerolles and Springer, 1992). One important means by which the interaction between LFA-1 and ICAM-1 is regulated is by inducing the expression of ICAM-1 upon inflammation (Dustin and Springer, 1988; Dustin *et al.*, 1988). ICAM-1 is induced on a wide variety of cells by inflammatory regulator molecules such as interleukin-1 (IL-1), TNF-α, γ-interferon (IFN), and lipopolysaccharide (reviewed in Springer, 1990). The upregulation of ICAM-1 expression is transcriptionally mediated and is first observed after 4 hours (Dustin *et al.*,

1988). However, cytotoxic T cells can interact with the appropriate target cells, deliver a lethal hit, and release the effected target in a far shorter time frame of 1 to 5 minutes (Poenie *et al.*, 1987). Thus, other regulatory mechanisms appear to be involved in cytotoxic T cell engagement. The LFA-1-dependent adhesive function of T lymphocytes can be stimulated by the crosslinking of the T cell receptor. Activation of resting T lymphocytes with either mAbs against the T cell receptor or by phorbol ester treatment transforms cellular LFA-1 from a low to a high avidity state, with no alteration in the surface expression or distribution (Dransfield and Hogg, 1989; van Kooyk *et al.*, 1989; Dustin and Springer, 1989; Figdor *et al.*, 1990). Furthermore, a mAb directed against LFA-1 designated NK1-L16 can also activate LFA-1 dependent binding in some T cells (Keizer *et al.*, 1988). It has also been proposed that cells regulate the adhesive function of LFA-1 by way of interactions between the cytoplasmic domain of LFA-1 and cytoskeletal elements (Hibbs *et al.*, 1991).

Another regulated integrin is the platelet integrin gpIIb-IIIa (CD41/CD61). On resting circulating platelets, this adhesion molecule is unable to bind its soluble ligand; however, it can adhere to immobilized fibrinogen. Platelet integrin gpIIb-IIIa can bind to soluble fibrinogen following treatment of the platelet with phorbol ester. Moreover, this activation of the molecule appears to involve intracellular signalling (Shattil and Brass, 1987). The adhesive function of this molecule can also be activated by treatment with certain mAbs directed against this integrin (O'Toole *et al.*, 1990) or by binding of fibrinogen (Du *et al.*, 1991). The activation of this adhesion molecule by the mAb does not appear to involve crosslinking of the proteins on the platelet cell surface as O'Toole and coworkers (1990) demonstrated that solubilized integrin could be induced to bind soluble ligand upon stimulation with Fab fragments. Furthermore, mAbs have been developed that recognize the activated molecule but not the molecule on resting cells (Shattil *et al.*, 1985). These data suggest that the adhesive property of gpIIb-IIIa is induced by mechanisms that cause a conformational change in the molecule. As with LFA-1, the regulation of platelet integrin appears to also involve intracellular interaction of the cytoplasmic domain of this molecule (O'Toole *et al.*, 1991).

Mac-1 (CD11b/CD18) is a β2 integrin also known as complement receptor 3 (CR3). It is expressed on monocytes, macrophages, granulocytes, large granular lymphocytes, and immature and CD5+ B cells (de la Hera *et al.*, 1988) This adhesion protein has been demonstrated to bind the complement component, C3bi (Beller *et al.*, 1982). When expressed on monocytes and neutrophils, the adhesive function of Mac-1 is rapidly induced by phorbol esters (Wright and Silverstein, 1982). This activation process is thought to involve the microclustering of the molecules on the cell surface (Detmers *et al.*, 1987). Similar to the platelet integrin, the activation of Mac-1 appears to involve a conformational shift as mAbs can be generated specifically against the activated form (Altieri and Edgington, 1988).

1.6 Potential Mechanisms Regulating the Adhesive Function of CD44

The adhesive function of CD44 does not appear to be regulated simply by expression of the protein. Other potential regulatory mechanisms may exist to control the binding of CD44 to its ligand, hyaluronan. These mechanisms may include interactions between the cytoplasmic domain and cytoskeletal components, conformational changes, different binding affinities for alternate CD44 isoforms, masking or shedding of the CD44 on the cell surface, or interaction with other regulatory molecules. Two or more of these regulatory pathways may also function in concert with each other or with other unknown mechanisms.

1.6.1 Involvement of the Cytoplasmic Domain in the Regulation of CD44-Ligand Interactions

The evidence that the cytoplasmic domain of CD44 may be involved in regulating the adhesion of CD44 to hyaluronan first came from deletional mutagenesis studies (Lesley *et al.*, 1992; Thomas *et al.*, 1992). A mutant CD44 protein was generated lacking all of the cytoplasmic domain except for the first six amino acid residues. Wild-type CD44 expressed in a CD44-negative T lymphoma line designated AKR1, was able to bind hyaluronan. The truncated

mutant expressed in the same cell line, however, was unable to bind soluble hyaluronan but did adhere to immobilized hyaluronan (Lesley *et al.*, 1992). Further studies by this group demonstrated that the mutant CD44 could be induced to bind hyaluronan by treatment with mAb IRAWB directed against CD44 (Lesley *et al.*, 1992). The use of Fab fragments of this mAb were unable to alter the adhesive function of CD44. These data suggest that aggregation of CD44 on the cell surface is important for the binding of hyaluronan, and that this clustering may involve the cytoplasmic domain of CD44.

Thomas and coworkers (1992) performed a similar study in which a cytoplasmic deletion mutant was transfected into melanoma cells. These transfectants were unable to bind immobilized hyaluronan whereas cells transfected with full-length CD44 bound avidly. The two studies suggest that the cytoplasmic domain of CD44 may be important for the regulation of CD44 adhesive function. Potentially, the cytoplasmic domain may regulate the cell surface distribution of CD44 or may alter the extracellular conformation into a state that can bind hyaluronan.

The cytoplasmic domain of CD44 has been demonstrated to bind cytoskeletal elements. Kalomiris and Bourguignon (1988) studied this interaction and showed that purified CD44 can bind to purified erythrocyte ankyrin. Furthermore, this group demonstrated that the cytoplasmic domain of CD44 can be phosphorylated *in vitro* by protein kinase C isolated from brain tissue, and that the addition of phosphate groups enhanced the ability of CD44 to bind ankyrin (Kalomiris and Bourguignon, 1989). The correlation between phosphorylation and the affinity of CD44 for cytoskeletal elements is, however, not absolute as Camp and colleagues (1991) observed that cytoskeletally-associated CD44 found in the detergent (Nonidet P-40) insoluble fraction of murine peritoneal macrophages was not phosphorylated, whereas non-cytoskeletally-associated detergent-soluble CD44 was phosphorylated.

Neame and Isacke (1992) studied the role of the cytoplasmic domain and phosphorylation in the localization of CD44 to the basolateral membrane of polarized epithelial (MDCK) cells. The localization of CD44 in these cells appeared to be regulated by the

intracellular domain, as cytoplasmic deletion mutants expressed in these cells demonstrated a scattered and unclustered distribution. Furthermore, point mutations were generated in which two serines residues (Ser-303 and Ser-305) implicated as targets of phosphorylation were replaced by either alanine or glycine. The CD44 distribution in cells transfected with the mutant constructs appeared normal, suggesting that cytoplasmic phosphorylation is not important for localization.

The interaction of CD44 with cytoskeletal elements is also affected by the cellular activation state. In human peripheral blood leukocytes, Geppert and Lipsky (1991) observed that following treatment with phorbol myristate acetate (PMA), the percentage of CD44 associated with the cytoskeleton decreased.

To date, the importance of cytoskeletal interaction and phosphorylation on the interaction between CD44 and hyaluronan remains largely undefined. Intracellular molecules however have been observed in other systems to regulate adhesive function. Pullman and Bodmer (1992) used a mammalian expression cloning system which enriched for collagen type I binding to isolate a regulator of integrin adhesive function. The cDNA clone isolated was designated cell adhesion regulator or CAR. This clone encoded a protein of 142 amino acids which contained an N-terminal myristoylation motif suggesting a cytoplasmic sub-membrane location for the protein. Moreover, this molecule had a consensus tyrosine-kinase phosphorylation site at the C-terminus. Site directed mutagenesis removing this tyrosine residue abolished the ability to enhance cell-matrix binding (Pullman and Bodmer, 1992).

1.6.2 Involvement of Extracellular Modification in the Regulation of CD44-Ligand Interactions

A wide variety of glycosylation patterns on the CD44 molecule have been observed in different human cell lines (Brown *et al.*, 1991; Jalkanen *et al.*, 1988). Changes in glycosylation pattern are influenced directly by the generation of alternative CD44 species as isoform specific additional peptide sequences often contain potential glycosylation signals.

Other adhesion molecules have also been found to be regulated by glycosylation of their extracellular domains. Diamond and colleagues (1991) have suggested that the extent of glycosylation on ICAM-1 may regulate adhesion to LFA-1 or Mac-1. Mutations in ICAM-1 that destroy consensus sequences for N-linked glycosylation enhanced binding to purified Mac-1 (Diamond *et al.*, 1991). Furthermore, Calvete and coworkers (1993) have demonstrated modification of the glycosylation pattern of boar spermadhesin served to modulate its ligand-binding capacity. This group observed that glycosylated boar spermadhesin was unable to bind seminal-plasma protease inhibitors as well as zona pellucida glycoproteins due to the presence of an oligosacharide chain on a conserved asparagine residue. Non-glycosylated forms of spermadhesin bind avidly to the appropriate ligands (Diamond *et al.*, 1993). Two groups showed an important role for glycosylation in the adhesive-function of human CD2 (Recny *et al.*, 1992; Parish *et al.*, 1993). The T-lymphocyte glycoprotein receptor, CD2, mediates cell-cell adhesion by recognizing and binding to the cell surface molecule, CD58 (LFA-3). Recny and colleagues (1991) demonstrated that high mannose oligosaccharides attached to Asn-65 on the CD2 molecule were required for CD2-CD58 interaction. To date, no evidence has been reported linking glycosylation of CD44 with altered adhesive-function. However, the fact that other adhesion molecules can be regulated in this manner suggests that this form of post translational modification may also regulate CD44.

1.6.3 Involvement of CD44 Isoforms in the Regulation of CD44-Ligand Interactions

The role of higher molecular mass CD44 isoforms in hyaluronan binding is controversial with numerous conflicting reports to date. CD44E, the major isoform expressed by the colon carcinoma cell line HT29 (Stamenkovic et al., 1991) was found to be unable to recognize and bind hyaluronan. This group further suggested that isoform specific sequences were responsible for the variable adhesive functions of CD44 (Stamenkovic *et al.*, 1991).

Conversely, the studies of He and colleagues (1992) suggest that the murine homologue of CD44E, although it contains a 132 amino acid insertion, is able to mediate attachment to hyaluronan upon transfection into a CD44-negative T lymphoma cell line. CD44R1, an isoform differing from CD44E by just 3 amino acid substitutions, was isolated from the human myelomonocytic leukemia cell line KG1a (Dougherty *et al.*, 1991). This isoform can, however, bind avidly and specifically to hyaluronan, whether expressed on the surface of transfected COS7 cells or as a soluble chimeric protein fused in-frame to the enzyme alkaline phosphatase (Dougherty *et al.*, 1994). These data suggest an important role for one or more of these amino acids in hyaluronan binding. One of the amino acid substitutions in CD44E (tyrosine at position 109) is present within the region of CD44 implicated in hyaluronan binding (Peach *et al.*, 1993). It is important to note that the serine residue found at this position in CD44R1 is also found at this position in all other human and animal CD44 cDNAs reported to date. The tyrosine at this position may explain the inability of CD44E to bind hyaluronan. However, the other amino acid substitution may be involved as well, perhaps to alter the conformation of CD44.

1.6.4 Involvement of CD44 Masking or Shedding in the Regulation of CD44-Ligand Interactions

The reduction of CD44 on the cell surface by shedding of the extracellular domain is a potentially rapid mechanism by which the function of the molecule may be regulated. Treatment of human neutrophils with TNF-α, PMA, calcium ionophore, and formyl-Met-Leu-Phe (fMLP) for 30 minutes downregulated the expression of CD44, presumably by proteolytic cleavage as this reduction was blocked by protease inhibitors (Campanero *et al.*, 1991). Shedding of CD44 also appears to be involved in the downregulation of CD44 on granulocytes following stimulation with PMA or ionomycin for 12 hours (Bazil and Horejsí, 1992). Shedding seems to be the mechanism by which CD44 is downregulated as a lower molecular

mass (compared to cell surface CD44) ^{125}I-labeled molecule reactive with mAbs directed against CD44 could be isolated from supernatants of ^{125}I-surface labeled cells. Shedding of cell surface molecules as a mechanism of regulating expression is not restricted to CD44. Other hemopoietic molecules downregulated following stimulation are CD23, CD6, L-selectin, TNF-α receptor, CD14, ICAM-1, and CD32 (reviewed in Lesley *et al.*, 1993).

1.6.5 Involvement of Cell-Specific Molecules in the Regulation of CD44-Ligand Interactions

There is increasing evidence that the functional activity of adhesion proteins can be regulated by interactions in the plane of the membrane with other cell surface molecules. Dougherty and coworkers (manuscript in preparation) demonstrated that the proteins defined by mAbs belonging to CD43 and CD44 co-localize with ICAM-1 (CD54) to uropods and sites of cell-cell contact on KG1a, a human myelomonocytic cell line that spontaneously forms large homotypic aggregates *in vitro*. In addition, mAbs directed against ICAM-1 (CD54), and an epitope present on a subpopulation of CD44 molecules, inhibited the homotypic aggregation of KG1a cells, suggesting a functional association between CD44 and ICAM-1 (CD54) in this adhesive interaction.

1.7 Cellular Functions Dependent on CD44

Several independent lines of investigation have implicated the adhesion protein CD44 in numerous functional roles in a variety of cell types. The adhesion-dependent cellular functions that involve CD44 include T cell activation, cell adhesion, lymphocyte recirculation, cell migration, hemopoeisis, and tumour metastasis. The role of CD44 in a selection of these processes will be discussed.

1.7.1 The Role of CD44 in Lymphocyte Homing

Lymphocytes circulate throughout the body in the constant process of immune surveillance. These immune cells migrate though the bloodstream, move into lymphoid organs and other tissues, and then enter the lymphatics to return to the circulatory system (Yednock and Rosen, 1989; Shimizu *et al.*, 1992). This process of lymphocyte homing and recirculation allows the full repertoire of antigenic specificities to be continuously represented throughout the body.

The tissues of the immune system can be functionally subdivided into primary, secondary, and tertiary lymphoid organs (reviewed in Picker and Butcher, 1992). Production of functional lymphocytes occurs in the primary tissues including the bone marrow and thymus. The antigen-mediated proliferation and differentiation of antigen-specific lymphocytes occurs in the secondary tissues to which the lymph node, Peyer's patch, and spleen belong. The tertiary lymphoid tissues consist of all other tissues of the body and represent sites for antigen restimulation of memory lymphocytes and effector precursor cells.

Lymphocytes exit the bloodstream and migrate into secondary lymphoid tissues via adhesion to a specialized endothelium designated high endothelial venules (HEV; reviewed in Woodruff *et al.*, 1987). Lymphocytes have the ability to distinguish between HEV of the lymph node and Peyer's patch (Hill *et al.*, 1977). The ability of circulating lymphocytes to specifically recognize and bind to HEV can be measured using an *in vitro* assay developed by Stamper and Woodruff (1976). This assay system used frozen sections of lymphoid tissues to which lymphocyte populations were added. The L-selectin mAb was defined using this assay system (Gallatin *et al.*, 1983). This mAb, which was later determined to recognize murine L-selectin (Lasky *et al.*, 1989), was able to block murine lymphocyte and lymphoid cell line adhesion to HEV. A cell surface marker on human lymphocytes designated Hermes antigen was crossreactive with the L-selectin mAb and thus named "lymphocyte homing receptor". Subsequently, mAbs generated against the Hermes antigen were found to recognize human CD44 (Gallatin *et al.*, 1989; Picker *et al.*, 1989; St. John *et al.*, 1990). The Hermes series of

antibodies (Hermes-1, -2, and -3) were developed by Jalkanen and colleagues (1986). Of the three antibodies that recognize CD44, only one (Hermes-3) can block human lymphocyte adhesion to HEV frozen sections (Jalkanen *et al.*, 1987). This antibody was not, however, effective in blocking the hyaluronan-dependent adhesion of a B cell line transfected with human CD44 to cultured rat endothelium (Stamenkovic *et al.*, 1991). Furthermore, the adhesion of murine lymphocytes to HEV is not susceptible to hyaluronidase treatment or to mAbs known to inhibit hyaluronan binding by CD44 (Culty *et al.*, 1990). These data support CD44's involvement in lymphocyte homing but CD44-hyaluronan interaction may not participate in lymphocyte adhesion to HEV.

1.7.2 The Role of CD44 in Lymphopoiesis

Miyake and colleagues (1990) demonstrated that mAbs directed against CD44 were able to completely block B cell lymphohemopoeisis in long-term bone marrow cultures. These antibodies were originally isolated for their ability to inhibit a B cell line to adhere to a cloned stromal cell line. Further analysis demonstrated that CD44 on the B cell line recognized hyaluronan on the surface of the stromal cells (Miyake *et al.*, 1990). Although the mechanism by which CD44 affects hemopoiesis is not well defined, this group has demonstrated an important role for this adhesion protein in this particular function.

1.7.3 The Role of CD44 in T Cell Activation

Several groups have demonstrated that the addition of anti-CD44 antibodies augments both CD2- and CD3-mediated T cell activation (Denning *et al.*, 1990; Huet *et al.*, 1989; Shimizu *et al.*, 1989; Rothman *et al.*, 1991; Seth *et al.*, 1991; Tan *et al.*, 1993; Pierres *et al.*, 1992). Seth and coworkers (1991) have demonstrated that the CD44-specific antibody 9F3 could trigger the lytic activity of cytotoxic T lymphocytes. The antibody H90 developed by Huet and

coworker (1989) was able to inhibit the adhesion of lymphocytes to HEV. Furthermore, H90 could enhance [^{3}H]TdR incorporation of peripheral blood lymphocytes when a primary stimulus of CD2 mAb or CD3 mAb linked to plastic culture plates was used. H90 however had no effect on [^{3}H]TdR incorporation when peripheral blood lymphocytes were stimulated with lectins, allogeneic cells, or CD3 mAb in the soluble phase. Denning and colleagues (1989) have demonstrated that the addition of antibodies against CD44 to purified T cells resulted in a 25-fold increase of anti-CD2-mediated T cell IL-2 secretion. The interaction of antibodies with CD44 was believed to mimic the possible effects of ligand binding to CD44 on stimulation through other receptor-ligand interactions such as T cell receptor binding to antigen or triggering via CD2 or CD3 surface molecules. The primary stimulus in the described studies however is often suboptimal. A contrasting study demonstrated that the anti-CD44 mAb 212.3 could completely inhibit T cell proliferation stimulated by the CD3-specific antibody OKT3 (Rothman *et al.*, 1991). The study further showed that this inhibition of CD3-mediated T cell activation was not caused by a reduction of cell viability. It is however associated with an inhibition of IL-2 production and receptor expression, and a reduction of OKT3-mediated increase in intracellular Ca^{2+} levels. This antibody was not able to inhibit T cell activation resulting from stimulation by the T cell mitogens phytohemagglutinin or pokeweed mitogen. A more recent study demonstrated that intracellular cAMP is rapidly increased following treatment with this anti-CD44 antibody (Rothman *et al.*, 1993). This elevation of cAMP is not dependent on activation state and is not observed with non-inhibitory mAbs against CD44. Paul-Eugene and colleagues (1992) also demonstrated an upregulation of cAMP in 20 to 30 minutes using anti-CD23 mAb to stimulate CD23 on CD23+ve monocytes. Rothman *et al* (1993) suggested that CD44 may be directly coupled to adenylate cyclase as the elevation of cAMP required only 1 to 2 min in T cells. Lokeshwar and Bourguignon (1992) characterized CD44 as a GTP-binding protein with GTPase activity in *in vitro* assays. Interestingly, the binding of GTP significantly enhanced the interaction of purified CD44 with ankyrin (Lokeshwar and Bourguignon, 1992).

The proliferation of peripheral blood T cells induced by CD2 antibodies appears to be dependent upon monocytes (Denning *et al.*, 1989). Furthermore, Denning and colleagues (1990) suggested that increasing the adhesion of either LFA-1 to ICAM-1 or CD2 to LFA-3 leading to a stronger interaction between T cell and monocyte may be the mechanism by which CD44 may enhance T cell activation. This study demonstrated that the CD44 antibodies A3D8 and A1G3 enhance CD2-mediated T cell triggering by binding to monocytes and augmenting monocyte-T cell adherence, by inducing monocyte IL-1 release, and by binding to T cells and stimulating T cell release of IL-2 (Denning *et al.*, 1990). The treatment of human T cells with antibodies against CD44 have been demonstrated to induce LFA-1-dependent homotypic aggregation (Pals *et al.*, 1989 Koopman *et al.*, 1990) that is sensitive to both 1-O-alkyl-2-O-methyl glycerol-3 phosphocholine (AMG) and 1-(5-isoquinolinyl sulfonyl-2 methyl piperazide) treatment, both of which prevent protein kinase C activation. Furthermore, cytochalasin B also inhibits this activation. These data suggest that protein kinase C activation and cytoskeletal interaction are critical for the activation of the LFA-1 pathway via CD44 (Koopman *et al.*, 1990).

1.7.4 The Role of CD44 in Tumour Metastasis

The most life threatening aspects of the oncogenic process are tumour invasion and metastasis. Metastasis is a complex process that consists of a cascade of sequential inter-dependent stages involving multiple host-tumour interactions (Fidler and Hart, 1982; Schirrmacher, 1985; Fidler 1990). Metastatic cells must first be able to exit from the primary tumour site, invade the local host tissue and enter the bloodstream or lymphatics. Once in the circulation, the variant cell or group of cells must be able to survive within this harsh environment and arrest in a distant vascular bed. Upon adhesion to a secondary site, the cells must then exit the vasculature and colonize in an ectopic organ site.

Studies have demonstrated an important role for hyaluronan in tumour cell migration. Knudson (1990) has demonstrated that carcinomas are commonly associated with local accumulation of hyaluronan. Tumour cells may directly release hyaluronan or may induce nearby fibroblasts to secrete hyaluronan. The free hyaluronan may be incorporated into the extracellular matrix (Yoneda *et al.*, 1988; Knudson and Knudson, 1991) and may function to separate tissues, partially degrade the collagenous fibrillar framework, or affect adhesive forces between the cells and their substrate (Docherty *et al.*, 1989).

Continued tumour cell growth requires that tumour cells secrete angiogenic factors that promote the vascularization of the tumour mass. The degradation of the extracellular matrix and in particular hyaluronan is thought to be important for angiogenesis (Blood and Zetter, 1990). West and colleagues (1985) have suggested that the degradation products of hyaluronan promote angiogenesis. The study by this group demonstrated that 4 to 25 disaccharide degradation products of hyaluronan can induce the formation of blood vessels on the chick chorio-allantoic membrane.

The evidence supporting the role for CD44 in tumour invasion and metastasis is mounting. Birch and coworkers (1991) demonstrated that clones of the human melanoma cell line LT5.1 expressing high levels of CD44 gave 10 to 20 times the number of lung colones in nu/nu mice than clones expressing low levels of CD44. CD44 has been demonstrated to function in the internalization and degradation of hyaluronan (Culty *et al.*, 1992). An analysis of 107 cases of non-Hodgkin's lymphoma of various histologic and immunophenotypic subclasses revealed a correlation between CD44 expression and the degree of dissemination (Pals *et al.*, 1989). Furthermore, the expression of CD44 on non-Hodgkin's lymphoma is related to the clinical stage, tumour spread, and poor response to treatment (Horst *et al.*, 1990; Jalkanen *et al.*, 1991).

The correlation between the expression of CD44 and tumour growth appears to be important. Many studies have demonstrated the expression of CD44 in human tumour cells (Picker *et al.*, 1989; Stamenkovic *et al.*, 1989; Dougherty *et al.*, 1991; Jackson *et al.*, 1992;

Koopman *et al.*, 1993). Conversely, Shtivelman and Bishop (1991) have demonstrated that the expression of CD44 is downregulated in the neuroblastoma cell, NBM.

Isoforms of CD44 also appear to play a role in metastasis. Gunthert and colleagues (1991) demonstrated that a variant form of CD44 designated pMeta-1 is expressed in the metastasizing rat pancreatic carcinoma cell line BSp73 and in the mammary adenocarcinoma 13762NF. This isoform is not however expressed in the nonmetastasizing BSp73AS cells, in other nonmetastatic derivatives of the same parental tumour or in most normal rat tissues (Gunthert *et al.*, 1991). The overexpression of this isoform in the nonmetastasizing BSp73AS cells confers full metastatic ability. Koopman and colleagues (1993) reported that this alternatively spliced isoform of CD44 contained sequences encoded by variant exon 6 (v6). Furthermore they demonstrated that many aggressive non-Hodgkin's lymphomas express a v6-containing CD44 isoform. This particular isoform is expressed at low levels on normal resting lymphocytes, whereas it is transiently expressed on activated lymphocytes (Koopman *et al.*, 1993; Arch *et al.*, 1992). It is interesting to note that antibodies specific for the peptide encoded by the v6-containing variant CD44 sequences can inhibit *in vivo* activation of both B and T cells (Arch *et al.*, 1992). Seiter and colleagues (1993) demonstrated that an antibody (1.1ASML) specific for peptide sequences determined by v6 can inhibit the growth of lymph node and lung metastases. This retardation of metastatic behavior does not result from a 1.1ASML-dependent downregulation of this isoform or an activation of an immune response (Seiter *et al.*, 1993). They suggest that this mAb interferes with the interaction between the tumour cells and other cells and/or the extracellular matrix (Seiter *et al.*, 1993).

Sy and colleagues (1991) demonstrated that two isoforms of CD44 have distinct effects on tumour growth *in vivo*. The two CD44 forms studied were CD44H and a higher molecular mass species CD44E that does not mediate adhesion to hyaluronan (Stamenkovic *et al.*, 1989, 1991). The cDNAs encoding these isoforms were stably expressed in the human Burkitt lymphoma Namalwa and injected into nu/nu mice. Only the transfectants expressing the CD44H form greatly enhanced both local tumour formation and metastatic capacity (Sy *et al.*,

1991). The *in vivo* tumour formation resulting from CD44H transfectants can be suppressed by treatment of the mice with a soluble human CD44H-immunoglobulin fusion protein (Sy *et al.*, 1992). The mechanism by which this inhibition occured was not known, however, this group suggested that binding of hyaluronan may be involved (Sy *et al.*, 1992).

As all CD44 isoforms except CD44E appear to bind hyaluronan, this adhesive event may not play a critical role in tumour metastasis. The ability of CD44 and the higher molecular mass isoforms to bind other ligands may be important in this regard. To this end, cell mixing studies have suggested that homotypic aggregation is mediated by the adhesive interactions between a determinant encoded by the inserted region present in CD44R1, and a common region shared by both CD44R1 and CD44H (Droll *et al.*, submitted). Cells transfected with CD44R1 were able to homotypically aggregate whereas cells expressing CD44H were not. Furthermore, cells transfected with CD44H were able to aggregate with cells transfected with CD44R1. Moreover, mAb blocking studies suggested that the determinant recognized by CD44R1 was located in a region of the CD44 molecule distinct from that involved in hyaluronan binding. The evidence discussed in this section supports the notion that CD44 and its isoforms are involved in tumour formation and metastasis. However, the mechanism by which CD44 affects this function is not well defined.

1.8 Thesis Objective

The major objective of this thesis was to identify and characterize molecules that may potentially play an important role in regulating the hyaluronan-binding function of CD44. Using an expression cloning strategy, several putative regulatory molecules were isolated. The structure and distribution of one of these molecules was determined which made possible further studies to begin to define the mechanism used by this molecule to regulate the function of CD44. The purpose of these studies was to provide a better understanding of the mechanisms that regulate the function of CD44.

CHAPTER II

MATERIALS AND METHODS

2.1 Cell Culture and Monoclonal Antibodies

2.1.1 Established Cell Lines

The Polyoma-transformed murine fibroblastoid cell line MOP8 (Muller *et al.*, 1984) was obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA). It was grown in Dulbecco's minimum essential medium (DMEM; Stem Cell Technologies Inc., Vancouver, Canada) supplemented with 10% fetal clone I (FCI; Hyclone Laboratories, Logan, UT, USA). The IL-3-dependent murine mast cell line B6SUtA (Greenberger *et al.*, 1983) was cultured in RPMI-1640 medium (Stem Cell Technologies Inc.) containing 20% fetal calf serum (FCS; Hyclone Laboratories), 5×10^{-5} M 2-mercaptoethanol, and 10% pokeweed mitogen-stimulated spleen cell-conditioned medium.

2.1.2 Monoclonal Antibodies

Hybridomas producing the mAbs TIB 241 and TIB242 were obtained from the ATCC. Both of these mAbs are directed against the hyaluronan binding domain of murine CD44 (Miyake *et al.*, 1990).

2.2 DNA Isolation and Analysis

2.2.2 Isolation of Plasmid DNA

Plasmid DNA was isolated using a modified alkaline lysis procedure. Bacterial cultures were grown overnight in a 14 ml polypropylene snap-cap tube (Falcon, Lincoln Park, New Jersey, USA) containing 5 ml of LB broth supplemented with the appropriate antibiotic in a shaking incubator at 37°C. The cultures were centrifuged for 5 min at 5000 rpm using a JA-21 rotor. The bacterial pellet was suspended in 200 μl of GTE buffer (50 mM glucose (BDH Inc., Toronto, Ontario), 25 mM (hydroxymethyl) aminomethane (Tris; Gibco BRL, Gaithersburg, MD, USA) pH 8.0, 10mM ethylenediaminetetraacetic acid (EDTA; Sigma Chemical Co., St. Louis, MO, USA). Following an incubation period of 5 min. at room temperature, a 200 μl mixture of freshly prepared 1% sodium dodecyl sulphate (SDS; BDH Inc.) and 0.2 M NaOH (BDH Inc.) was added. After leaving the mixture on ice for 5 min., 300 μl of 7.5 M potassium acetate (BDH Inc.) was added. After incubating on ice for 10 min., the mixtures were centrifuged for 3 min. at 10000 rpm using the JA-21 rotor. The clear supernatant was removed to a 1.5 ml Eppendorf tube containing 0.6 volumes of isopropanol (usually 500 μl; BDH Inc.). Plasmid DNA was isolated by precipitating on ice for 10 min. and microfuging at 15000 rpm for 10 min. The DNA pellet was washed with 70% ethanol and resuspended in 100 μl TE buffer (10 mM Tris pH 7.5/1 mM EDTA) containing 100 μg/ml RNase A (Sigma) and 500 units/ml RNase T1 (Boehringer Mannheim, Germany). The typical yield of was approximately 1 μg of plasmid DNA.

Higher quality and quantity of DNA was isolated by a large scale DNA preparation protocol as follows. 500 ml of bacterial culture was grown overnight at 37°C. The culture was transferred into two 250 ml centrifuge bottles and centrifuged at 5000 rpm for 5 min. using the JA-14 rotor. The pellets was resuspended in 40 ml 10 mM EDTA and pooled into one 250 ml centrifuge bottle. 80 ml of freshly prepared 1%SDS/0.2 M NaOH was added

followed by the addition of 40 ml 5 M potassium acetate (2.5 M KOAc/2.5 M HOAc). The mixture was shaken semivigorously and centrifuged at 5000 rpm for 5 min. The supernatant was filtered through a cheesecloth into a clean 250 ml centrifuge bottle. The bottle was then filled with isopropanol and the nucleic acids allowed to precipitate for 10 min. at room temperature. The DNA pellet was isolated by centrifugation in the JA-14 rotor at 10 krpm for 10 min. The pellet was carefully rinsed with 70% EtOH and allowed to air dry. The DNA was resuspended in an appropriate volume of autoclaved water (100 - 500 μl depending on yield). The typical yield from this protocol is 500 μg.

2.2.3 Restriction Enzyme Digestion

The restriction enzymes used in this study were purchased from Bethesda Research Laboratories (Gibco BRL, Burlington, Ontario). The recommended conditions, including React buffers, for each restriction enzyme were used. Typically, 1 μl of a 100 μl mini-prep was digested with 0.5 μl enzyme (5 units) in a total reaction volume of 10 μl for 1 hr at 37°C for restriction analysis. For the isolation of DNA fragments and probes, approximately 10 μg DNA was digested with 3 μl (30 units) enzyme for a single enzyme digestion or 2 μl of each enzyme (20 units) for a double enzyme digestion. In either situation, the incubation period was 1 hr. at 37°C.

2.2.4 Gel Electrophoresis and DNA Fragment Isolation

After the addition of 1/10 volume of loading dye (0.25% bromophenol blue (Bio-Rad Laboratories, Richmond, CA, USA), 0.25% xylene cyanol (BDH Inc.) and 50% glycerol (BDH Inc.), the digested DNA was loaded into the wells of a 1% (w/v) agarose gel prepared in 1 x TAE buffer (40 mM Tris, 20 mM sodium acetate (BDH Inc.), and 1 mM EDTA, pH 7.2). Typically, electrophoretic runs would last for 1 - 2 hr. at a constant voltage setting ranging

from 80 - 120 V. DNA fragments were isolated from the agarose gels by Geneclean (BIO/CAN Scientific, Mississauga, Ontario).

2.2.5 cDNA clones

pCDM8.CD44H (clone 2.7) and pCDM8.CD44R1 (clone 2.3) encode respectively the 90 kD CD44H and 130 kDa CD44R1 isoforms of CD44 (Dougherty *et al.*, 1991). pCDM8.cjun was generated by isolating a full length NotI/HindIII human c-jun fragment from pUC18.cjun and subsequently ligating this into pCDM8. The pUC18.cjun and CD44 clones were generous gifts from Dr. Bill McBride, UCLA and Dr. Graeme Dougherty, Terry Fox Laboratory, respectively.

2.3 RNA Isolation and Analysis

2.3.1 RNA Isolation

RNA was isolated from various primary tissues and cultured cell lines by guanosine isothyocyanate (GIT) solublization method (Greenberger *et al.*, 1983). To isolate RNA from murine tissues, mice were sacrificed and various organs removed. Each organ was finely minced using a razor blade and forced through a wire mesh into a 50 ml tube with approximately 20 ml Hanks' balanced salt solution (HBSS; Stem Cell Technologies Inc.). This tissue sample was washed twice with HBSS and resuspended in 7.5 ml of GIT buffer containing 4 M GIT (Gibco BRL), 25 mM sodium acetate pH 6, and 0.84% (v/v) β-mercaptoethanol (Sigma). The GIT solublized samples were stored in a 15 ml polypropylene tube at -70°C until ready to use. To isolate RNA from cultured cells, the cells were harvested and approximately 5 x 10^7 cells were placed in a 15 ml polypropylene tube with 7.5 ml of GIT buffer and stored at -70°C.

When ready to isolate RNA, the frozen solublized tissue and cell samples were rapidly thawed and then carefully layered over 4 ml of CsCl buffer containing 5.7 M CsCl (Gibco BRL), and 25 mM sodium acetate pH 6 in a 14 x 89 mm Polyallomer centrifuge tube (Beckman). The samples were ultracentrifuged using an SW41 rotor at 32000 rpm and 20°C. After 21 hours of ultracentrifugation, the samples were taken out of the rotor and the supernatant carefully removed. The RNA pellet was then resuspended in 300 µl of 0.3 M sodium acetate and transfered to a 1.5 ml microfuge tube. The RNA was precipitated with 750 µl ethanol and stored at -70°C overnight. The RNA was then pelleted by microfuging for 10 min. The supernatant was removed and the pellet washed with 300 µl of 80% ethanol. The RNA was resuspended in autoclaved distilled water, quantitated by O.D. at 260 nM and stored at -70°C.

2.3.2 Northern Blotting

10 µg of total RNA were electrophoresed through a 1% (w/v) agarose gel containing 5% (v/v) formaldehyde (BDH Inc.), transfered to a Zeta-Probe blotting membrane (Bio-Rad) and crosslinked by exposure to ultraviolet irradiation. Filters were prehybridized for 2 hour at 42°C in 50% (v/v) formamide (Sigma), 5% SDS, 500 mM NaH_2PO_4 (BDH Inc.), pH 7.2, 1 mM EDTA and 1 mg/ml bovine serum albumin (BSA), and then hybridized for 16 hours at 42°C in the same solution denatured probe. ^{32}P-labelled probes were prepared using the Ready-To-Go DNA Labelling Kit from Pharmacia (Baie d'Urfé, Quebec). The probes used in these studies were a full length B6F1.3 fragment prepared from pCDM8.B6F1.3 by partial digestion with XbaI or a full length murine c-jun probe removed from pUC18.cjun using NotI and HindIII. Filters were washed at 55°C for 30 min twice with a solution of 2 x SSPE/0.3% SDS, twice with 1 x SSPE/0.5% SDS and finally two times with 0.3 x SSPE/1% SDS. Solutions containing SSPE are diluted from a 20 x stock containing 3 M NaCl (BDH Inc.), 0.3 M NaH_2PO_4 and 20 mM Na_2EDTA, pH 7.4. To confirm that approximately equal

amounts of RNA were loaded in each lane, filters were stripped and reprobed with 1.8 kb actin probed produced by Pst-I digestion of plasmid pA1-actin (Greenberger *et al.*, 1983).

2.4 cDNA Cloning and Sequencing

2.4.1 cDNA Library

The cDNA library was prepared by Dougherty and colleagues (1989). Using the GIT solublization method (Greenberger *et al.*, 1983), total cellular RNA was isolated from the B6SUtA cell line. An oligo(dT)-cellulose column was used to select poly(A)$^+$ mRNA. The cDNA was then synthesized according to the method of Gubler and Hoffman (1983) and non-self-complementary BstXI oligonucleotide linkers (Seed and Aruffo, 1987) were added. Larger than 1 kb cDNAs were size selected on a continuous 5-20% potassium acetate gradient. The pooled cDNAs were ligated into the BstXI-digested pCDM8 expression vector (Seed and Aruffo, 1987), and transformed into competent *Escherichia coli* strain MC1061/p3. The resulting library consisted of approximately 5 x 10^5 recombinants and had a mean cDNA insert size of 2 kb.

2.4.2 Isolation of Clone B6F1.3

The pCDM8-based cDNA library prepared using mRNA isolated from B6SUtA (Dougherty *et al.*, 1989) was introduced into MOP8 by spheroplast fusion as previously described (Greenberger *et al.*, 1983). Following 48 h incubation to allow replication of introduced plasmids and expression of encoded protein products, the transfected cells were harvested by brief incubation with phosphate-buffered saline (PBS; Stem Cell Technologies Inc.) containing 2.5 mM EDTA. Transfected cells in which the adhesive function of CD44 was "activated" were enriched by panning on 10 cm plastic Petri dishes that had been coated by overnight incubation

at 4°C with potassium hyaluronate isolated from human umbilical cord (5 mg/ml in PBS; Sigma). Plasmid DNA was recovered from hyaluronan-adherent cells using a modified alkaline lysis procedure (Kay and Humphries, 1991), transformed back into *E. coli* strain MC1061/P3. Following two rounds of selection, a clone (designated B6F1.3) demonstrating significant enhancement of CD44-inhibitable MOP8 adhesion to hyaluronan, was isolated. Figure 4 shows an outline of this protocol.

2.4.3 DNA Sequencing

Restriction fragments of the cDNA clone, B6F1.3 were subcloned into pUC19 and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using denatured double stranded templates and T7 DNA polymerase (Sequenase, United States Biochemical Corp., Cleveland, OH, USA).

2.5 Functional Analysis of cDNA clones

2.5.1 MOP8 Cell Transfection

2.5.1.1 Transient Expression Using pCDM8

MOP8 were transfected with either pCDM8 (Figure 5), pCDM8.CD44 cl 2.2 encoding human CD44H (Dougherty *et al.*, 1991), cDNA clone B6F1.3 in pCDM8, or B6F1.3ΔSH2 in pCDM8 (described below) by electroporation using the Bio-Rad Gene-Pulser system (Bio-Rad). Briefly, cells were trypsinized and resuspended in cold PBS at a final concentration of 1 x 10^7 cell/ml. 0.4 ml aliquots were mixed with 2.5 μg of plasmid DNA, transferred to a 0.4 cm cuvette and electroporated at 280 volts with a capacitance setting of 250 μF. The time constants obtained ranged from 11.3 to 11.9 msecs. After electroporation, cells were incubated

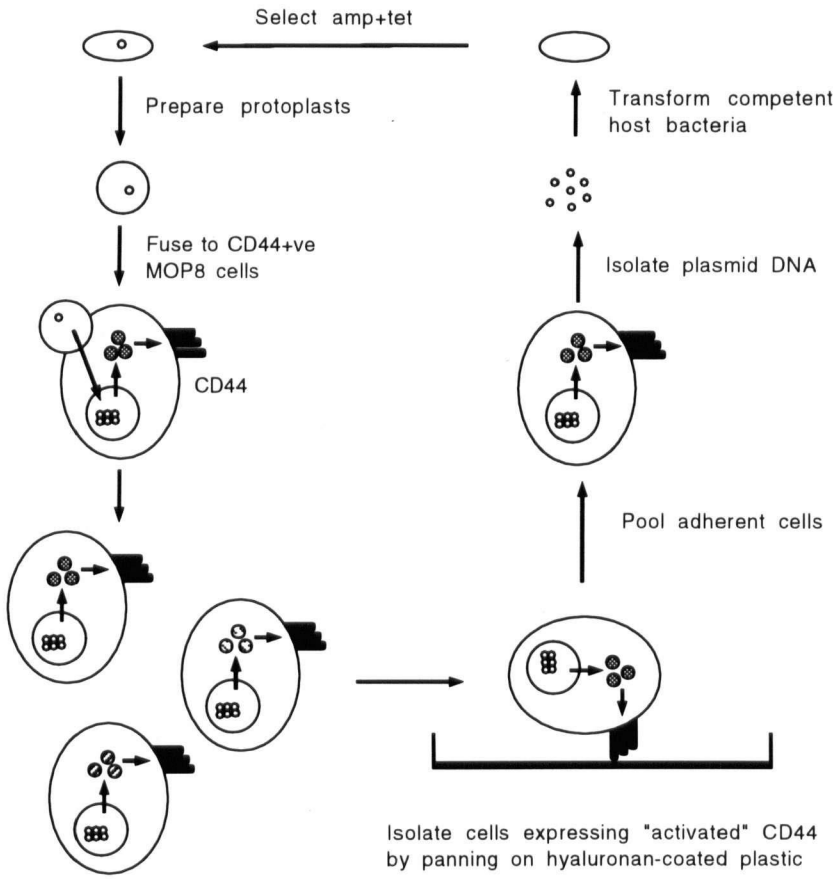

Figure 4: Experimental approach used to clone regulators of CD44-mediated adhesion to hyaluronan.

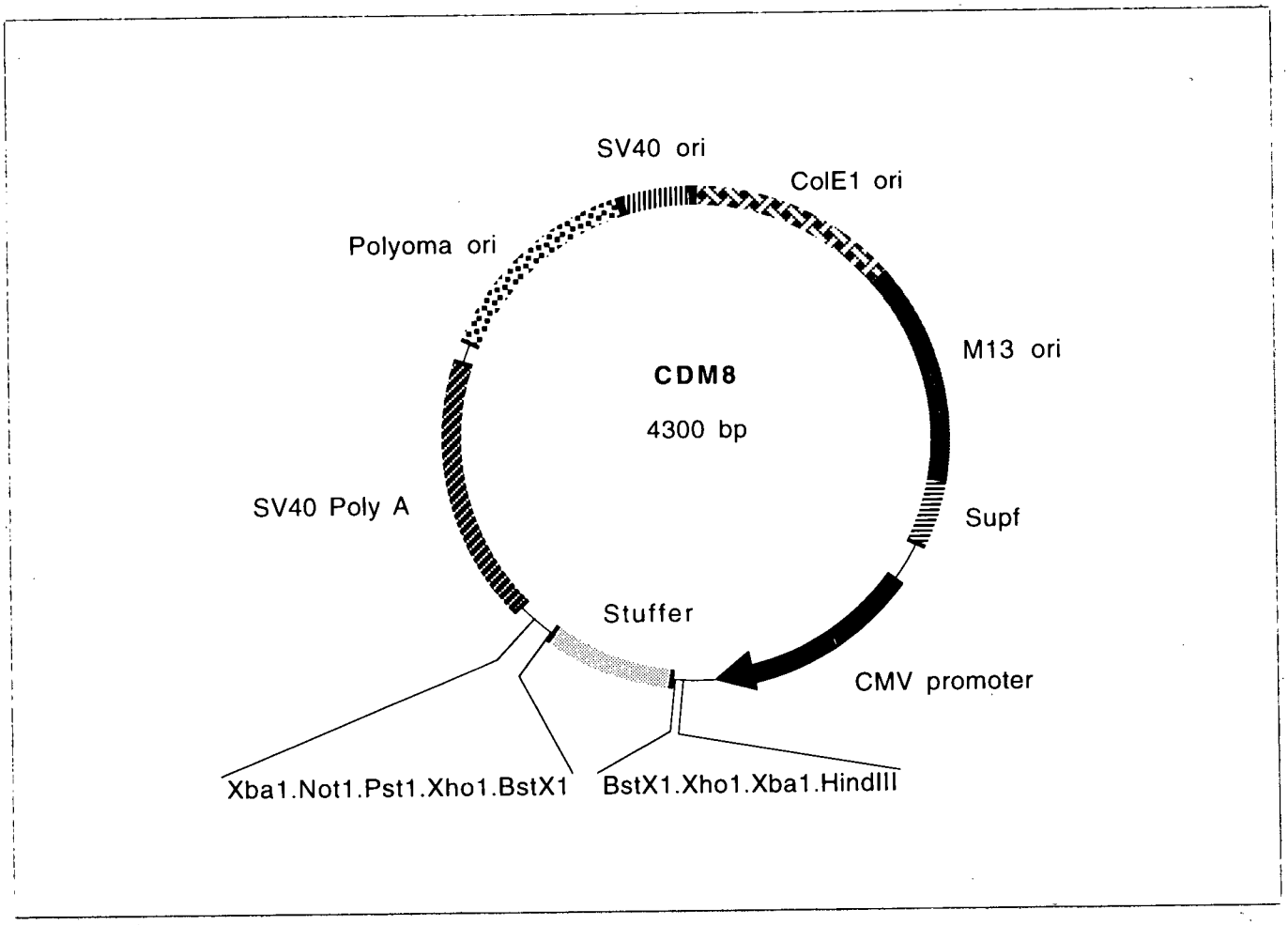

Figure 5: A schematic representation of the pCDM8 vector. This expression vector is used to transiently express cDNAs in the MOP8 cells. The basic features of this shuttle vector are an SV40 origins of replication, SV40 splice and poly A addition signals, a synthetic tyrosine suppressor tRNA gene (sup F) for selection, a human cytomegalovirus promoter, and a stuffer fragment with polylinker sites. The cDNA of interest replaces the stuffer fragment and upon transfection into MOP8 cells, the construct replicates extrachromosomally via the SV40 origin. Transcription of the cDNA is driven off the CMV promoter.

on ice for 5 minutes, then diluted in 30 ml DMEM + 10% FCI, plated in a 15 cm Integrid tissue culture dish (Falcon, Lincoln Park, NJ, USA) and incubated for 3 days to allow replication and expression of the introduced cDNAs.

2.5.1.2 Retroviral-mediated Gene Transfer for Stable Expression

The Moloney murine leukemia virus-based vector Jzen.1 (Laker *et al.*, 1987) was used to introduce and express the cDNA clone B6F1.3 encoding murine IL-2Rγ in MOP8 cells. A retroviral construct, designated Jzen.B6F1.3, was constructed by inserting a full-length XbaI fragment of B6F1.3 cDNA isolated by partial digestion from plasmid pCDM8.B6F1.3 into the XbaI site of plasmid pTZ19RTkneo. A SmaI and HindIII digestion producing a fragment containing both the B6F1.3 gene and the thymidine kinase promoter (Tk neo), was isolated and subcloned into Jzen.1 digested with HpaI and HindIII. The plasmid generated (Figure 6), was transfected into the ecotropic packaging cell line GP+E-86 (Markowitz *et al.*, 1988) by calcium phosphate precipitation. Transfected cells were selected in medium containing G418 (0.5 mg/ml active weight; Gibco Laboratories, Grand Island, NY, USA). MOP8 cells were infected with Jzen.B6F1.3 retrovirus as described by McBride and colleagues (1992). Approximately 1 x 10^5 MOP8 cells were plated in a 10 cm dish and incubated at 37°C overnight to allow the cells to adhere and to begin to proliferate. The medium was then removed and replaced with 5 ml of filtered (0.22 mm Millex GV; Millipore, Marlborough, MA, USA) cell-free supernatant conditioned by the appropriate packaging cell line. Polybrene (Sigma) was added to the final concentration of 4 μg/ml and the cultures incubated for 4 hours at 37°C in 5% CO_2. This infection protocol was repeated 3 times and infected cells selected and maintained in medium containing G418 (0.3 mg/ml active weight; Gibco BRL).

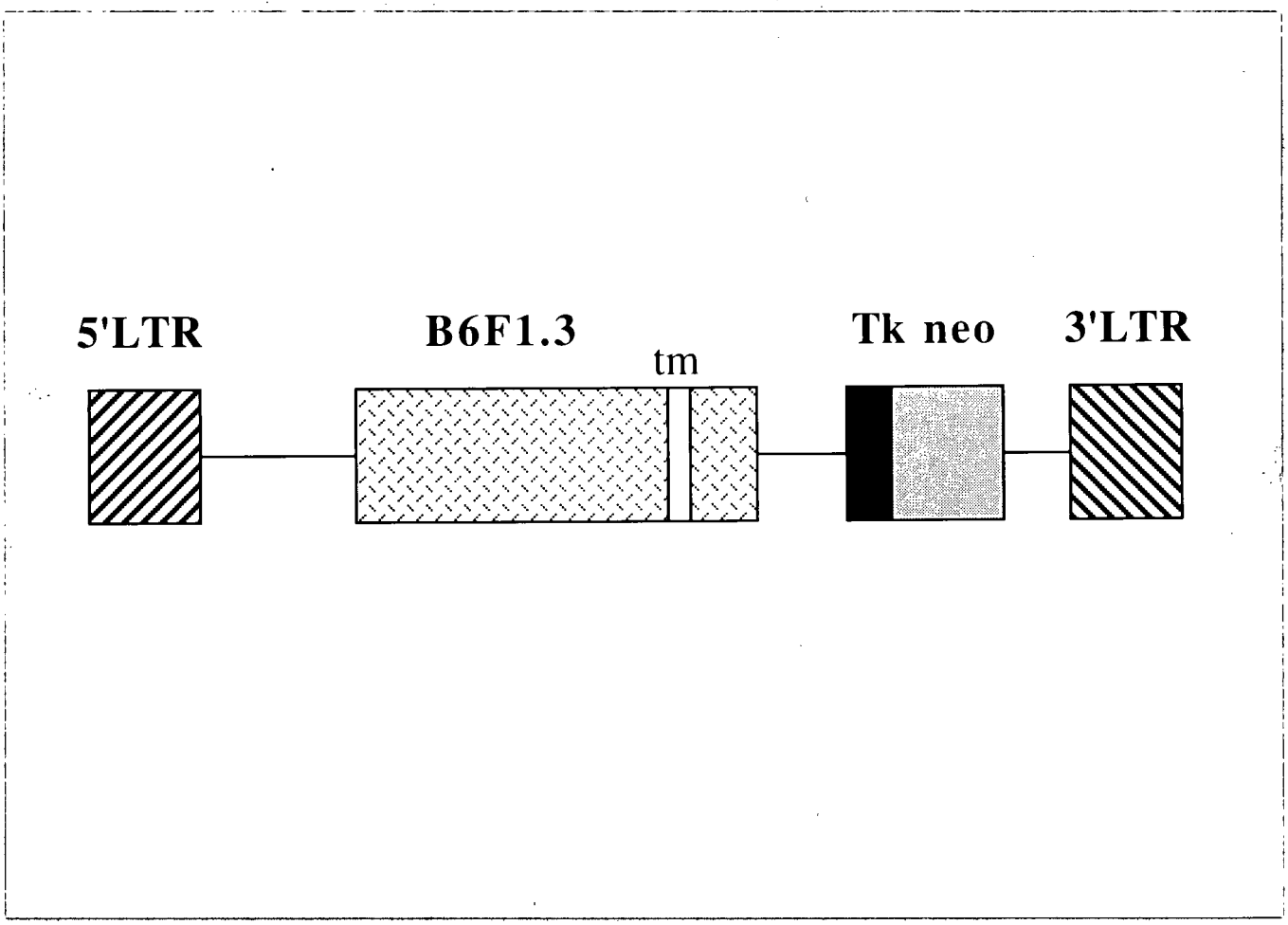

Figure 6: Retroviral vector Jzen.B6F1.3. The expression of the B6F1.3 cDNA is controlled by the retroviral 5' LTR. The contruct contains a selectable neomycin resistance marker which is driven off a thymidine kinase promoter.

2.5.2 Hyaluronan Binding Assay

Transfected cells were harvested with 2.5 mM EDTA in PBS, and 2 x 10^6 cells were labelled with Calcein (Molecular Probes, Eugene, OR, USA) at a final concentration of 5 μg/ml by incubating for 20 min at 37°C in HBSS. The fluorescently-labelled cells were washed with 50 ml HBSS and resuspended in 200 μl HBSS. 100 μl aliquots of each cell suspension were added to either 1 ml DMEM + 10% fetal calf serum (FCS, Hyclone) or an equivalent volume of hybridoma TIB 241 tissue culture supernatant containing mAbs directed against the hyaluronan-binding domain of murine CD44. After a 30 min incubation on ice, cells were washed 3 times with warm HBSS and resuspended in 1.6 ml HBSS. 400 μl aliquots of each cell suspension (approximately 2.5 x 10^5 cells) were then added to the wells of a 24 well plate (Falcon) that had been coated overnight at 4°C with human placental hyaluronan (5 mg/ml in PBS). After 10 min incubation at room temperature, non-adherent cells were removed by gently washing each well 5 times with HBSS. The number of cells adherent to hyaluronan was determined using a fluorescence plate reader (Millipore). The error bars were calculated using the formula for standard deviation: $SD = \sqrt{(\sum(xi\text{-mean})/(n-1))}$; where xi = fluorescence of each sample and n = sample size.

2.5.3 Western Blot Analysis

Transfected MOP8 cells were harvested by brief incubation in PBS containing 2.5 mM EDTA, washed extensively with PBS and resuspended at 2 x 10^7 cells/ml in PBS containing 1% (v/v) NP40 (Sigma), 5 mM EDTA and 10 mM phenylmethyl-sulphonyl fluoride (PMSF). Lysates were incubated on ice for 15 minutes, microfuged for 5 minutes to pellet nuclei and other insoluble cell debris, and stored at -70°C until required. Aliquots were rapidly thawed, added to an equal volume of non-reducing sample buffer containing 125 mM Tris, 20% (v/v) glycerol, 4.6% (w/v) SDS, pH 6.8, and incubated at 100°C for 5 minutes. Total cellular

proteins were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH, USA) as previously described. Non-specific binding sites were blocked by incubating the filters at 4°C overnight in PBS containing 5% (w/v) milk protein. After washing in HBSS, filters were incubated at room temperature for 4 hours with mAb tissue culture supernatant or a 1:200 dilution of mAb ascites in DMEM+10% FCI, washed with HBSS, and incubated for a further 1 hour at room temperature with a 1:100 dilution of horseradish peroxidase-conjugated goat anti-rat IgG (Dako) in DMEM+10% FCI. After extensive washing in HBSS, the reaction was developed in PBS containing 0.06% (w/v) 3-3'-diaminobenzidine (Sigma) and 0.012% (v/v) hydrogen peroxide (Sigma).

2.5.4 Reverse Trancription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the First-Strand cDNA Synthesis Kit from Pharmacia. 5 μg of total RNA isolated by GIT solubilization (described above) was combined with 0.2 μg of random hexadeoxynucleotides and supplied reagents to a volume of 15 μl in a 500 μl eppendorf tube. Following incubation at 37°C for 1 hour, the mixture was prepared for PCR reaction. The completed first-strand reaction was heated to 90°C for 5 min to denature the RNA-cDNA duplex and to inactivate the reverse transcriptase. 30 pmol of upstream and downstream primers, 2.5 units of Taq polymerase (BRL), and water to 50 μl were added to the total cDNA reaction. The 30 cycle PCR reaction consisted of 95°C for 1 min, 55°C for 30 sec, and 72°C for 30 sec. 5 μl of the reactions were loaded onto a 1% agarose gel as previously described. The primers used were 5'mcjun, 3'mcjun, B6F1.3SSTF (5'GAT TAC TTC TGG CTG TCA G3') and B6F1.3X/X (5'CCG TTC ACT GTA GTC TGG C3').

2.5.5 Flow Cytometric Analysis of Transfected Cells

The cells to be analyzed were harvested and transferred to wells of a 96-well plate (Falcon). The cells were then incubated for 30 min at 4°C with DMEM supplemented with 10% FCS, or an equivalent volume of hybridoma TIB 241 or TIB 242 tissue culture supernatant. The antibody treated cells were washed 3 times with cold HBSS containing 2% FCS. This was followed by staining with fluorescein isothiocyanate-conjugated goat anti-rat IgG $F(ab')_2$ fragment (Sigma) for 30 min at 4°C. Three washes with ice-chilled (4°C) HBSS with 2% FCS removed unbound secondary antibodies. To distinguish viable from nonviable cells, the stained cells were further treated with 1 μg/ml propridium iodide (PI) in HBSS with 2% FCS immediately before analysis. The relative fluorescence intensity was measured on a FACScan (Becton Dickinson, Immunocytometry Systems, San Jose, California, USA).

2.5.6 Generation of B6F1.3 Deletion Mutant

A deletion mutant of B6F1.3 was constructed and designated B6F1.3ΔSH2. pCDM8.B6F1.3 was digested with XbaI and the resulting 1 kb fragment isolated and subsequently religated into XbaI cut pCDM8. Sequence encoding the last 68 amino acids present within the cytoplasmic domain of the protein encoded by B6F1.3 transcript were removed. The stop codon is generated at position 932 in the nucleotide sequence (Figure 10).

CHAPTER III

RESULTS

3.1 Isolation of cDNA Clone B6F1.3

3.1.1 Screening of Cell Lines

The expression cloning scheme used is a potentially powerful tool that can be utilized to isolate novel adhesion molecules or their regulators, for which mAbs are not available. The system used the polyoma-transformed murine fibroblastoid cell line MOP8, which supports extrachromosomal relication of the pCDM8 vector. Although MOP8 cells expressed high levels of CD44 (Figure 7), the cell line did not constitutively adhere to hyaluronan (Figure 8). The murine mast cell line, B6SUtA also expressed high of CD44 (Figure 7) but this particular cell line did bind constitutively to hyaluronan (Figure 8). Furthermore, the adhesion was mediated by CD44 as the binding could be readily inhibited by mAb directed against CD44. In order to use this cloning scheme, the assumption that the expression of a single gene product can cause a non-binding cell line to adhere to hyaluronan, was necessarily made. The major limitation of this protocol is the selection of a suitable cell type for the transfection of cDNAs. The appropriate cell type must allow high-copy number plasmid replication and permit selection in an adhesion assay with minimal constitutive binding. MOP8 was a suitable choice as the enhancement of binding was readily distiguished from background binding.

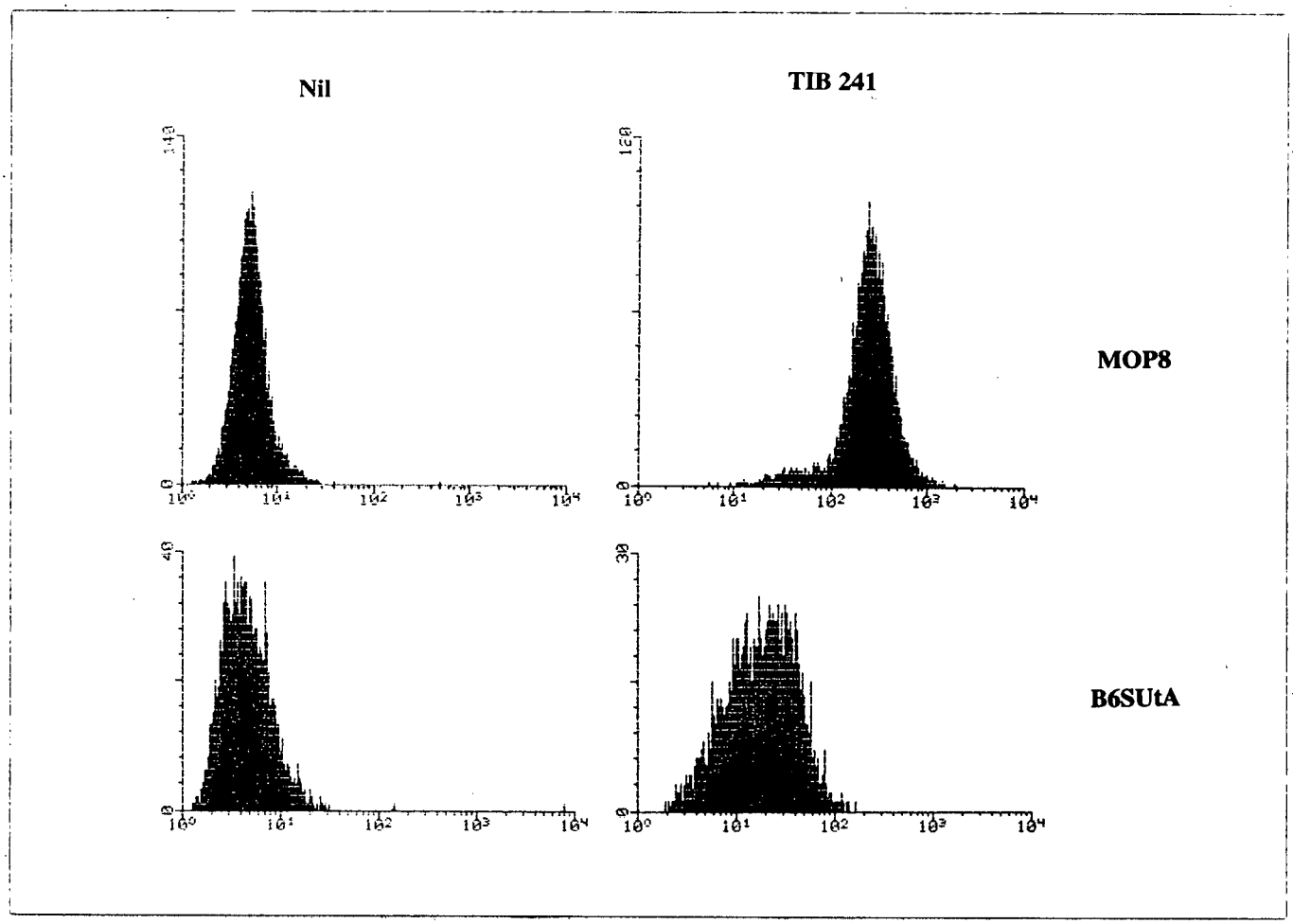

Figure 7: Expression of CD44 by MOP8 and B6SUtA. The Polyoma-transformed murine fibroblastoid cell line MOP8 expressed high levels of CD44 as demonstrated by FACS analysis using the mAb TIB 241. The IL-3 dependent murine mast cell line, B6SUtA expressed CD44 but at a relatively lower level.

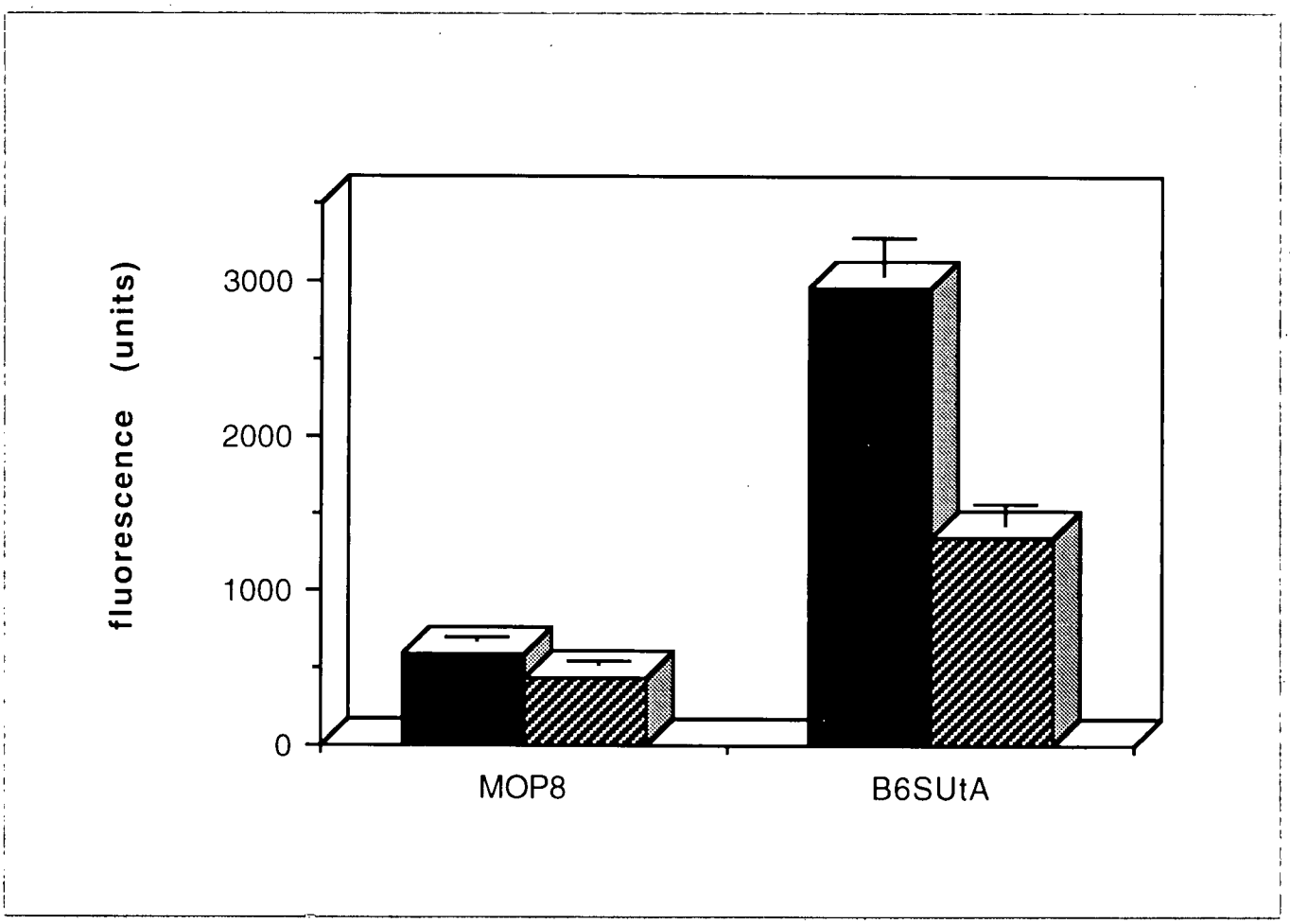

Figure 8: Adhesion to hyaluronan by MOP8 and B6SUtA. B6SUtA constitutively binds to hyaluronan in an anti-CD44 inhibitable manner. Although expressing high levels of CD44, MOP8 does not show binding to hyaluronan. Binding in the absence (black bars) or presence (shaded bars) of antibodies against CD44 is reported. The binding assay was performed in duplicate.

3.1.2 Isolation of Putative Positive Regulators

In order to isolate molecules that might be important in the regulation of CD44-mediated adhesion to hyaluronan, MOP8 cells were transfected with a cDNA library made from B6SUtA mRNA and panned over hyaluronan-coated plastic to enrich for high affinity hyaluronan binding. Following two rounds of selection, two cDNA clones designated B6F1.3, and B6F2.4 were isolated. The retransfection of these clones into MOP8 by electroporation was necessary to quantitate the level of adhesion to hyaluronan. The transfectants expressing cDNA clones B6F1.3 and B6F2.4 demonstrated relatively high levels of hyaluronan binding. The clone B6F2.4 was demonstrated to be approximately 4 kb by agarose gel electrophoresis. Clone B6F1.3 migrated at approximately 1.6 kb. Since the protein encoded by the clone B6F1.3 confered the greatest binding to hyaluronan, my research concentrated on it and formed the basis of the remainder of my thesis work. To determine whether the enhancement of adhesion to hyaluronan induced by the transfection of B6F1.3 was mediated by CD44, anti-CD44 inhibition studies were performed (Figure 9). Again, as demonstrated in Figure 9, the B6F1.3-transfectants bound to immobilized hyaluronan. This adhesion was readily inhibited with antibodies against the hyaluronan binding domain of murine CD44 (Figure 9). This evidence suggests that the binding to hyaluronan is mediated by CD44 and is not dependent on another hyaluronan-binding protein.

3.1.3 Nucleotide and Amino Acid Sequence Analysis of Clone B6F1.3

In order to gain insight into the structure and possible function of the protein encoded by the clone B6F1.3, sequence analysis was performed. The nucleotide and predicted amino acid sequence of B6F1.3 is shown in Figure 10 and 11, respectively. The 5' untranslated region is 37 bp whereas the 3' untranslated region containing a canonical polyadenylylation signal, is 476 bp

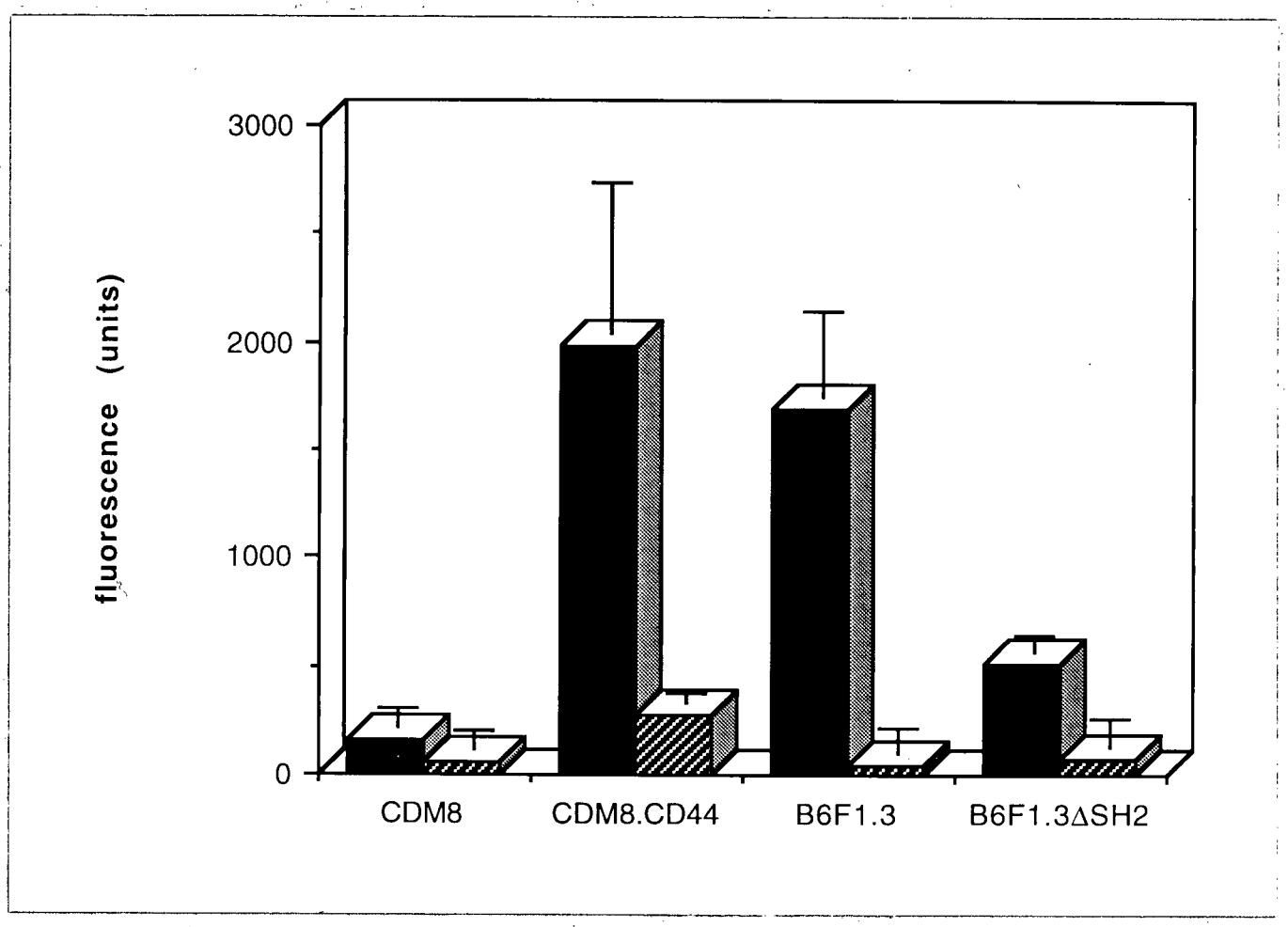

Figure 9: B6F1.3 transfection induces MOP8 cells to bind to hyaluronan. Adhesion of MOP8 cells transfected with cDNA clone B6F1.3, and deletion mutant B6F1.3ΔSH2 in pCDM8, to hyaluronan in the presence (shaded) or absence (black) of antibodies against CD44. Expression of the protein encoded by the cDNA clone B6F1.3 induces MOP8 cells to bind in a CD44 inhibitable fashion to hyaluronan. A deletion mutant in which the last 68 amino acids of the cytoplasmic tail including the SH2 subdomain is removed does not mediate the same effect. Overexpression of human CD44 enhances MOP8 adhesion and thus was used as the positive control. The binding assay was performed in duplicate.

a)

```
1       gacacagact acacccagag aaagaagagc aagcaccatg ttgaaactat
51      tattgtcacc tagatccttc ttagtccttc agctgctcct gctgagggca
101     gggtggagct ccaaggtcct catgtccagt gcgaatgaag acatcaaagc
151     tgatttgatc ctgacttcta cagcccctga acacctcagt gctcctactc
201     tgccccttcc agaggttcag tgctttgtgt tcaacataga gtacatgaat
251     tgcacttgga atagcagttc tgagcctcag gcaaccaacc tcacgctgca
301     ctataggtac aaggtatctg ataataatac attccaggag tgcagtcact
351     atttgttctc caaagagatt acttctggct gtcagataca aaaagaagat
401     atccagctct accagacatt tgttgtccag ctccaggacc cccagaaacc
451     ccagaggcga gctgtacaga agctaaacct acagaatctt gtgatcccac
501     gggctccaga aaatctaaca ctcagcaatc tgagtgaatc ccagctagag
551     ctgagatgga aaagcagaca tattaaagaa cgctgtttac aatacttggt
601     gcagtaccgg agcaacagag atcgaagctg gacggaacta atagtgaatc
651     atgaacctag attctccctg cctagtgtgg atgagctgaa acggtacaca
701     tttcgggttc ggagccgcta taacccaatc tgtggaagtt ctcaacagtg
751     gagtaaatgg agccagcctg tccactgggg gagtcatact gtagaggaga
801     atccttcctt gtttgcactg gaagctgtgc ttatccctgt tggcaccatg
851     gggttgatta ttaccctgat ctttgtgtac tgttggttgg aacgaatgcc
901     tccaattccc cccatcaaga atctagagga tctggttact gaataccaag
951     ggaacttttc ggcctggagt ggtgtgtcta aagggctgac tgagagtctg
1001    cagccagact acagtgaacg gttctgccac gtcagcgaga ttccccccaa
1051    aggaggggcc ctaggagagg ggcctggagg ttctccttgc agcctgcata
1101    gcccttactg gcctccccca tgttattctc tgaagccgga agcctgaaca
1151    tcaatccttt gatggaacct gaaagtccta tagtcctaag tgacgctaac
1201    ctcgggtact caccttggca atctggatcc aatgctcact ggcttccttg
1251    gggctaaggt aagtttcgat ttcctgtccc atgtaactgc ttttctgttc
1301    catatgcgct acttgagagt gtcccttgcc ctctttccct gcacaagccc
1351    tcccatgccc agcctaacac ctttccactt tctttgaaga gagtcttacc
1401    ctgtagccca gggtggctgg gagctcacta tgtaggccag gttggtccaa
1451    ctcacaggct atcctcccac ctctgcctca taagagttgg ggttactggc
1501    atgcaccacc acacccagca tggtccttct cttttatagg attctccctc
1551    ccttttcta cctatgattc aactgtttcc aaatcaacaa gaaataaagt
1601    ttttaaccaa tgataaaaaa aaa
```

b)

```
899     cctccaattc cccccatcaa gaatctagag gatctggtta ctgaatac
        P  P  I  P   P  I  K   N  L  E   D  L  V  T   E  Y

899     cctccaattc cccccatcaa gaatctagag gatcttttg
        P  P  I  P   P  I  K   N  L  E   D  L  stop
```

Figure 10: The nucleotide sequence of cDNA clone B6F1.3. Clone B6F1.3 was sequenced and determined to be 1623 nucleotides long (a). The nucleotide and predicted amino acid sequence for the region with homology to SH2 subdomains is shown (b; top) and compared with the sequence generated for B6F1.3ΔSH2 (b; bottom). The XbaI site used to generate the deletion mutant B6F1.3ΔSH2 is shown in underlined italics.

```
B6F1.3     1    MLKLLLSPRSFLVLQLLLLRAGWSSKVLMSSANEDIKADL
hIL2Rγ             PS PFT L F   P  GV LNTTI TPNG   TT  F

B6F1.3    41    ILTSTAPEHLSAPTLPLPEVQCFVFNIEYMNCTWNSSSEPQ
hIL2Rγ          F  TMPTDS VS             V

B6F1.3    82    ATNLTLHYRYKVSDNNTFQECSHYLFSKEITSGCQIQKEDI
hIL2Rγ          P       W  N   DKV K       E       L  KE

B6F1.3   123    QLYQTFVVQLQDPQKPQRRAVQKLNLQNLVIPRAPENLTLS
hIL2Rγ          H            RE R Q T M K       W      H

B6F1.3   164    NLSESQLELRWKSRHIKERCLQYLVQYRSNRDRSWTELIVN
hIL2Rγ          K        N NN FL-NH  EH     TDW H    QS D

B6F1.3   205    HEPRFSLPSVDELKRYTFRVRSRYNPICGSSQQWSKWSQPV
hIL2Rγ          YRHK       GQ          F      A H  E  H I

B6F1.3   246    HWGSHTVEENPSLFALEAVLIPVGTMGLIITLIFVYCWLER
hIL2Rγ              N SK   F       V S  S     S LC  F

B6F1.3   287    -MPPIPPIKNLEDLVTEYQGNFSAWSGVSKGLTESLQPDYSE
hIL2Rγ          T  R  TL          H            A

B6F1.3   328    RFCHVSEIPPKGGALGEGPGGSPCSLHSPYWPPPCYSLKPEA
hIL2Rγ           L L               A   NQ     A    TL   T
```

Figure 11: Deduced amino acid sequence of the protein encoded by B6F1.3 and comparison with the human interleukin-2 receptor γ chain. Clone B6F1.3 was sequenced and shown to encode a single long open reading frame of 369 amino acids. Sequence comparison suggests that B6F1.3 constitutes the murine homologue of the human interleukin-2 receptor γ chain (hIL-2Rγ). The identity between coding regions of clone B6F1.3 and hIL-2Rγ is 80% at the nucleotide level and 81% at the amino acid level. The signal peptide is underlined and the membrane spanning domain boxed. Potential sites of N-linked glycosylation are marked by a stipled line. The 4 conserved cysteines and the WS motif characteristic of the cytokine receptor supergene family are indicated repectively by the symbols * and #. The region with homology to an SH2 subdomain is shown in shadowed type.

(not including the stop codon). Clone B6F1.3 was observed to encode a single long open reading frame of 369 amino acids corresponding to a typical type I integral membrane protein (Figure 11 and 11).

A search of Genbank revealed striking sequence identity (80% at the nucleotide level, and 81% at the amino acid level) to the coding region of human interleukin-2 receptor γ chain (hIL-2Rγ; Takeshita *et al.*, 1992) (Figure 11). The sequence identity is greatest in the cytoplasmic domain where 82% amino acid identity is observed. Both hIL-2Rγ and B6F1.3 appear to be encoded as 369 amino acid proproteins and have N-terminal hydrophobic signal peptides. The cysteine-rich region (positions 62, 72, 102, and 115) and the WSXWS motif (positions 238 to 242) characteristic of the cytokine receptor supergene family (Cosman *et al.*, 1990) are conserved between species. Furthermore, a region with limited homology to the fourth and fifth SH2 subdomains present within a number of proteins involved in signal transduction (Koch *et al.*, 1991), is observed at position 288 to 321 (Figure 13). However, it is interesting to note that this region is less well conserved in the mouse, particularly Arg-289 and Thr-292 which are conserved in Lck, Hck, Lyn, and Blk, but not in mIL-2Rγ. Six N-linked glycosylation sites (Asn-X-Ser/Thr) are found in the extracellular domain. A leucine zipper motif was noted to exist in the hIL-2Rγ sequence (formed by Leu-165, -172, -179, and -186), although no clear functional role for this was demonstrated. In the protein encoded by B6F1.3, Leu-165, and -172 are conserved, Leu-179 is replaced by Ile, and the final Leu at position 187 is shifted to position 186. Although this particular region differs, these data suggest that the B6F1.3 constitutes the murine homologue of IL-2Rγ (mIL-2Rγ). It should be noted that following the isolation of the cDNA clone B6F1.3, the cloning of the mIL-2Rγ was reported (Cào *et al.*, 1993). Furthermore, the mIL-2Rγ demonstrated 100% identity to clone B6F1.3 .

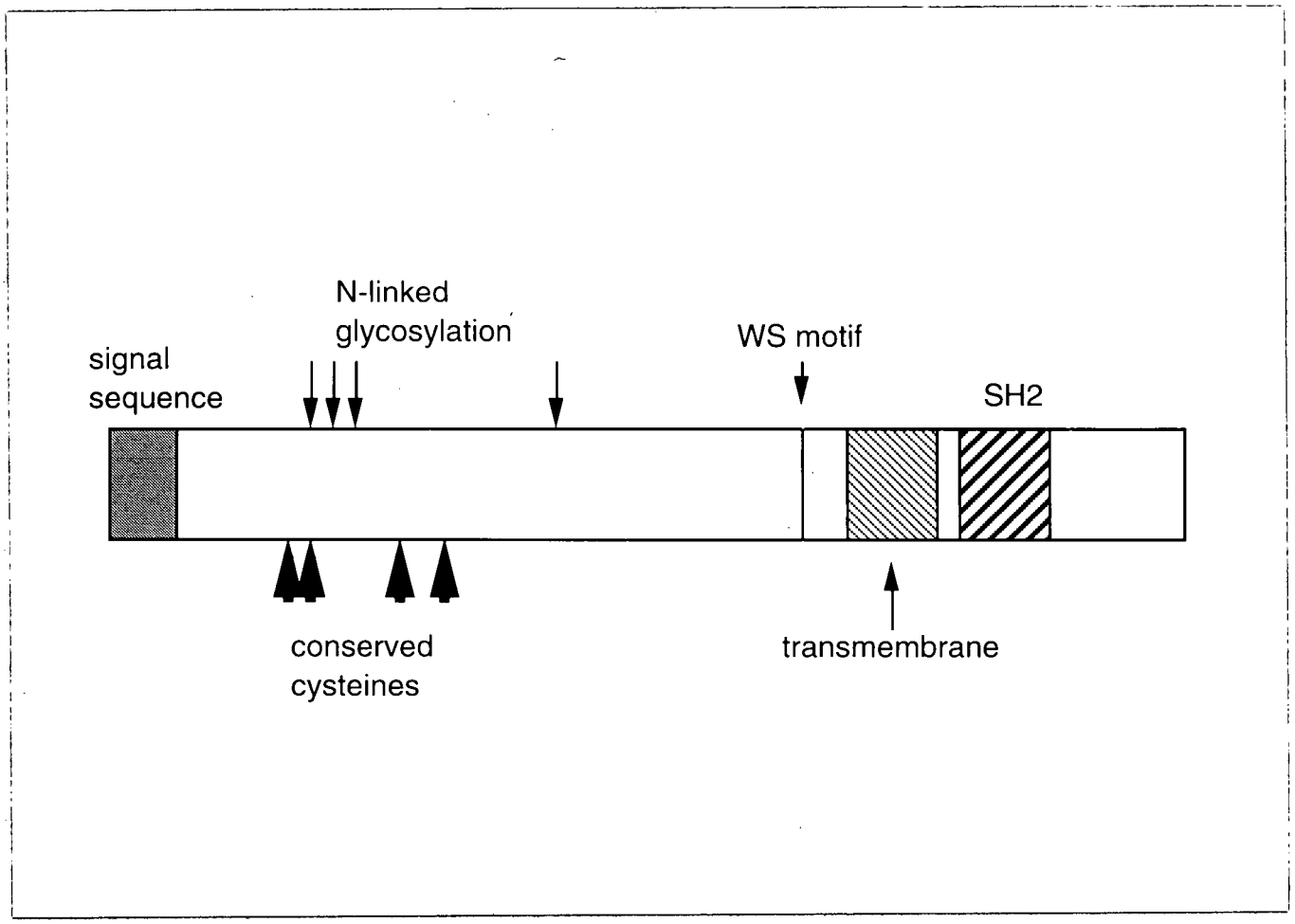

Figure 12: Diagramatic representation of mIL-2Rγ. The salient features of the mIL-2Rγ protein are labelled. It is important to note that the leucine zipper motif observed in the hIL-2Rγ molecule is not conserved in the corresponding murine protein. The region marked SH2 denotes the domain with limited homology to the fourth and fifth subdomain of Src Homology Region 2.

```
B6F1.3   PPIPPIKNLEDLVTEYQGNFSAWSGVSKGLTESL
hIL2Rγ   PRIPTLKNLEDLVTEYHGNFSAWSGVSKGLAESL
Lck      PRI-TFPGLHDLVRHYTNASD-------GLCTKL
Hck      PRS-TGSTLQELVDHYKKGND-------GLCQKL
Lyn      PRI-TFPCISDMIKHYQKQAD-------GLCRRL
Blk      PRI-TFPTLQALVQHYSKKGD-------GLCQKL
```

Figure 13: Sequence similarity between mIL-2Rγ, hIL-2Rγ and other proteins containing SH2 domains. Both murine (protein encoded by B6F1.3) and human IL-2Rγ are aligned with the appropriate SH2 subdomains of Lck, Hck, Lyn and Blk. Amino acid residues which are conserved in SH2 domains are boxed. The dashes represent absent amino acids.

3.2 Analysis of B6F1.3 Expression

The distribution of B6F1.3 expression was determined by Northern blot analysis. Total RNA from various hemopoietic and fibroblastoid cell lines as well as numerous murine tissues were isolated. Upon probing the filter with full length B6F1.3, high expression levels were observed in the thymus, lymph node, peritoneal cavity and spleen whereas the bone marrow, brain, kidney, lung, and heart contained little or no message (Figure 14a). All hemopoietic cell lines tested were positive with the exception of Sp2/0, while the fibroblastoid cell lines Fsa-N, Fsa-R and MOP8 were negative (Figure 14b).

3.3 Functional Analysis of B6F1.3

3.3.1 Stable Expression of the B6F1.3 cDNA in MOP8 Cells

Whether stable expression of B6F1.3 produces similar "activation" of CD44 function was unknown. To address this issue, an ecotropic retrovirus (Jzen.B6F1.3, Figure 6) was constructed to allow for chromosomal integration into MOP8. MOP8 cells infected with cell-free Jzen.B6F1.3 viral supernatant were selected and maintained in G418. Expression of the transfected B6F1.3 cDNA was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) analysis of total RNA isolated from MOP8.Jzen.B6F1.3 cells. Following first strand DNA synthesis, internal B6F1.3 primers were used to amplify a 655 bp fragment. Agarose gel electrophoresis and ethidium bromide staining demonstrated the DNA band to be of the appropriate size DNA segment (Figure 15).

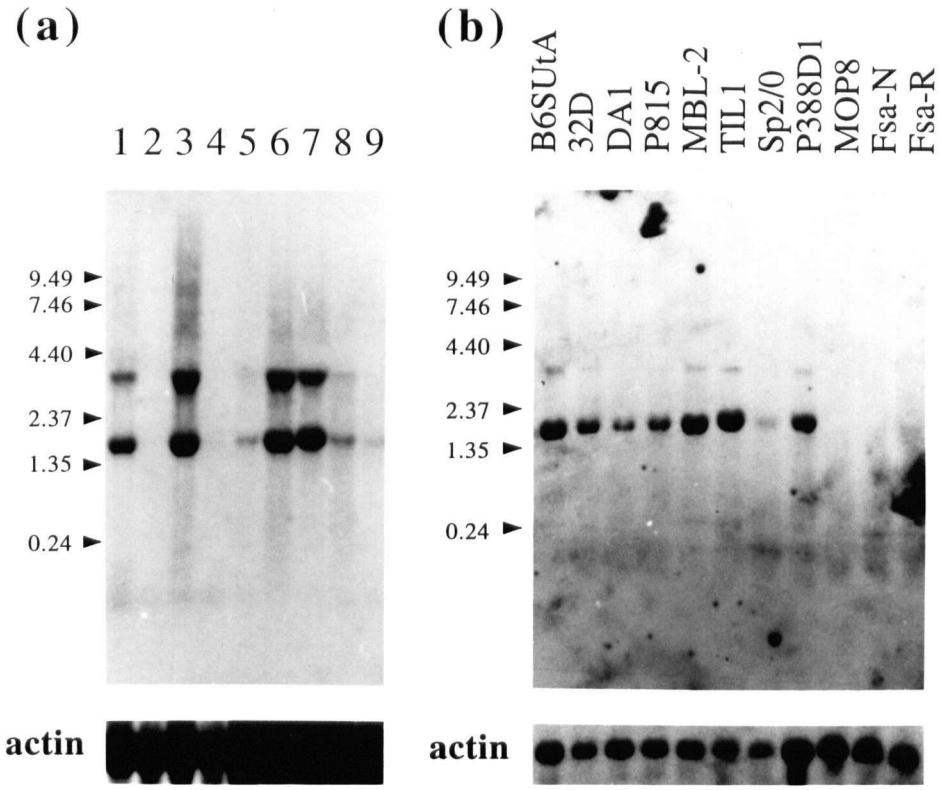

Figure 14: Expression of B6F1.3 transcripts in primary tissues (a), and cultured cell lines (b). The expression pattern of B6F1.3 transcripts was determined by Northern blot analysis. (a) High expression levels are observed in the spleen (lane 1), thymus (lane 3), lymph node (lane 6), and peritoneal cavity (lane 7) whereas the brain (lane 2), kidney (lane 4), bone marrow (lane 5), lung (lane 8), and heart (lane 9) contained little or no message. (b) All hemopoietic cell lines tested were positive while the fibroblastoid cell lines Fsa-N, Fsa-R, and MOP8 were negative. Thus, expression of the protein encoded by B6F1.3 appears to be restricted to cells of the hemopoietic lineage with the bone marrow being an exception.

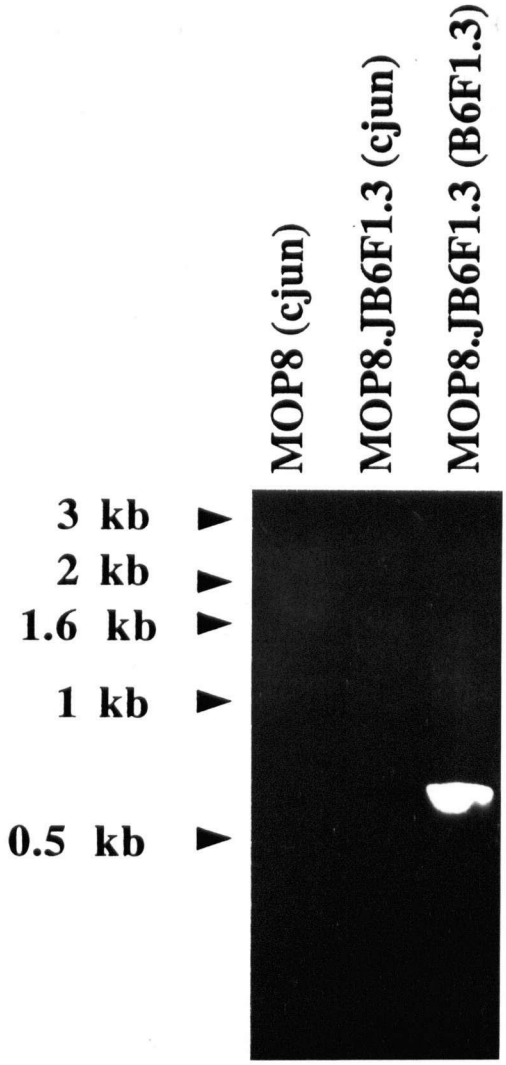

Figure 15: B6F1.3 is expressed in Jzen.B6F1.3 infected MOP8 cells. Ethidium bromide stained agarose gel of RT-PCR reactions. The first two samples were amplified using murine c-jun primers. The third lane represents MOP8.JzenB6F1.3 DNA amplified using internal B6F1.3 primers. The expected sequence length is 655 bp.

3.3.2 Binding of MOP8.Jzen.B6F1.3 Cells to Hyaluronan

MOP8 cells stably expressing the B6F1.3-encoded protein were tested for their ability to bind to hyaluronan-coated dishes. The infected cells were labelled with Calcein, and then incubated with or without anti-CD44 antibodies. The Jzen.B6F1.3 infected cells demonstrated marked enhancement of binding as compared to the control of uninfected MOP8 cells (Figure 16). Furthermore, the pretreatment with anti-CD44 antibody significantly inhibited this binding back to the level of the control cells (Figure 16). This further demonstrated that cDNA clone B6F1.3 "activates" CD44 to bind hyaluronan.

3.3.3 Effect of B6F1.3 on the Expression of CD44

Since overexpression of human CD44 can enhance MOP8 adhesion to hyaluronan (Figure 9), it was important to determine whether B6F1.3 mediates its effects simply by upregulating the expression of CD44 on transfected cells. MOP8 cells transiently expressing the protein encoded by B6F1.3 were stained with anti-CD44 antibodies and analyzed by FACS analysis, and Western blot analysis. By FACS analysis, both the untransfected and B6F1.3-transfected cells demonstrated high fluorescence compared to unstained controls (Figure 17). This result is not unexpected as MOP8 cells express high levels of CD44. It is important to note, however, that the expression pattern of CD44 on these cells appeared to be unaltered. Moreover, Western blot analysis (Figure 18) and indirect immunoperoxidase staining also showed no significant difference.

Jzen.B6F1.3 infected MOP8 cells were also tested by FACS analysis. Both uninfected and Jzen.B6F1.3-infected cells also failed to show an increase in the level of CD44; in fact the CD44 expression level was distinctly decreased (Figure 19). Although the mechanism by which B6F1.3 "activates" CD44 is unknown, induction of CD44 on transfected MOP8 does not appear to be involved.

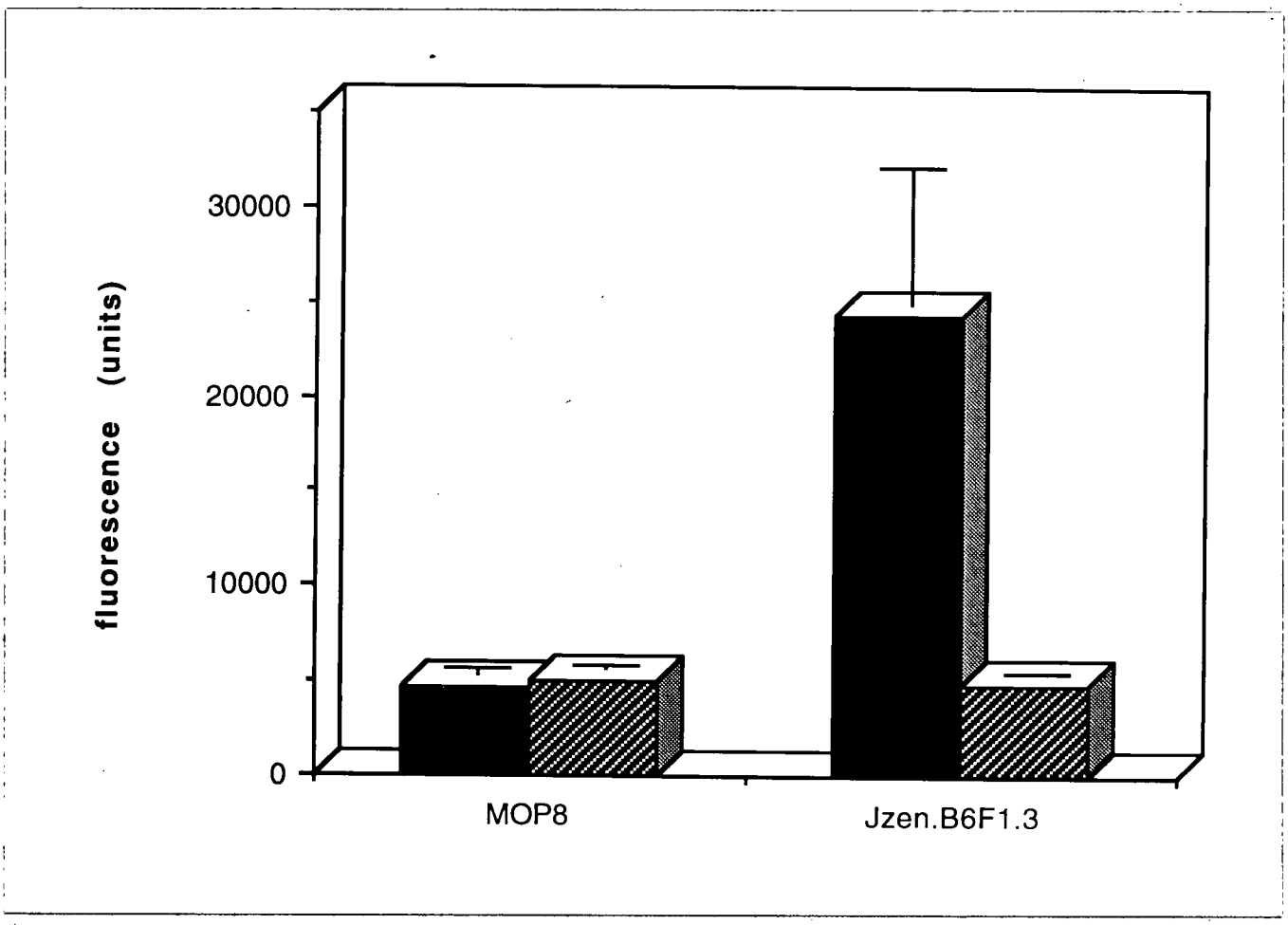

Figure 16: Stable expression of B6F1.3 induces MOP8 cells to bind to hyaluronan. Adhesion of MOP8 cells infected with the Jzen.B6F1.3 retrovirus compared to uninfected MOP8 cells. The binding assay was performed in the presence (shaded) and absence (black) of anti-CD44 mAb TIB 241. The binding assay was performed in duplicate.

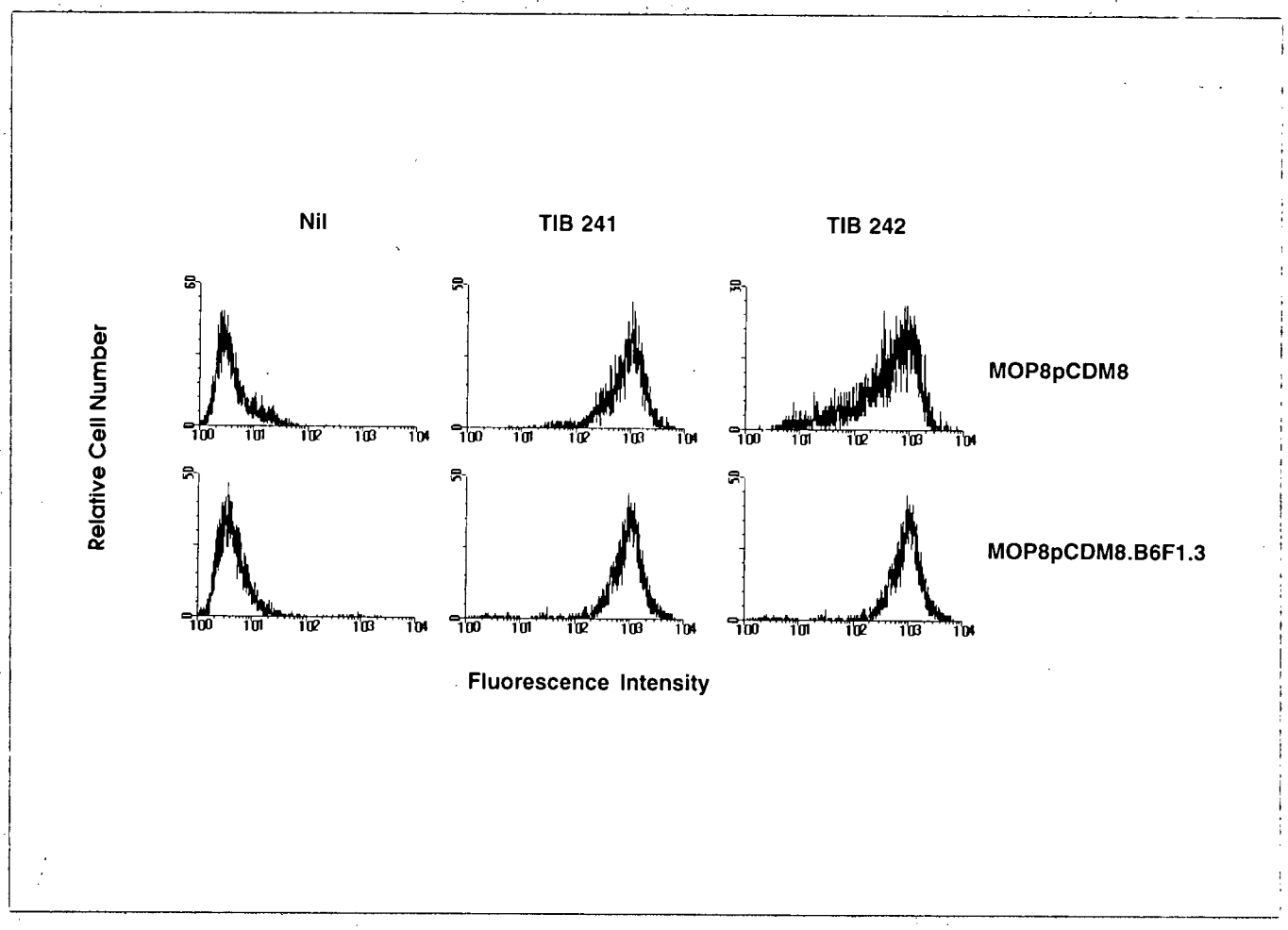

Figure 17: Transient expression of the B6F1.3-encoded protein does not upregulate CD44 expression. FACS analysis of MOP8 cells transfected with pCDM8, pCDM8.B6F1.3. The cells were stained with TIB 241, TIB 242 or media alone (nil).

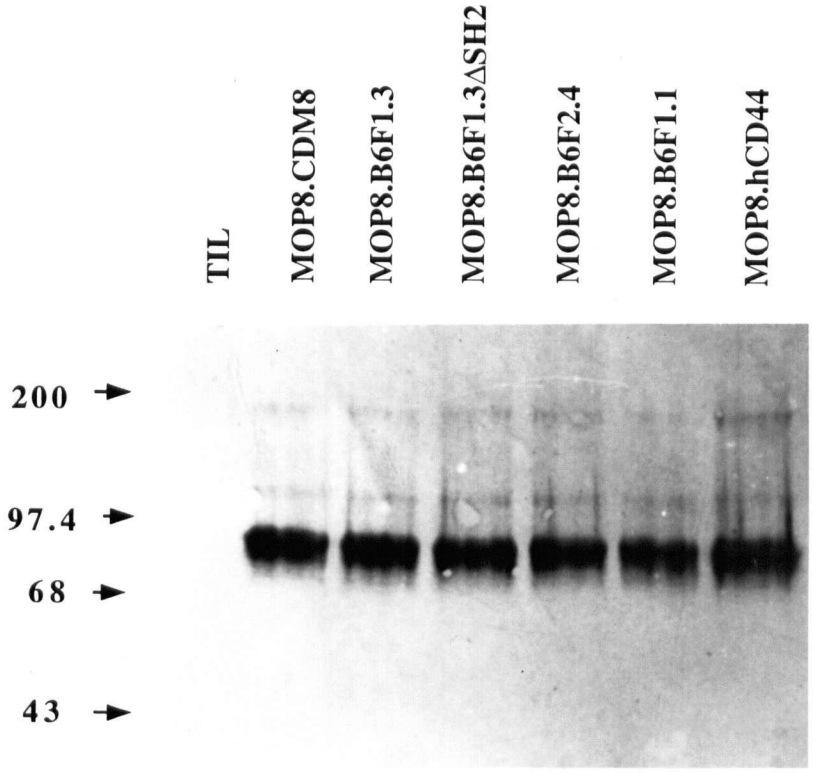

Figure 18: Transient expression of the B6F1.3-encoded protein does not alter CD44 expression. Western blot analysis of various MOP8 transfectants. The primary antibody used was TIB 241. The secondary antibody was a horseradish peroxidase-conjugated goat anti-rat IgG.

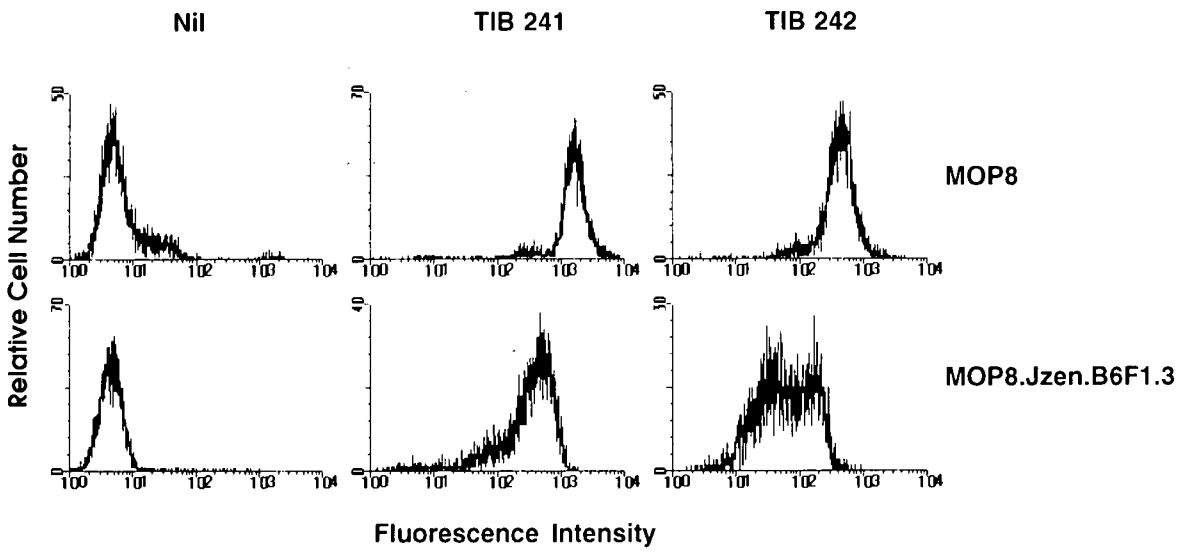

Figure 19: Stable expression of the protein encoded by B6F1.3 fails to show an increase in CD44 expression. FACS analysis of MOP8 cells infected with Jzen.B6F1.3 compared to unifected MOP8 cells. The cells were stained with TIB 241, TIB 242 or media alone (nil).

3.3.4 Mutational Analysis

Studies were undertaken to begin to define the mechanism by which mIL-2Rγ "activates" CD44-mediated adhesion. As stated above, B6F1.3 contains a region with homology to SH2 subdomains (Figure 11 and 11), a motif involved in signal transduction. Thus the existence of this domain suggested a potential role for this cytoplasmic element in the "activation" of CD44. To study this possibility, a deletion mutant (B6F1.3ΔSH2) in which sequence encoding the last 68 amino acids present within the cytoplasmic tail of the mIL-2Rγ molecule including 20 of the 34 residues that make up the SH2 subdomain were removed, was constructed and inserted into the pCDM8 vector. An adhesion assay was performed in the same manner as with the B6F1.3 cDNA clone. B6F1.3ΔSH2 transfected cells failed to demonstrate induction of binding to hyaluronan (Figure 9). It is important to note, however, that the protein encoded by B6F1.3ΔSH2 may not have been expressed. The cell surface expression of this mutant protein could not be determined as mAbs directed against the mIL-2Rγ were not in our possession. If the mutant protein is not found on the cell surface then CD44-mediated adhesion to hyaluronan would not be expected. These data suggest an important role for signal transduction events mediated by the cytoplasmic domain of mIL-2Rγ in the "activation" of CD44.

3.4 Involvement of the Proto-oncogene c-jun

Previous studies have demonstrated that the proto-oncogenes c-myc, c-fos and c-jun may play important roles as downstream mediators for the signal transduction pathway regulating events triggered by activation of the IL-2 receptor complex (Asao *et al.*, 1993). These three proto-oncogenes are induced following the addition of IL-2 to fibroblasts expressing the high- or intermediate-affinity IL-2 receptor complexes. In the absence of exogenous IL-2, low levels of only the proto-oncogene c-jun are induced (Asao *et al.*, 1993). To test whether c-jun is involved in the B6F1.3-mediated activation of CD44, the hyaluronan binding ability of

pCDM8.cjun transfectants was determined. Furthermore, the expression of c-jun messages in MOP8 cells infected with the retrovirus Jzen.B6F1.3 was also studied.

3.4.1 Expression of c-jun Message in MOP8.Jzen.B6F1.3 Cells

The studies by Asao and colleagues (1993) demonstrated a correlation between c-jun induction and IL-2Rγ in the human system. In order to determine whether c-jun can be induced in murine fibroblastoid cells by mIL-2Rγ, a retrovirus carrying the B6F1.3 cDNA was constructed and used to infect MOP8 cells (Figure 6). After growing MOP8 cells infected with the retrovirus designated Jzen.B6F1.3, in selectable medium for several weeks, total RNA was isolated. Concurrently, total RNA was isolated from uninfected MOP8 cells. As a positive control, RNA was harvested from MOP8 cells treated with X-rays (10 Gy), as this treatment does induce c-jun expression.

Northern blot analysis of the Jzen.B6F1.3 retrovirally infected cells was performed. The filter was probed using a full length murine c-jun probe. An increase in c-jun expression was demonstrated by an induction of c-jun mRNA in the Jzen.B6F1.3-infected cells as compared with uninfected cells (Figure 20). However, the elevated levels of c-jun mRNA may not be significant, or may have arisen during the selection process. The MOP8 cells pretreated with X-ray showed high levels of expression. These findings suggested that c-jun may act as a downstream signal following stimulation of the IL-2Rγ leading to the "activation" of CD44 such that a hyaluronan binding state is achieved.

3.4.1 Transient Expression of c-jun "Activates" CD44

Although induction of c-jun expression was demonstrable upon mIL-2Rγ stable expression in MOP8 cells, the role of c-jun in the activation of CD44 could not be inferred from this observation. To test whether this proto-oncogene can enhance MOP8 cell binding to hyaluronan coated plastic, c-jun in pCDM8 was transiently expressed in MOP8. Following an

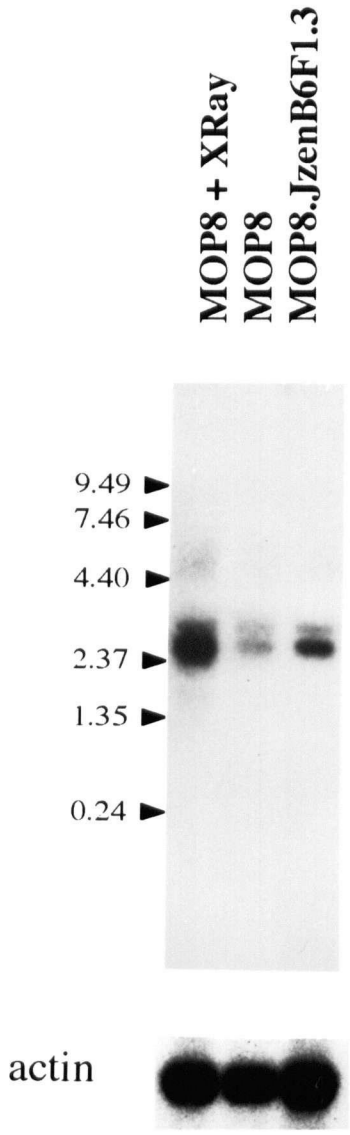

Figure 20: B6F1.3 induces c-jun mRNA. Northern blot analysis of RNA isolated from MOP8 cells pretreated with X-Rays, or infected with Jzen.B6F1.3. The filter was probed with full length murine c-jun.

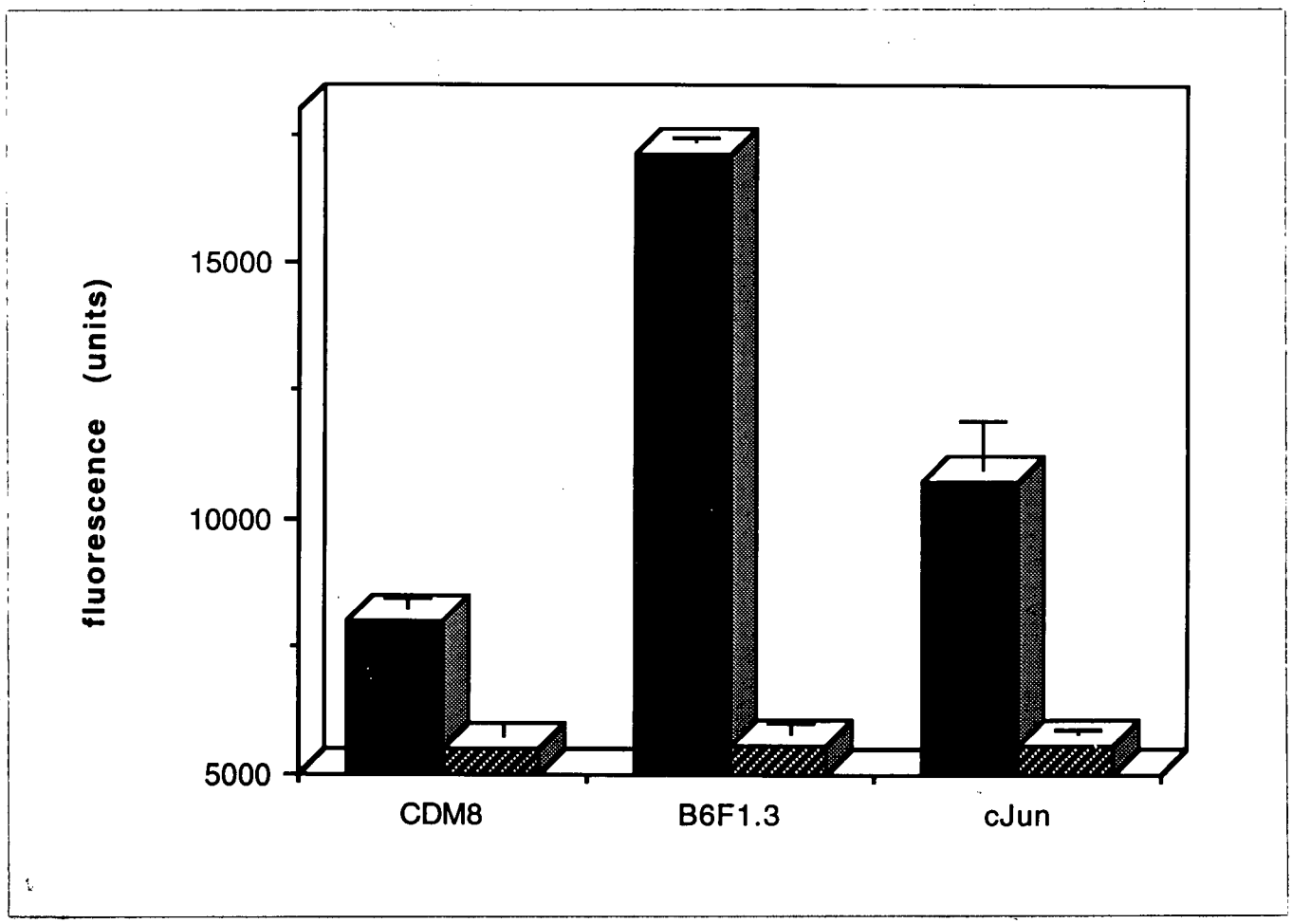

Figure 21: c-jun induces MOP8 cells to bind to hyaluronan. Adhesion of MOP8 cells transiently transfected with murine c-jun in pCDM8 as compared to pCDM8 alone. The binding assay was performed in the presence (shaded) and absence (black) of anti-CD44 mAb TIB 241. The binding assay was performed in duplicate.

appropriate amount of time to allow for replication of the plasmid, cells transfected with pCDM8, pCDM8.c-jun, or pCDM8.B6F1.3 by electroporation were tested for their ability to bind to hyaluronan-coated dishes. As demonstrated in Figure 21, B6F1.3 transfected cells demonstrated significant hyaluronan binding as determined by fluorescence. The pCDM8 transfectants showed little or no binding as compared to the background. pCDM8.c-jun transfectants also bound to hyaluronan, however, at levels somewhat lower than that of B6F1.3 (Figure 21). Moreover, this induction could be inhibited by a pretreatment of the transfectants with mAbs directed against the hyaluronan binding domain of murine CD44 (Figure 21). Therefore, transient expression of c-jun appeared to enhance MOP8 cell adhesion to hyaluronan in a CD44-inhibitable fashion.

To determine whether the enhancement of hyaluronan binding of the MOP8 cells transfected with pCDM8.c-jun was caused by an upregulation of CD44 molecules on the cell surface, FACS analysis using anti-CD44 antibodies was performed. Figure 22 demonstrates that no obvious alteration in the CD44 levels appeared. CD44 is expressed at high levels on untransfected control MOP8 cells but no further increase above the unstained control was observed in either the pCDM8.c-jun or the pCDM8.B6F1.3 transfected cells.

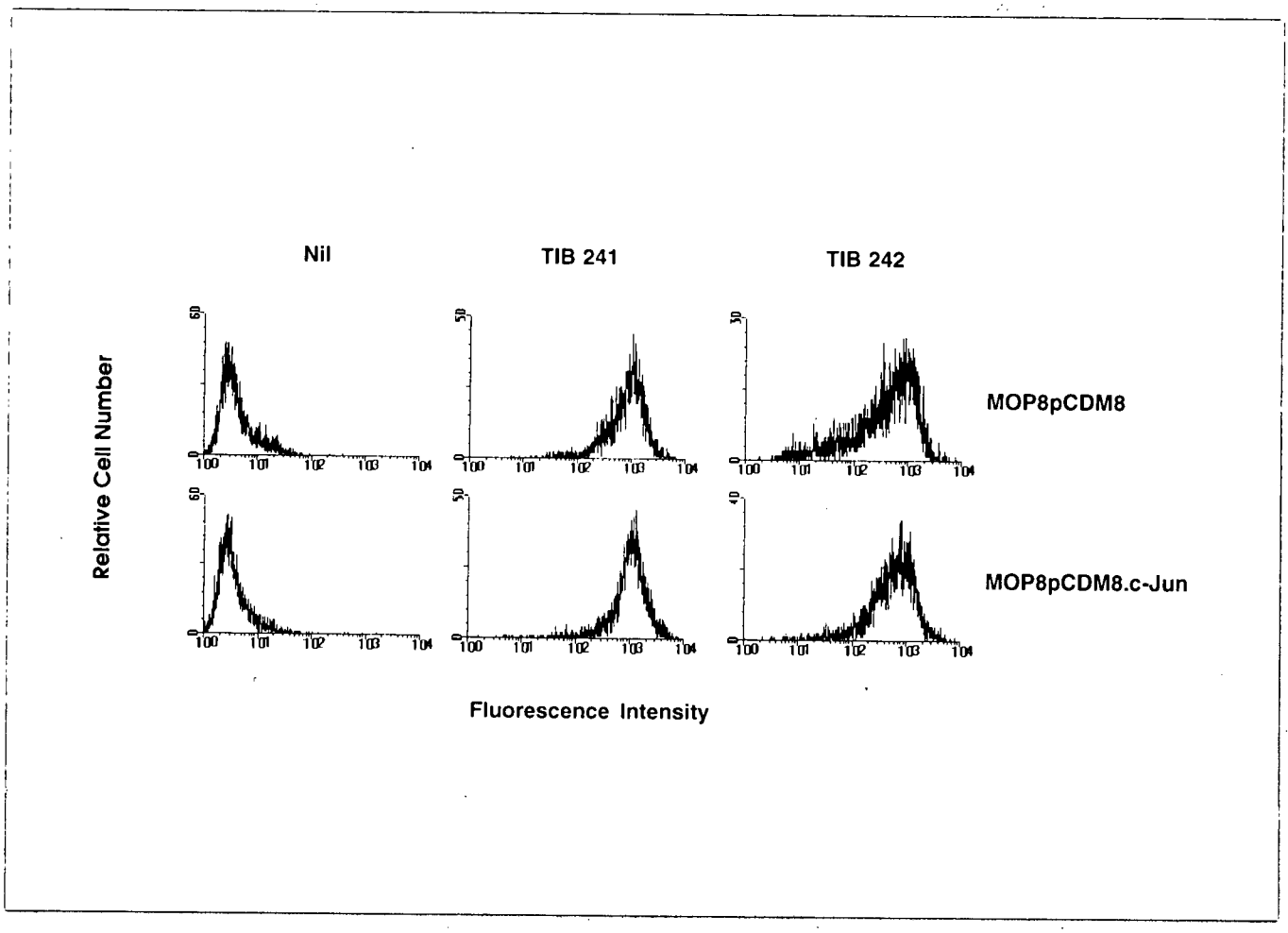

Figure 22: Transient expression of the protooncogene, c-jun does not alter CD44 expression levels. FACS analysis of MOP8 cells transfected with pCDM8, pCDM8.B6F1.3, or pCDM8.cjun. The cells were stained with the mAbs TIB 241, TIB 242 or media alone (nil).

CHAPTER IV

DISCUSSION

Considerable interest has been focused on understanding the molecules involved in cell adhesion. One of these proteins is the widely distributed glycoprotein CD44. This adhesion protein has been demonstrated to play a significant role in a variety of cellular processes. Some of these functions involve the interaction of CD44 with its ligand, hyaluronan. This polymer is a common component of extracellular matrices and extracellular fluids. The observation that not all cells expressing CD44 can bind to hyaluronan led to the notion that the adhesive function of CD44 may be strictly regulated. To date, the molecular mechanisms that regulate the adhesive function and ligand-binding specificity of CD44 are not well defined. Several isoforms of CD44 which share extensive sequence homology have been identified (Dougherty *et al.*, 1991; Stamenkovic *et al.*, 1991; He *et al.*, 1992; Screaton *et al.*, 1992; Günthert *et al.*, 1991). These higher molecular mass CD44 isoforms are generated by the insertion of additional peptide sequences of varying length encoded by a contiguous series of at least 10 alternatively spliced exons, into a single site within the extracellular domain of the molecule proximal to the membrane spanning domain (Dougherty *et al.*, 1991; Cooper *et al.*, 1992; Jackson *et al.*, 1992; Screaton *et al.*, 1992; Tölg *et al.*, 1993). Some of these isoforms have been demonstrated to exhibit altered ligand-binding specificity and cellular distribution (Stamenkovic *et al.*, 1991; Dougherty *et al.*, 1994; Dougherty *et al.*, 1991; Günthert *et al.*, 1991; He *et al.*, 1992). Cytoplasmic phosphorylation and subsequent interactions with cytoskeletal elements, may regulate CD44 function and expression. One other potential mechanism may be the existence of cell-specific regulatory molecules.

The studies presented in this thesis have focussed on the isolation, identification and functional analysis of the cDNA clone B6F1.3, a potential positive regulatory molecule for CD44-mediated adhesion to hyaluronan. Moreover, attempts to delineate the mechanism of

CD44 "activation" by B6F1.3 have been reported including mutational analysis, CD44 expression and the involvement of the proto-oncogene c-jun.

Sequence analysis has identified clone B6F1.3 as identical to the mIL-2Rγ. The cDNA for mIL-2Rγ was recently cloned (Cao *et al.*, 1993), however, most of the studies of IL-2Rγ have been performed in the human system. The human molecule was originally isolated as the third subunit of the IL-2 receptor complex (Takeshita *et al.*, 1992). Comparisons within Genbank demonstrated that mouse and human IL-2Rγ showed a high degree of sequence identity. 80% identity at the nucleotide level and 81% at the amino acid level was observed for the coding regions (Figure 9). The region with the highest conservation between mouse and human, is the cytoplasmic domain. This suggests an important role for this region in IL-2Rγ function. Interestingly, although the cytoplasmic domain of the human molecule contains a region with limited homology to the fourth and fifth SH2 subdomains (Takeshita *et al.*, 1992), this region is not as well conserved in the mouse. The residues Arg-289 and Thr-292 in hIL-2Rγ, is conserved in Lck, Hck, Lyn and Blk, but is not found in mIL-2Rγ. Moreover, the leucine-zipper motif is not conserved in mouse. Therefore, the lack of rigorous conservation of SH2 subdomains and leucine-zipper motifs, tends to lessen the probability that they play critical roles in mIL-2Rγ function.

The SH2 domain was originally identified as sequences observed in an approximately 100 amino acid noncatalytic region of Src-related tyrosine kinases (Sadowski *et al.*, 1986). Subsequently, this domain has been determined to bind specifically to tyrosine-phosphorylated cellular proteins (reviewed in Koch *et al.*, 1991). In general, the motif that binds to the SH2 domain consists of a phosphotyrosine followed by two nonbasic polar residues and a large hydrophobic amino acid (Songyang *et al.*, 1993). By crystal structure analysis, the interaction between the Src SH2 domain and the phosphopeptide was determined to resemble a two-pronged plug (phosphopeptide) engaging a two-holed socket (SH2 domain; Waksman *et al.*, 1993). Two regions within the SH2 domain are functionally important for interacting with the

phosphopeptide. One such region is primarily concerned with binding to the phosphotyrosine. The second region supplies sites for binding the three residues immediately following the phosphotyrosine (Waksman *et al.*, 1993). The portion of the SH2 domain found in IL-2Rγ contains only the second binding region and thus is unable to bind phosphotyrosines. The data provided from crystal structure analysis coupled with the degree of conservation between human and mouse IL-2Rγ suggest that this SH2 subdomain homology is unlikely to be functionally important.

The expression pattern of mIL-2Rγ mRNA was determined by Northern blot analysis on various murine tissues and cell lines. The analysis revealed messages of approximately 1.6 kb corresponding to B6F1.3. A second unknown species was also detected at approximately 4 kb on some of the tested tissues and cell lines. This second less intense band was only observed in lanes that contained the 1.6 kb message. The origin of this higher molecular mass species is unknown however a similar finding was observed in the original studies in which the hIL-2Rγ was cloned (Takeshita *et al.*, 1992). The authors of that report also did not know the origin or identity of the second higher molecular mass species.

The expression of B6F1.3 mRNA appeared to be limited to the hemopoietic tissues and cell lines. The one exception observed was the low level of B6F1.3 expression in the bone marrow, a tissue compartment rich in immature hemopoietic cells. This finding agrees with the observation that cells isolated from the bone marrow while strongly CD44-positive, do not readily adhere to hyaluronan (data not shown).

The cellular response to IL-2 appears to be dependent upon the presence of IL-2Rγ (Taniguchi and Minami, 1993; Voss *et al.*, 1993; Asao *et al.*, 1993). While various combinations of the three distinct components that make up the IL-2 receptor complex can bind IL-2, only the high (Kd=10^{-11}M)- and intermediate (Kd=10^{-9}M)-affinity receptors consisting respectively of αβγ heterotrimers and βγ heterodimers can internalize IL-2 and transduce IL-2-mediated signals (Taniguchi and Minami, 1993; Voss *et al.*, 1993; Asao *et al.*, 1993).

Furthermore, Noguchi and colleagues (1993) mapped the gene encoding the human IL-2Rγ chain to chromosome Xq13 and demonstrated that mutations in this gene resulting in the generation of premature stop codons are responsible for XSCID (Noguchi *et al.*, 1993). Individuals with this condition have greatly reduced numbers of circulating T cells and suffer from severe and persistent infections (Cooper and Butler, 1989). Interestingly, despite being severely immunocompromised, IL-2 knockout mice appear relatively normal with respect to thymocyte and peripheral T cell subset composition (Schlore *et al.*, 1991). Thus, it is appears unlikely that the XSCID phenotype results simply from a loss of cellular responsiveness to IL-2.

Two independent groups attempted to find a link between the IL-2Rγ and the receptor for IL-4. Russell and coworkers (1993) defined this link on the basis of chemical cross-linking data, the ability of IL-2Rγ to augment IL-4 binding affinity, and the requirement for IL-2Rγ in IL-4-mediated phosphorylation of insulin receptor substrate-1. Kondo and colleagues (1993) produced mAbs that reacted with IL-2Rγ. One such mAb designated TUGm2, was able to inhibit IL-2 to bind to the functional IL-2 receptors. TUGm2 also inhibited IL-4-induced cell growth and the high-affinity binding of IL-4 to the CTLL-2 mouse T cell line. One other mAb known as TUGm3, was isolated based on its ability to react with the IL-2Rγ chain cross-linked with IL-2. Interestingly, TUGm3 also immunoprecipitated the γ chain when cross-linked with IL-4. The combined studies of these two research groups strongly suggest that the IL-2Rγ chain is a functional component of the IL-4 receptor complex (Russell *et al.*, 1993; Kondo *et al.*, 1993).

Two reports also described an association of the IL-2Rγ with the receptor for IL-7. Noguchi and colleagues (1993) used chemical cross-linking data to demonstrate that IL-2Rγ was physically associated with the IL-7 receptor. This group further showed that the presence of IL-2Rγ enhanced both IL-7 binding affinity and the efficiency of IL-7 internalization. Kondo and coworkers (1994) again used the mAb TUGm3 for immunoprecipitation studies.

This experiment immunoprecipitated the IL-2Rγ chain when cross-linked with IL-7. These studies suggested a sharing of the IL-2Rγ chain with the IL-7 receptor complex.

Although the increase of CD44 adhesion by IL-2Rγ may or may not contribute to the phenotype of XSCID, the fact remains that regulation of CD44 binding activity is likely to be significant for many adhesion-dependent cellular processes *in vivo*. Therefore, the mechanism by which this regulation is mediated, is an important area of study. A positive control used in the hyaluronan adhesion studies was human CD44. The transfection of human CD44 into MOP8 cells demonstrated that simply overexpressing CD44 allowed the transfectants to bind to hyaluronan. To rule out the possibility that B6F1.3 transfection enhances cellular adhesion to hyaluronan simply by upregulating CD44 expression, several studies were performed. FACS analysis, and Western blot analysis were used to determine the levels of CD44 upon mIL-2Rγ expression. Both studies failed to demonstrate an increase in CD44 expression with mIL-2Rγ transient expression. Furthermore, Western blot analysis revealed that the molecular mass of CD44 was not changed. This observation suggested that higher molecular mass CD44 isoforms were not upregulated. A similar result was observed using FACS analysis on Jzen.B6F1.3-infected MOP8 cells. It is intriguing to note that for these retrovirally infected cells, CD44 expression appeared to be even somewhat lower than that on non-infected MOP8. This finding strongly suggests that upregulation of CD44 is not the mechanism by which mIL-2Rγ induces CD44-mediated adhesion to hyaluronan. Therefore, mIL-2Rγ must somehow "activate" CD44 in order to induce this adhesion to hyaluronan.

Since the cytoplasmic tail has the highest degree of sequence identity between the human and mouse IL-2Rγ molecules and contains the regions with homology to SH2 subdomains, it seemed likely that the intracellular domain may play a critical functional role in regulating CD44. Furthermore, studies of XSCID patients have demonstrated that a mutation in hIL-2Rγ resulting in the removal of as few as 62 amino acids from the carboxy-terminal end of the protein, was sufficient to be linked to XSCID (Noguchi *et al.*, 1993). To determine whether the cytoplasmic domain of mIL-2Rγ is important in the regulation of CD44-mediated

adhesion to hyaluronan, deletional mutagenesis was performed. A cytoplasmically truncated molecule designated B6F1.3ΔSH2 was produced and used to transfect MOP8 cells using the pCDM8 system described above. Incidentally, most (20 of 34 residues) of the region with homology to the SH2 subdomain were included in the deletion. Upon transfection, B6F1.3ΔSH2 failed to induce hyaluronan binding in MOP8 cells. Thus this finding implicated the cytoplasmic domain of mIL-2Rγ as a critical player in regulating the function of CD44.

Previous studies by Asao *et al* (1993) have demonstrated that in fibroblasts, co-expression of the IL-2Rγ chain with IL-2Rα and IL-2Rβ or IL-2Rβ alone can activate tyrosine kinases and induce expression of the protooncogenes c-myc, c-fos and c-jun following IL-2 stimulation. Importantly, in the absence of exogenous IL-2, co-expression of IL-2Rγ with both IL-2Rα and IL-2Rβ or IL-2Rβ alone induced the expression of low levels of c-jun (Asao *et al.*, 1993). Such induction required the presence of the SH2 subdomain of IL-2Rγ and did not occur if this was deleted (Asao *et al.*, 1993). Whether c-jun is involved in the activation of CD44 in B6F1.3 transfected MOP8 cells remained unclear.

To delineate a possible connection between the proto-oncogene c-jun and the induction of hyaluronan binding by mIL-2Rγ, the effect on the expression of c-jun in MOP8 cells upon infection with Jzen.B6F1.3 was determined. Northern blot analysis demonstrated that an approximately 2.5 kb c-jun message was observed in the RNA of the retrovirally-infected cells. Only low level expression of c-jun was seen in the RNA of non-infected MOP8 cells. This finding suggests that c-jun may act as a downstream signal following stimulation of the IL-2Rγ leading to the "activation" of CD44 such that a hyaluronan binding state is achieved.

To determine if the protooncogene c-jun alone can also induce hyaluronan binding, human c-jun was inserted into the expression vector pCDM8, and transfected into MOP8 cells. These transfectants demonstrated significant induction of hyaluronan binding that was readily inhibited by anti-CD44 antibodies. Furthermore, the expression of CD44 molecules on the cell surface was not altered suggesting that upregulation of the adhesion protein was not the mechanism by which enhancement occurs.

These finding suggest a link between the proto-oncogene c-jun and the enhancement of CD44-mediated hyaluronan binding. c-jun is a member of a group of cellular genes termed "immediate early genes" along with c-myc, c-fos and c-rel that is rapidly induced when resting cells are treated with mitogens. It has been suggested that the proteins encoded by these genes are required to initiate a cascade of gene induction events that will allow a cell to enter S phase of the cell cycle (reviewed in Hunter, 1991).

The c-Jun molecule is a sequence-specific DNA binding protein that together with c-Fos, forms a component of the AP-1 transcription factor. This transcription factor recognizes a short sequence (TGACTCA) that can be found in the promoter/enhancer region of many genes (Angel *et al.*, 1987; Bohmann *et al.*, 1987; Bohen and Curran, 1988; Lee *et al.*, 1987; Piette and Yaniv, 1987; Rauscher *et al.*, 1988b; Struhl, 1987; Vogt *et al.*, 1987). This heptameric sequence was originally found in the enhancer of SV40, but since has been identified in promoters and enhancers of cellular genes including CD44 (Shtivelman and Bishop 1991). AP-1 is a nuclear factor required to mediate transcription induced by phorbol ester tumour promoters (such as TPA) via the protein kinase C pathway (Angel *et al.*, 1987; Lee *et al* 1987). Furthermore, an AP-1 binding site confers TPA inducibility upon a target gene (Angel *et al.*, 1987; Lee *et al.*, 1987) and thus this binding site is designated TPA response element or TRE sequence.

c-Jun was initially isolated as a component of the AP-1 nuclear factor by the demonstration that antibodies to its viral counterpart v-Jun, cross-react with AP-1. The cloning of the c-jun gene and the demonstration that its product is present in AP-1 preparations soon followed (Bohmann *et al.*, 1987). A deletion of a sequence of 27 residues called the δ region from c-Jun as well as individual point mutations in the remaining region generated v-Jun. Furthermore, an extensive 3' untranslated region that is present in c-jun mRNA is missing in v-jun transcripts.

The c-Jun protein has several features that are important for c-Jun to activate transcription. Three essential regions are located at the C-terminal end of the protein. A

leucine zipper motif is found and may allow for dimerization to occur. Dimer formation is critical for binding to DNA. The second significant structure is a basic region immediately adjacent to the leucine zipper. This region is also required for DNA binding. Thus a mutation in either of these domains would abolish the ability of c-Jun to bind DNA. The final important domain designated A2, is a proline-rich region that is necessary to activate transcription.

The transcription factor AP-1 is one of the best known examples of cooperating oncogenes. AP-1 is composed of a heterodimeric combination of c-Jun and c-Fos proteins or a homodimeric pairing of c-Jun proteins. The ability of c-Jun to cooperate with c-Fos to activate transcription has been shown by cotransfection experiments (Angel *et al.*, 1987; Bohmann *et al.*, 1987; Curran and Franza, 1988; Franza *et al.*, 1988; Lee *et al.*, 1987; Piette and Yaniv, 1987; Rauscher *et al.*, 1988a; Struhl, 1987; Vogt *et al.*, 1987). Furthermore, the heterodimers produced by the combination of c-Jun and c-Fos have been demonstrated to activate transcription more effectively that the c-Jun homodimer (Chiu, R. *et al.*, 1988; Sassone-Corsi *et al.*, 1988). c-Fos alone cannot dimerize and thus does not have the ability to bind DNA. This suggests that the role of c-Fos may be to increase the DNA-binding efficiency of c-Jun.

The mechanism by which the IL-2Rγ molecule functions to "activate" CD44 is still not well defined. Several potential models may exist leading to the activation of CD44 including a direct association between IL-2Rγ and CD44 on the cell surface or a possible pathway involving the upregulation of c-jun expression by IL-2Rγ causing the induction of other regulating proteins. The direct association model can be tested simply by immunoprecipitating with anti-CD44 or anti-IL-2Rγ antibodies or chemical crosslinking studies. This mechanism is unlikely to occur as structural motifs involved in coupling are absent in the two proteins.

The second model is more complex as the proto-oncogene c-Jun is involved. Since c-Jun and IL-2Rγ are found in the nucleus and on the surface of cells respectively, intermediate molecule must exist to deliver linking signals. The identity of these signal transduction molecules are unknown, however, a cascade of kinases and phosphatases are likely to be involved. Upon transmitting this IL-2Rγ induced signal across the nuclear membrane,

upregulation the c-Jun protein could then produce a Jun-Fos complex capable of activating transcription of a variety of genes. The proteins generated subsequently may directly or indirectly "activate" CD44. Proteins that phosphorylate the cytoplasmic domain of CD44 would directly influence the function of CD44. c-Jun-induced transmembrane proteins could also mediate the activity of CD44 by association on the cell surface. Coimmunoprecipitation experiments using anti-CD44 could identify molecules that act in this manner. Western blot analysis using anti-phosphotyrosine could also be important to resolve messengers in this pathway.

The major difference between the two mechanisms is the absence of c-Jun in the direct association model. Whether or not c-Jun plays an important role in this regulation can be investigated by using other procedures to induce the expression of this proto-oncogene and the subsequent influence on CD44-mediated hyaluronan binding tested. One particular method to upregulated c-jun would be to X-ray treat the MOP8 cells.

It is important to note that the "activation" of CD44 by IL-2Rγ may be solely due to the ectopic expression of the γ chain in the fibroblastoid cell line. The IL-2Rγ protein is observed in a variety of hemopoietic cell types, however, these cells may not mediate adhesion to hyaluronan. The studies presented in this thesis do not provide evidence to suggest any effect by IL-2Rγ on the interaction of hemopoietic cells to hyaluronan.

In summary, a novel expression cloning strategy demonstrated that the mIL-2Rγ chain regulates CD44-mediated adhesion to hyaluronan. This cloning system has the potential to be used to isolate additional regulatory molecules involved in the activation of CD44 and other adhesion molecules. The present studies suggest that induction of hyaluronan binding may involve the cytoplasmic domain of IL-2Rγ as well as induction of the proto-oncogene c-jun. The findings presented in this thesis may prove to be important in future studies regarding the function of CD44 in its various roles including tumour metastasis, lymphocyte homing and hemopoiesis.

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