MOLECULAR INTERACTIONS AND SIGNALING MEDIATED BY THE CELL ADHESION MOLECULE CD44

By

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Date September 19, 2001
ABSTRACT

CD44 mediates cell adhesion and cell migration in leukocytes. Most of these functions involve the interaction of cell surface CD44 with one of its most characterized ligands, hyaluronan or HA, a component of the extracellular matrix. The ability of CD44 to bind HA is strictly regulated in leukocytes such that not all CD44-expressing cells will bind HA. All three domains of CD44, the extracellular domain, the transmembrane domain, and the cytoplasmic domain, regulate HA binding. The extracellular domain of CD44 has been studied extensively for its regulation on HA binding, however, the mechanisms by which the transmembrane and the cytoplasmic domains of CD44 influence HA binding are poorly understood. In this study, therefore, I have focused on the roles for the transmembrane and cytoplasmic domains of CD44 in regulating HA binding and signaling in leukocytes.

Using chimeric CD44 molecules, the transmembrane domain of CD44 was shown to be responsible for the non-covalent association of CD44 in the plasma membrane of T cells. This self-association of CD44 was found to facilitate HA binding. In addition, CD44 was also observed to associate with the cytoskeletal components, ezrin/radixin/moesin (ERM), with Src-family kinases, Lck and Fyn, and with an adapter protein, Grb-2. CD44 interacted with ERM proteins in T cells and myeloid cells. In vitro, this interaction required a low ionic strength and the presence of EDTA, as well as the integrity of actin filaments. The interactions of CD44 with Src-family kinases and Grb-2 took place in T cells. CD44 interacted with the Src-family kinase Lck in the membrane lipid microdomains, which are important for receptor signal transduction. In BW5147 T cells, this CD44-Lck interaction, as well as the distribution of Lck in lipid microdomains, is negatively regulated by CD45, a leukocyte specific tyrosine phosphatase. More strikingly, in the absence of CD45, BW5147 T cells underwent cell spreading concomitant with the tyrosine phosphorylation of Pyk2 and FAK upon stimulation by an anti-CD44 antibody. Inhibition of the activity of Src-family kinases abolished CD44-
mediated cell spreading as well as the tyrosine phosphorylation of Pyk2 and FAK. Therefore, signals via cell surface CD44 may induce the tyrosine phosphorylation of Pyk2 or FAK through the interaction with Src-family kinases, which then triggers downstream cellular events, leading to cell spreading. CD45 acts as a negative regulator of this process by inhibiting the molecular interaction of CD44 and Src-family kinases.

In summary, this study identified a regulatory mechanism for HA binding by the transmembrane domain of CD44, as well as the molecular interactions and the signaling events mediated by CD44 in leukocytes.
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<tr>
<th>Abbreviation</th>
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>b-FGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney cell</td>
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<tr>
<td>bp</td>
<td>base-pair</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CD44H</td>
<td>CD44 standard or haematopoietic isoform</td>
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<td>cDNA</td>
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<tr>
<td>cyt</td>
<td>cytoplasmic domain</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DTSSP</td>
<td>dithiobis(sulfosuccinimidylpropionate)</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGTA</td>
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<td>ERM</td>
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</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<td>ICAM-1, 2, 3</td>
<td>intercellular cell adhesion molecule 1, 2, 3</td>
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<td>MLC</td>
<td>myosin light chain</td>
</tr>
<tr>
<td>MLR</td>
<td>mixed lymphocyte reaction</td>
</tr>
<tr>
<td>ND</td>
<td>not determined</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OG</td>
<td>n-octyl-β-D-glucopyranoside</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PTPase</td>
<td>phosphatase</td>
</tr>
<tr>
<td>PY</td>
<td>phosphotyrosine</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated upon activation, normally T cell expressed and secreted, a chemokine</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SVEC</td>
<td>small vascular endothelial cell</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TCS</td>
<td>tissue culture supernatant</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>TSG-6</td>
<td>tumor necrosis factor stimulated gene-6</td>
</tr>
<tr>
<td>TX-100</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>Ura</td>
<td>uracil</td>
</tr>
<tr>
<td>Val</td>
<td>valine</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
</tbody>
</table>
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DEDICATION

To Jiucheng
For being an understanding and helpful husband

To Kelly and Justin
For great pleasure after daily experiments and for reminding me of
what a lovely family I have!

To Mum and Dad
For everything you give me

To Qiangqiang
Brother and friend
CHAPTER ONE

Introduction

1.1 Cell adhesion and cell migration

Cell adhesion and cell migration are two fundamental biological processes, both of which are highly regulated in multicellular organisms. Cell adhesion includes cell-cell adhesion and cell-extracellular matrix (ECM) adhesion. Cell migration refers to the movement of cells towards their designated destinations. The cells in vertebrates are usually in contact with a complex network of secreted extracellular macromolecules known as the ECM. ECM helps to hold cells together, and in animals it provides an organized lattice within which cells can interact with each other and migrate. By cell adhesion and cell migration, most of the cells in the embryos of multicellular organisms are organized into cooperative assemblies known as tissues, which are then associated in various combinations to form larger functional units known as organs. The processes of cell adhesion and cell migration are not random events, but involve strictly controlled mechanisms. In vertebrate embryos, for example, cells from the neural crest break away from the epithelial neural tube with which they are initially associated and migrate along specific paths to many other regions. There they assemble with other cells and with one another and differentiate into a variety of tissues, including those of the peripheral nervous system. In a second example, during tissue regeneration or tissue remodeling in wound healing and angiogenesis, the cells do not simply accumulate as a disorderly pile. Instead, the tissue architecture is actively maintained by selective adhesions that the cells make and progressively adjust. Thus, a key concept in cell adhesion and cell migration is its regulation by which only selective cell adhesions and cell migrations can occur. This is also manifested by leukocyte adhesion and migration in the immune system.
1.2 Cell adhesion and cell migration in the immune system

The interaction of leukocytes with other cell types during haemopoiesis, lymphocyte activation, lymphocyte recirculation, and leukocyte extravasation to the sites of inflammation, is a typical example of the cell-cell or cell-ECM adhesion, and can therefore, provide excellent opportunities to study the cell biology of these dynamic processes. The most striking characteristic of leukocyte adhesion and migration is its regulation. For example, as a subset of leukocytes, circulating lymphocytes rapidly interconvert between a nonadherent state in circulation and an adherent state in tissues (Figure 1.1). This cycle is repeated many times over the life span of lymphocytes in order to first, support their survival and differentiation, and second, target them to sites of antigenic or microbial invasion. Disruption of the well-controlled adherent or nonadherent state of lymphocytes can lead to the development of many pathological conditions, including inflammatory disorders and atherosclerosis (reviewed in refs. 1, 2). Therefore, to study how leukocytes are regulated to interact with other cell types in the immune system will substantiate our knowledge on cell adhesion, and will help delineate the signals required to initiate cell migration. Some key events of leukocyte adhesion and migration are those that occur during the processes of haemopoiesis, T lymphocyte activation and leukocyte extravasation at the site of inflammation.

1.2.1 Adhesion between haemopoietic precursor cells and bone marrow stromal cells

The bone marrow provides an essential microenvironment for the generation and development of blood cells. Haemopoietic stem cells, precursor cells and different types of blood cells are intermingled with one another, as well as with the stromal cells that produce a delicate supporting meshwork of collagen fibers and other ECM components. The cellular interaction between primitive haemopoietic progenitor cells and bone marrow stromal cells is mediated by cell adhesion molecules, and is required for the development of haemopoietic cells
Figure 1.1. Diagram of lymphocyte circulation in the immune system. Lymphocytes circulating in each place are indicated. Arrows indicate the flow of circulation.
In B cell development, for example, the contribution of the stromal cells is twofold. First, they form specific adhesion contacts with the developing B-lineage cells by interactions between cell adhesion molecules and their ligands. Second, they provide growth factors for the differentiation of B cells. For instance, the membrane-bound stem cell factor (SCF) is recognized by the surface receptor kit on early B-lineage cells, whereas the secreted interleukin-7 (IL-7) is recognized by late pro-B and pre-B cells (4). The relationship between B cell precursors and the bone marrow microenvironment provides a model system for cell-cell interactions, which may be applicable to progenitor development in other haemopoietic lineages.

1.2.2 Adhesion between T cells and antigen presenting cells (APCs)

The initial interaction of T cells with APCs is achieved by cell-cell adhesion, which facilitates the activation of T cells through the antigen receptor. When naïve T cells migrate through the cortical region of the lymph node, they bind transiently to each APC they encounter. Professional APCs, and dendritic cells in particular, bind naïve cells very efficiently through interactions between cell adhesion molecules including integrins and immunoglobulin (Ig) superfamily molecules. These cell adhesion molecules will be introduced in detail in 1.3. The transient binding of naïve T cells to APCs is crucial in bringing T cells and APCs into close proximity (around 15 nm), and in providing time for T cells to sample large number of MHC molecules on the surface of APCs for the presence of specific peptide (reviewed in ref. 5). In those cases where a naïve T cell recognizes its specific peptide: MHC ligand on the APC, signaling through the T cell receptor (TCR) is triggered. Subsequent TCR signaling can induce a conformational change in cell adhesion molecules, and these changes stabilize the association between the antigen-specific T cells and APCs. The association can persist for several days during which the naïve T cell proliferates and its progeny, which also adhere to the
APC, differentiate into armed effector T cells (reviewed in refs. 6, 7). Therefore, the adhesion of T cells to APCs plays a pivotal role in T cell activation.

1.2.3 Leukocyte adhesion and transmigration across the endothelial vascular wall

The process of lymphocyte homing requires the adhesion of lymphocytes to specialized endothelial cells called high walled endothelial venules (HEVs) in the lymph nodes. Lymphocytes are first slowed down by the interaction of cell adhesion molecules called selectins (will be introduced in detail in 1.3) expressed on lymphocytes and selectin ligands which are expressed on HEVs. This “rolling” step then allows the firm adhesion between lymphocytes and HEVs via integrins and Ig superfamily cell adhesion molecules (the processes of “adhesion” and “arrest”). Upon attachment to the HEVs, signals are sent to the lymphocyte to facilitate its migration through the endothelial layer, a process known as diapedesis (reviewed in ref. 8).

Similar to the process of lymphocyte homing, during an inflammatory response, neutrophils, monocytes, and activated T lymphocytes need to leave the circulation and enter tissues. This extravasation process also requires the interaction between leukocytes and endothelial cells. Inflammatory agents, such as cytokines, chemokines, or bacterial toxins released at the inflammatory site, cause the nearby microvascular endothelium to become activated. This results in the increased cell surface expression or activation of several types of cell adhesion molecules, such as Ig superfamily molecules and selectins. The binding of adhesion molecules on leukocytes to their ligands expressed on endothelial cells then recruits the activated leukocytes from circulation to the microvasculature by mediating leukocyte rolling, adhesion, arrest and diapedesis (Figure 1.2 and ref. 8).
Figure 1.2. Cell adhesion molecules and their ligands involved in leukocyte-endothelial cell interactions. The molecules implicated in leukocyte adhesion to endothelial cells in the lymph node or at inflammatory sites are illustrated. N-linked oligosaccharides are indicated by (○), O-linked oligosaccharides by (~~), and molecules that are known to be sulfated are indicated with a (★). Adapted from ref. 11.
1.3  Cell adhesion molecules in the immune system

Four classical families of cell adhesion molecules mediate the general process of cell adhesion and cell migration including those that occur in the immune system. They are cadherins, selectins, integrins, and molecules of the immunoglobulin superfamily. CD44 is a novel member of the cell adhesion molecule family, as mounting evidence suggests that it participate in similar biological events as other cell adhesion molecules (reviewed in refs. 9-11).

1.3.1  Cadherins

Cadherins are responsible for Ca\(^{2+}\)-dependent cell-cell adhesion in vertebrate tissues, such as the cell-cell junctions. They mediate cell-cell adhesion by a homophilic mechanism (molecules on one cell bind to other molecules of the same kind on adjacent cells). It has been well established that cadherins exert more important functions in embryonic development and maintenance of solid tissue architecture than in mediating leukocyte adhesion (reviewed in ref. 12). However, a recent study provided some novel evidence that N-cadherin may be involved in early haemopoietic cell differentiation by mediating interactions between haemopoietic precursor cells and bone marrow stromal cells (13).

1.3.2  Selectins

Selectins function in a variety of Ca\(^{2+}\)-dependent transient cell-cell interactions in the bloodstream. They are expressed on leukocytes (L-selectin), platelets (P-selectin) and endothelial cells (P- and E-selectin). Selectins bind carbohydrate moieties on the surface of leukocytes or endothelial cells, including the tetrasaccharide sialyl Lewis\(^x\) and the heavily glycosylated mucin-like proteins GlyCAM-1, CD34 and MAdCAM-1 (14). The interaction of selectins with their carbohydrate ligands enables leukocytes to bind transiently to endothelial
cells lining small blood vessels (the “rolling” process) in order to prepare for the transmigration across the HEVs during lymphocyte homing, as well as for the leukocyte extravasation at sites of inflammation.

1.3.3 Integrins and immunoglobulin (Ig) superfamily molecules

Integrins mediate the Ca\(^{2+}\)- or Mg\(^{2+}\)-dependent attachment of cells to the extracellular matrix (ECM). Some integrins can also bind cells together through heterophilic interactions (molecules on one cell bind to molecules of a different kind on adjacent cells). The cell adhesion molecules of the Ig superfamily are responsible for most Ca\(^{2+}\)-independent cell-cell adhesion in vertebrates (reviewed in refs. 15, 16). Integrins and Ig superfamily molecules are expressed on most tissues in vertebrates. They comprise a large number of cell surface proteins that mediate adhesion in immune and inflammatory responses in addition to their important roles in tissue organization during embryonic development. For example, the interaction of lymphocyte function-associated antigen-1 (LFA-1), an integrin expressed on T lymphocytes, to intercellular adhesion molecule-1 (ICAM-1), a member of the Ig superfamily that is expressed on APCs, is critical for the initial interaction of T cells and APCs (reviewed in ref. 5). Moreover, the interaction of LFA-1 and ICAM-1 is also important for the firm adhesion of leukocytes to the endothelial vascular wall after the “rolling” step (reviewed in ref. 8). In another in vitro study, the interaction of very late antigen-4 (VLA-4), another integrin with the vascular cell adhesion molecule-1 (VCAM-1), a member of the Ig superfamily, has been demonstrated to be essential for the adhesion of haemopoietic progenitor cells to bone marrow stromal cells during B cell development (4, 17). Therefore, integrins and Ig superfamily molecules carry out a broad range of functions in the immune system that regulate leukocyte adhesion and migration in different developmental stages and activation states.
1.3.4 CD44

Compared to cadherins, selectins, integrins and Ig superfamily molecules, CD44 is a newly identified cell adhesion molecule. It interacts with components from the ECM in a Ca²⁺-independent manner. In the immune system, the functions of CD44 overlap with those mediated by selectins, integrins and Ig superfamily molecules. For example, CD44 has been implicated in the process of leukocyte “rolling” along the endothelial vascular wall, mimicking the function of selectins (18-21). Like integrins and Ig superfamily molecules, CD44 is also involved in the adhesion of haemopoietic progenitor cells to bone marrow stromal cells during haemopoiesis (22-29). Furthermore, similar to the interaction of LFA-1 and ICAM-1, signals transmitted through CD44 cooperate with anti-CD3 stimulation in the process of lymphocyte activation (30-34). However, unlike other cell adhesion molecules, which have been well characterized, CD44 is a new cell adhesion molecule, and much remains to be learned about its functions, signaling pathways, and the regulation of its ligand binding ability. By comparing the similarities and differences of CD44 with other cell adhesion molecules such as selectins, integrins, or Ig superfamily molecules, insight can be gained into the functions of CD44.

1.4 CD44 structure

CD44 is a transmembrane glycoprotein. It is broadly distributed not only on haematopoietic cells, but also on non-haematopoietic cells, including keratinocytes, chondrocytes, epithelial cells, endothelial cells, and neuronal cells. First synthesized as a 37 kDa core protein, CD44 can undergo extensive modifications by N- and O-linked glycosylation, sulfation, and addition of glycosaminoglycan (GAG) side chains, leading to a size ranging from 80 to 200 kDa (reviewed in refs. 9, 35). In addition, CD44 can also be modified by insertion of up to 10 alternatively spliced exons that encode portions of the extracellular domain (36). The predominant isoform of CD44 is the 85-90 kDa glycoprotein (CD44H) that does not contain any of the differentially spliced exons. CD44 isoforms
containing these spliced exons are usually referred as CD44v. CD44H consists of a ~270 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 72 amino acid cytoplasmic domain (Figure 1.2 and ref. 37).

1.4.1 The extracellular domain

The N-terminal 168 amino acids of the CD44 extracellular domain are highly conserved among mammalian species, showing 80-90% sequence identity. Within this region, there are 6 cysteine residues, which are disulfide bonded, and 5 conserved N-glycosylation consensus sequences. It also contains the “link module”, a region that is ~35% identical to cartilage link proteins and other HA-binding proteins (38, 39). The proposed structure of the link module on CD44 is based on the solution structure of this region from the tumor necrosis factor stimulated gene-6 (TSG-6) that was determined by Nuclear Magnetic Resonance (NMR). This revealed the structural similarity of the link module from TSG-6 or CD44 to that of the calcium-dependent (C-type) lectin, such as the mannose-binding protein and the selectins (40, 41). The structural similarity of CD44 to selectins may explain why CD44 binds carbohydrate moieties on its ligands and participates in the “rolling” process during lymphocyte homing or leukocyte extravasation. Mutagenesis studies using a CD44 immunoglobulin fusion protein (CD44-Ig) showed that the critical residues whose mutation abolished HA binding were two pairs of amino acids, Arg 41 and Tyr 42, and Arg 78 and Tyr 79. These 4 amino acids form a cluster at the center of the proposed HA binding site. Other important residues, such as Lys 38, Lys 68, Asn 100, Asn 101 and Tyr 105, reside near the center of the HA binding site, and form a continuous surface along a ridge on the protein surface (40). In another study, Young et al. proposed that the two B(X7)B motifs (where B is Arg or Lys, X is any non-acidic amino acid, and X7 includes one additional Arg or Lys) in the N-terminal 168 amino acids were required for HA binding (see figure 1.3 and ref. 38). However, one of the B(X7)B motifs is outside of the link module, providing an alternative view for the structural basis for HA binding. The
Regions and sites of interest are represented as follows:

- +++++ Site of glycosaminoglycan addition
- • Potential O-linked glycosylation site
- ≥ N-linked glycosylation site
- s—s Potential disulfide bond
- ♂ Potential site of phosphorylation

**Figure 1.3. Schematic diagram of the CD44H molecule.** B(X₇)B is a motif known to play a role in HA binding. B represents a basic amino acid, and X is a neutral or basic amino acid. The amino acids are numbered according to the human CD44H sequence. Adapted from ref. 11.
membrane proximal region of the extracellular domain is less conserved, showing only ~35% sequence identity among mammalian species. It contains potential sites for carbohydrate modification, addition of GAG side chains (42-45) and a site for insertion of up to 10 alternately spliced exons (44, 46, 47).

1.4.2 The transmembrane domain

The transmembrane domain of CD44 is highly conserved, showing 80-90% sequence identity in human, mouse, rat, baboon, cow and hamster. This domain was shown to be responsible for the insolubility of CD44 upon cell lysis with the nonionic detergent, Triton X-100 (TX-100). The detergent insolubility of CD44 was interpreted as an association of CD44 with actin cytoskeleton (48), or with lipid rafts (49). These lipid rafts are specialized membrane microdomains enriched in glycosphingolipids, cholesterol, and glycoposphoinositol (GPI)-anchored proteins, and are believed to play important roles in membrane receptor signaling. In addition, the cysteine residue in the transmembrane region was also shown to mediate a disulfide bonded dimerization of CD44 upon PMA stimulation in Jurkat T cells (50).

1.4.3 The cytoplasmic domain

The cytoplasmic domain of CD44 (CD44cyt) is also highly conserved among mammalian species mentioned in 1.4.2 (~80-90% sequence identity). It does not contain any tyrosine residues, but has six serines, four of which are conserved (37). As for other cell adhesion molecules, this domain does not exhibit any enzymatic activity. It was shown in some cases that the CD44cyt was required for HA binding (18, 35). In addition, the CD44cyt is also needed for proper sorting of CD44 in polarized cells. In MDCK cells, for example, CD44 was excluded from the apical region, and localized to the basolateral surface of the cell. This
preferential distribution of CD44 to the basolateral surface was mediated by a di-peptide leucine-valine (LV) motif in the cytoplasmic domain of CD44 (51, 52).

1.5 CD44 ligands

Most CD44 ligands are components of the ECM. Hyaluronan (HA), is the best-characterized ligand for CD44 (23, 53-55). It is a high molecular weight GAG found in the ECM of most vertebrate tissues. Serglycin, another ligand of CD44, is a haematopoietic cell lineage-specific proteoglycan, and binds to CD44 via its chondroitin sulfate side chains (56-58). Another GAG, chondroitin-4-sulfate, once attached to a protein backbone, also binds to certain isoforms of CD44 (59). Free chondroitin-4-sulfate binds to CD44 poorly, however, binding avidity can be increased when multiple chondroitin-4-sulfate side chains are presented in a protein such as serglycin and the invariant chain of class II (56, 57, 60). Osteopontin is a cytokine-like molecule present in the ECM. It associates with CD44 in a Ca\(^{2+}\) and Mg\(^{2+}\)-independent manner, whereas it associates with \(\alpha v\beta 1\) and \(\alpha v\beta 5\) integrins in a Ca\(^{2+}\)- and Mg\(^{2+}\)-dependent manner (61). Certain types of fibronectin (62) and collagen (63) have also been demonstrated to bind CD44, in addition to binding to integrins. Some cytokines (i.e. MIP-1\(\beta\) and bFGF) can be immobilized by cell surface CD44 through association with GAGs attached to the CD44 external domain (64-66).

HA has received considerable attention in recent years due to its profound influence on cell behavior. It consists of repeating disaccharide units composed of \(\beta(1\rightarrow4)\)-D-glucuronic acid-\(\beta-(1\rightarrow3)\)-N-acetyl-D-glucosamine (Figure 1.4). The HA level within the ECM is strictly regulated by cellular hyaluronidase and receptor-mediated endocytosis (67). The ECM becomes enriched in HA during periods of rapid cell proliferation, aggregation, and migration in the processes of embryogenesis, tissue regeneration and remodeling, inflammation, as well as tumor cell invasion (68, 69). These HA-induced effects on cells are in turn supported and directed by a wide variety of HA-binding proteins like CD44 (70). The CD44-HA interaction
Hyaluronan (HA)

Figure 1.4. Structure of the repeating disaccharide of hyaluronan. $n$ is the number of repeating disaccharides and can range from 1 to over 1000.
is implicated in most CD44-mediated cell adhesion and migration including the interaction
between haemopoietic progenitor cells and bone marrow stromal cells (22, 23) and the initial
attachment of leukocytes to endothelial cells in vitro (18-21, 71). However, in vivo, the role of
HA in mediating lymphocytes to HEV still remains to be clarified. This is because some anti-
CD44 antibodies, which recognize the membrane proximal region of CD44 other than the HA
binding site, block the attachment of lymphocytes to HEV (72, 73). Moreover, treatment of
HEV with hyaluronidase or with an anti-CD44 mAb that blocks the HA binding site on CD44,
do not abolish binding of murine lymphocytes (55). Of note, even though CD44 is widely
expressed on the surface of many cell types, not all CD44-bearing cells can adhere to HA (74,
75). Based on their state of HA recognition, CD44-containing cells are divided into three
groups: (i) those able to bind HA constitutively; (ii) those that can be induced to bind HA by
various stimuli or anti-CD44 mAbs; and (iii) those unable to bind or be induced to bind HA
(76). These varied states of HA binding reflect a strict regulatory characteristic of CD44-
mediated cell adhesion to HA, which also mimics the adhesion of other cell adhesion molecules
(i.e. selectins and integrins) to their ligands.

It is important to mention here that other HA receptors on the cell surface in addition to
CD44, also play important roles in HA-mediated cell adhesion, motility and proliferation.
RHAMM, for instance, is another receptor for HA (77). It is expressed at very low levels in
quiescent cells but becomes markedly up-regulated during cell migration. In T lymphocytes,
fibroblasts and malignant cells, the HA-RHAMM interaction promotes cell locomotion via
signaling pathways that involves pp60\(^{src}\) and p125\(^{FAK}\) (70, 78). LYVE-1, a new CD44
homologue identified two years ago, is another lymph-specific receptor for HA. Like CD44,
the LYVE-1 binds both soluble and immobilized HA. However, unlike CD44, LYVE-1 co-
localizes with HA on the luminal face of the lymph vessel wall and is completely absent from
blood vessels. The HA-LYVE-1 interaction was shown to be important for the uptake of HA
by lymphatic endothelial cells (79).
1.6 CD44 function

CD44 is implicated in a variety of biological processes including embryonic development, wound healing, angiogenesis, haemopoiesis, T lymphocyte activation, lymphocyte homing, leukocyte extravasation at the inflammatory site, and tumor metastasis (reviewed in refs. 9, 11, 35, 80). In the immune system, as mentioned previously in 1.3.4, accumulating evidence indicates that CD44 plays important roles in the process of leukocyte adhesion at various stages of development and activation. These processes include the adhesion between haemopoietic progenitor cells and bone marrow stromal cells during haemopoiesis, the cellular interactions during T lymphocyte activation, and the interaction of leukocytes and endothelial cells during leukocyte extravasation and lymphocyte homing. Here, I will focus on the functions of CD44 in the immune system. However, since the distribution of CD44 is not entirely restricted to haemopoietic cells, it cannot be neglected that CD44 also mediates other important functions, such as cell adhesions during embryonic development, tumor metastasis, tissue regeneration and remodeling.

1.6.1 A Role for CD44 in bone marrow cell adhesion

Like integrins and Ig superfamily molecules, CD44 has been shown to mediate the adhesion of haemopoietic progenitor cells to bone marrow stromal cells. When CD44 was first identified as a cell surface receptor for HA in 1990, the CD44-HA interaction was found to be important for the adhesion of immature B cells to bone marrow stromal cells (22, 23). Subsequent in vitro studies showed that anti-CD44 antibodies, which block the interaction of progenitor cells and bone marrow stromal cells, also negatively regulate the development of CD34+ bone marrow-derived cells (24-27). In contrast, anti-CD44 antibodies, which enhance the interaction of progenitor cells and stromal cells, positively influence stromal cell-supported haemopoiesis (28, 29). The underlying mechanisms that regulate CD44-mediated adhesion...
between progenitor cells and bone marrow stromal cells are not clear, but possibly involve the generation of different isoforms of CD44 on progenitor cells. Rosel et al. reported an intermolecular interaction of CD44 variant isoform v10 on a progenitor cell and an adjacent stromal cell. This intermolecular interaction was shown to influence progenitor maturation, particularly of the B cell lineage (81). Additionally, another in vitro study showed that by mediating the interaction of progenitor cells with bone marrow stromal cells, the v7 isoform of CD44 played an important role in homing of these haemopoietic progenitor cells (82). In a third example, generation of CD44v4-v10, a ligand binding isoform of CD44, was shown to be a key regulatory event during human myelopoiesis (83). In accordance with these findings, a deficit in the egress of myeloid progenitor cells from bone marrow has been described in CD44 knockout mice (84). Clearly, CD44 exerts important functions in the interaction of haemopoietic progenitor cells with bone marrow stromal cells during haemopoiesis, and this field is currently being actively investigated.

### 1.6.2 A role for CD44 as a co-stimulatory molecule in T lymphocyte activation

Another functional aspect of CD44 is its involvement in T lymphocyte activation. In a murine Th1 cell line and in freshly isolated murine splenic CD4+ T cells, co-ligation of CD44 and CD3 by monoclonal antibodies (mAb) enhances IL-2 production (85). Similarly, in human T lymphocytes, cross-linking CD44 by a mAb synergizes with anti-CD3 stimulation, and up-regulates IL-2 gene transcription and secretion (86, 87). The co-stimulatory effect of CD44 in TCR-induced T cell activation is thought to be mediated by the CD44-HA interaction. This is because pretreatment of T cells with an anti-CD44 mAb, which blocks the HA binding site on CD44, also inhibits the IL-2 receptor expression, the intracellular calcium mobilization, and the G1→S transition of cell cycle triggered by anti-CD3 stimulation (88). In addition to these in vitro studies, some in vivo studies using anti-CD44 antibodies also provided evidence that CD44 is involved in a number of immunological disorders involving chronic inflammation.
These include murine arthritis (89-91), inflammatory bowel disease, experimentally induced colitis (92), and experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (93). It was thought that in these diseases CD44 might transmit signals that contribute to the activation of effector T cells, resulting in tissue damage. Therefore, in summary, like CD2 and CD28, CD44 may act as a co-stimulatory molecule that delivers additional signals to enhance signal transduction via the TCR/CD3 complex (reviewed in ref. 94).

1.6.3 A role for CD44 in leukocyte-endothelium adhesion

Compared to the roles of CD44 in bone marrow cell adhesion and in T cell activation, the function of CD44 in leukocyte-endothelium interaction has attracted more attention. The first evidence that CD44 may play a role in this interaction was from an in vitro study. In order to identify a lymphocyte homing molecule in humans, monoclonal antibodies were generated that could block the adhesion of leukocytes to frozen sections of mucosal lymph node. This resulted in the generation of the Hermes-3 mAb, an anti-CD44 mAb (72), and suggested that CD44 may participate in the process of lymphocyte homing. In 1993, another interesting in vivo observation was made in a murine model of delayed-type hypersensitivity. The administration of anti-CD44 antibodies (which resulted in the loss of CD44 from the cell surface) delayed leukocyte infiltration at the cutaneous site, but had no effect on lymphocyte recirculation (95). This result implied that CD44 might participate in vivo in the initial phase of the delayed-type hypersensitivity response, possibly by facilitating leukocyte extravasation into the inflammatory sites. More recently, in vitro studies looking at leukocyte rolling under flow conditions identified an interaction between CD44 on activated lymphocytes and one of its ligands, HA, on an endothelial cell line (SVEC4-10) (19). Both anti-CD44 antibodies and exogenous HA could block lymphocyte rolling in these studies. Further experiments by Siegelman's group indicated that binding of CD44 to HA could be induced transiently in T
cells upon \textit{in vivo} superantigen stimulation. These cells exhibited CD44- and HA-dependent rolling and extravasation at the inflammatory site (20, 21). This is the strongest evidence to date for a role of CD44 and HA in T cell extravasation at an inflammatory site. Recent work by Maiti \textit{et al.} suggested that the CD44- and HA-mediated interaction of the myeloid cell line SR91 to the endothelial cell line SVEC4-10 is regulated in a sulfate-dependent manner upon stimulation of SR91 cells by TNF\(\alpha\) (71). This therefore provides insights on the regulation of leukocyte-endothelial cell adhesion by CD44.

\textbf{1.6.4 CD44 knockout mice}

Surprisingly, in spite of the high expression levels of CD44 during embryogenesis and its numerous roles in cell adhesion and cell migration, CD44 knockout mice appear to be normal in embryonic development and neurologic functions (84). When lymphocyte functions were evaluated in these CD44\textsuperscript{-/-} mice and compared to their littermates, CD44\textsuperscript{-/-} mice were found to have normal total serum Ig levels and normal levels of the various Ig subclasses. This suggested that B cells undergo normal class switching, and that cognate T:B interactions are not critically altered by the absence of CD44. Similarly, no difference was seen between CD44\textsuperscript{+/-} and CD44\textsuperscript{-/-} T lymphocytes in \textit{in vitro} lymphocyte proliferative assays in responses to various challenges such as soluble anti-CD3 mAb, cross-linked anti-CD3 mAb, staphylococcal enterotoxin B, and concanavalin A (ConA). Moreover, no defect was observed in the ability of CD44\textsuperscript{-/-} T lymphocytes to respond in mixed lymphocyte reactions (MLRs), primary \textit{ex vivo} cytotoxicity assays, delayed-type hypersensitivity tests, and tests of memory T lymphocyte development. However, a minor hematological impairment due to defective myeloid progenitor egress from the bone marrow was observed. In what was either a compensatory response to CD44 deficiency or an immuno-regulatory defect, CD44\textsuperscript{-/-} mice also develop an exaggerated granuloma response to \textit{Cryptosporidium parvum} infection. This reflects dysfunctions in monocytes and macrophages in these animals. One possible explanation for the
minimal immunological deficiency in mice lacking CD44 is that there may be some compensation by other adhesion molecules with functional similarities to CD44. These molecules include selectins, integrins and other lectins. Alternatively, other HA-binding proteins with structural similarities to CD44 may substitute for CD44. These proteins include RHAMM, LYVE-1, neurocan, versican, aggrecan, link proteins, and TSG-6.

1.7 Regulation of CD44-HA interaction in leukocytes

Most CD44-mediated functions involve an association with HA. Examples of these are the interaction of haemopoietic progenitors with bone marrow stromal cells (22, 23), the interaction of leukocytes with the endothelium (18-21, 71), and the cell migration process during tumor invasions (80, 96). Like other cell adhesion molecules, the binding of CD44 to HA is tightly regulated in a cell type-, differentiation stage-, and activation state-specific manner, such that not all cells that express CD44 will bind HA (35, 74). In resting leukocytes, CD44 is normally present in its non-binding form. It can be, however, converted to a ligand binding form upon appropriate activation, such as activation of T cells by antigen or PMA (21, 97-99), or activation of monocytes by inflammatory cytokines (100, 101). Thus, the regulated CD44-HA interaction may have profound impact on CD44-mediated leukocyte adhesion and migration. To date, it has been demonstrated that three domains of CD44, the extracellular domain, the transmembrane domain, and the cytoplasmic domain all contribute to regulate the CD44-HA interaction.

1.7.1 Regulation by the extracellular domain of CD44

One mechanism by which the extracellular domain of CD44 influences the CD44-mediated cell adhesion to HA is through post-translational modifications, including glycosylation, sulfation, addition of GAG side chains, and sialylation. For example, induction of HA binding after culturing B cells in IL-5 correlates with a decrease in N-glycosylation of
CD44 (102). Differences in N-linked glycosylation of CD44 have also been observed between resident (normal) and elicited (activated) macrophages, although in this case HA binding was not examined (103). In another study, however, no dramatic molecular weight changes of CD44 were observed in superantigen activated lymph node T cells which were induced to bind HA, indicating that glycosylation of CD44 might not occur during T cell activation (20). A less common post-translational modification, sulfation, was shown to be responsible for the CD44-mediated HA binding induced by TNFα in a myeloid cell line (71). This induction of HA binding is also responsible for adhesion of the myeloid cells to an SVEC4-10 endothelial monolayer, indicating a potential role of sulfation in CD44-mediated leukocyte-endothelium interaction. Changes in the addition of GAG side chains to CD44 have also been reported to regulate HA binding in monocytes. For example, modifications of the heparan sulfate and chondroitin sulfate on CD44 occur upon induction of cells by inflammatory cytokines, such as IL-1α, IL-4 and TNFα. These modifications then lead to an altered HA binding ability of the cell (101, 104). In one study, sialylation of CD44 was shown to negatively regulate HA binding in monocytes. Neuraminidase treatment of the human monocytic cell Line THP-I and peripheral blood monocytes increases receptor activity after overnight culture with LPS. The sialidase inhibitor, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, completely blocks the LPS-induced recognition of HA by THP-1 cells (105). Thus all these post-translational modifications can regulate the ability of CD44 to bind HA.

Another mechanism by which the extracellular domain of CD44 affects HA binding is through alternative splicing. In vivo, antigenic stimulation induces a transient expression of CD44 isoforms containing v6 in T cells, B cells, and macrophages (106). CD44v isoforms were also reported to be up-regulated after antigen or mitogen stimulated T cells (107, 108). It was suggested that CD44 isoforms containing v4-7 exhibit an increased HA binding capacity in monocytes (109), which also confers a metastatic potential to a non-metastatic cell line (110).
One possibility for the role of the variant exons in HA binding may be to provide potential sites for further glycosylation and GAG modification.

1.7.2 Regulation by the transmembrane domain of CD44

There are only a small number of studies that have looked at the function of CD44 transmembrane domain in regulating HA binding. However, results derived from these studies are somewhat contradictory. Liu et al. reported that in Jurkat T cells, the conserved cysteine residue in the transmembrane domain of CD44 is required for HA binding induced by anti-CD3 or anti-CD44 antibody stimulation. Mutation of this cysteine to alanine abolishes the HA binding capacity (111). Subsequent studies from the same group revealed that this cysteine residue mediates the dimerization of CD44 molecules through disulfide bond formation upon PMA stimulation. This dimerization of CD44 correlates with the induced HA binding ability in these cells (50). In a mutational study, however, a CD44 mutant bearing the CD45 transmembrane region, which contains no cysteine residue, exhibit similar levels of HA binding compared to the wild type CD44 when transfected into T cells (49). This observation questions the role of the cysteine residue in regulating HA binding. One the other hand, results from other research groups indicate that the transmembrane region of CD44 is responsible for the association of CD44 with lipid rafts (49, 112). The localization of CD44 to lipid rafts, however, does not seem to affect the ability to bind HA since substitution of the transmembrane domain of CD44 with that of CD45 excluded CD44/CD45 chimeras from lipid rafts, but had no effect on HA binding (49). Therefore, the precise role for the transmembrane domain of CD44 in regulating HA binding remains to be determined.

1.7.3 Regulation by the cytoplasmic domain of CD44

The cytoplasmic domain of CD44 (CD44cyt) has been demonstrated to be important for optimal HA recognition, and for CD44-mediated melanoma cell migration (76, 113, 114).
Mutagenesis studies showed that truncated CD44 mutant lacking all but the first 6 amino acids of the cytoplasmic domain failed to bind soluble HA when expressed in AKR T lymphoma cells (18). Similarly, in Jurkat cells transfected with CD44H, HA binding was induced upon stimulation with an anti-CD3 mAb, PMA, or an anti-CD44 mAb, and such inductions also required the presence of the first 16 amino acids of the CD44cyt (98). Melanoma cells transfected with a truncated CD44 lacking the cytoplasmic tail failed to migrate on HA coated plate, suggesting a role of the CD44cyt in cell migration (115). Taken together, these results provide evidence that the CD44cyt can influence HA binding and cell locomotion.

How CD44cyt regulates HA binding or cell migration is not clear. Since no enzymatic activity was detected for the CD44cyt, it is proposed that the CD44cyt may mediate protein-protein interactions in order to regulate its ligand binding ability or the CD44-induced cytoskeletal changes (35). Subsequent studies have identified some molecules that are associated with CD44 in T cells, PBMCs, endothelial cells and epithelial cells. These molecules include Src-family tyrosine kinases (Lck, Fyn and Lyn, refs. 116, 117), Rho GDP dissociation inhibitor (Rho-GDI, ref. 118), as well as cytoskeletal proteins ezrin/radixin/moesin (ERM, ref. 48), ankyrin (119-123), and protein 4.1 (124). However, no functional study has been carried out to examine the correlation between these interactions and HA binding.

In addition to protein-protein interactions, serine phosphorylation of CD44 has also been suggested as a regulatory mechanism by which the CD44cyt can influence cell migration on an HA substratum. Mutational studies showed that Ser 323 and Ser 325 are the only residues of the CD44cyt that are phosphorylated in vivo (114, 125). Even though Ser 325 accounts for 90% of phosphorylation on the CD44 molecule, Ser 323 is required for the phosphorylation of Ser 325 (125, 126). Currently, it is not clear which kinase phosphorylates CD44, although there is some evidence that protein kinase C (PKC) may be involved (119). Phosphorylation of Ser 325 was shown to regulate melanoma cell migration on HA coated substrates as well as wound closure, but not the ability to bind soluble HA. Mutation of Ser
325 to glycine completely inhibits the melanoma cell migration (127). These data, therefore, suggest that serine phosphorylation of the CD44cyt may play an important role in CD44-mediated cell locomotion, but not the adhesion to soluble HA.

1.7.4 Regulation of selectin- and integrin-mediated leukocyte adhesion, a comparison to CD44

The interaction between CD44 and HA shares many similarities to that of selectins with their carbohydrate ligands. First, both N-terminal regions of CD44 and the selectins are likely to be structurally related; CD44 contains a “link module”, which is structurally similar to the C-type lectin domain expressed by all selectins (40, 41). Both CD44 and the selectins use this lectin-like domain to bind carbohydrate moieties. This lectin-carbohydrate interaction can also mediate *in vitro* leukocyte-endothelial cell interactions and leukocyte rolling under flow conditions (14, 20, 21). Both CD44 and selectins are cell adhesion molecules that can be proteolytically shed from the cell surface as a potential means to regulate transient adhesion (91, 128-132). In addition, sulfation has been implicated in the regulation of HA binding by CD44 and is also required for high affinity interaction of selectins with their ligands (71, 133-135). Lastly, for both selectins and CD44, the cytoplasmic domain in some cases, optimizes the ligand binding function (14, 18, 97, 136).

The optimal ligand binding function of integrins and CD44 is also regulated in a similar fashion. The levels of cell surface integrins and CD44 increase after cellular activation (15, 74, 137). Both integrins and CD44 are cell adhesion molecules that can undergo conformational change upon activation, resulting in enhanced capability for ligand binding. For integrins and the human CD44 molecule, this activated conformation can be detected by monoclonal antibodies (15, 36, 137, 138). Both integrins and CD44 exhibit a low intrinsic affinity for ligands and depend on multivalency to increase binding avidity. For integrins, this multivalency is achieved by receptor clustering via the association of the cytoplasmic domain
of integrins with actin cytoskeleton (1, 139, 140). For CD44, this receptor clustering is mediated by the extracellular domain through the association of carbohydrates or GAG side chains (141), or by the transmembrane domain through disulfide bonds or non-covalent interactions (50, 142). Again, for both integrins and CD44, the cytoplasmic domain is indispensable for ligand binding (15, 18, 137).

1.8 Regulation of CD44-mediated cell adhesion and signaling

Currently, little is known about the signaling events mediated by CD44. However, it has been proposed that, like other cell adhesion molecules, CD44 can signal through the cell membrane in either direction. The extracellular binding activity of CD44 is regulated from the inside of the cell (inside-out signaling), while the binding of the ECM elicits signals that are transmitted into the cell (outside-in signaling).

1.8.1 CD44 outside-in signaling

A number of experiments using anti-CD44 mAb or the natural ligands of CD44 established that CD44 could signal into the cell. First, in vitro studies show that adhesion of lymphocytes to immobilized anti-CD44 antibody, which were believed to mimic the engagement of CD44, induces cell spreading. In human peripheral T cells activated by PMA, both anti-LFA-1 mAb and anti-CD44 mAb induces a dendritic phenotype, which is associated with cytoskeletal rearrangement (143, 144). Similarly, in B cells activated by anti-IgM and IL-4, several anti-CD44 mAbs also promote cell spreading (145, 146). These data indicate that CD44 can transmit signals culminating in cytoskeletal changes. Second, ligation of CD44 by anti-CD44 antibodies stimulates leukocyte adhesion. In several haemopoietic cell lines, cross-linking CD44 with anti-CD44 antibodies or with soluble HA induces homotypic aggregation of the cells (53, 147). More interestingly, triggering of CD44 by an anti-CD44 mAb also activates integrin-mediated cell-cell adhesion in T cells and in colon cancer cells, suggesting a
connection of signaling pathways between CD44 and integrins (148, 149). This process appears to require protein kinase C activity and an intact actin cytoskeleton. Third, as mentioned previously in 1.5.2, activation of CD44 by antibodies augments T cell activation via TCR and CD3, supporting the role of CD44 as a co-stimulatory molecule for T lymphocyte activation (30, 85-88). Fourth, cytokine production after anti-CD44 antibody stimulation has also been observed as a consequence of CD44 outside-in signaling. Cross-linking of CD44 on rheumatoid synovial cells induces IL-6 production (150). Similarly, in cultured dendritic cells (DC), anti-CD44 antibodies or HA also induce the secretion of IL-8, TNFα, IL-1β, GM-CSF, IL-10 and IL-12 (151). Lastly, a natural ligand of CD44, HA, has been demonstrated to signal to cells via its binding to CD44. In activated macrophages, HA fragments induce the secretion of cytokines and chemokines, up-regulate integrin functions, and induce nitric-oxide synthase via a NF-κB-dependent mechanism (152-154). Thus, it is now evident that cell surface CD44 molecules can transmit signals from the outside to the inside of cells.

The signaling cascade initiated by CD44 has just started to be elucidated, but much awaits further investigation. In 1996, the first study on CD44 signaling by Taher et al. found that cross-linking CD44 on Jurkat T cells with anti-CD44 mAbs activates the protein tyrosine kinase Lck, which then recruits ZAP-70 (116). Subsequent studies by Ilangumaran et al. confirmed this finding, and also showed that Lck and Fyn are both associated with CD44 that was isolated from the low-density fractions of a sucrose gradient (117). These fractions, known as the lipid rafts, are enriched in molecules important for cell surface receptor signaling (reviewed in refs. 155, 156). More recently, Foger et al. showed that cross-linking of CD44 on a T helper cell line is accompanied by adhesion, spreading and actin bundle formation. The CD44-initiated reorganization of the cytoskeleton is associated with the recruitment of CD44 and the associated Lck and Fyn into lipid rafts. This process also involves the small GTPase Rac as shown by the absence of spreading in cells over-expressing a dominant negative form of Rac (157). Similar to this finding, fragmented HA was also shown to activate Rac1 upon
binding to CD44, and to promote the formation of lamellipodial protrusion (158). These data, therefore, suggest that CD44 activates Rac1 in a Src-family kinase-dependent manner, and leads to the rearrangement of cytoskeleton.

1.8.2 CD44 inside-out signaling

The ligand binding ability of CD44 can also be regulated from inside of the cell. As introduced in 1.6, the external domain, the transmembrane domain, as well as the cytoplasmic domain of CD44 all contribute to regulate HA binding via different mechanisms. These mechanisms include alternative splicing of CD44 variants, post-translational modifications of CD44 by glycosylation, addition of GAG side chains, sulfation, sialylation and serine phosphorylation, as well as the disulfide bond formation via the transmembrane region of CD44. However, intracellular events directing these modifications or changes to CD44, for example, regulations of the glycosyl-transferases, sulfo-transferases, or sialidases upon appropriate stimuli, are currently not understood.

1.8.3 Role of Src-family protein tyrosine kinases and CD45 in integrin-mediated leukocyte adhesion, an example

It is generally accepted that Src-family tyrosine kinases and CD45 play pivotal roles in T cell antigen receptor (TCR) signaling. Upon receptor engagement, Src-family kinases are recruited to the receptor. This enhances the phosphorylation of characteristic sequences within the cytoplasmic domain of the antigen receptor complex, known as the immunoreceptor tyrosine-based activation motif (ITAM). This results in the recruitment and phosphorylation of ZAP-70/Syk tyrosine kinases, which subsequently phosphorylate downstream substrates, leading to the activation of the signal transduction cascade. For the initiation process to proceed, the leukocyte-specific transmembrane tyrosine phosphatase CD45 is required to dephosphorylate the inhibitory site of Src-family kinases in order to prepare or prime the
kinases to be activated (reviewed in refs. 159-162). However, neither Src-family kinases nor CD45 are unique to lymphocytes. CD45 is expressed in leukocytes (lymphoid and non-lymphoid cells), whereas Src-family kinases are expressed in an even broader range of cell types, such as leukocytes, epithelium, endothelium and fibroblasts. This indicates that both Src-family kinases and CD45 may exert other profound cellular functions in addition to antigen receptor signaling.

Src-family kinases have been demonstrated to participate in cell adhesion triggered by either integrins or the antigen receptor (reviewed in refs. 163-165). For integrin-mediated adhesion, upon engagement with components of ECM, integrins activate various tyrosine kinases, including Src-family kinases, focal adhesion kinase (FAK), Pyk2, Abl and integrin-linked kinase (ILK). Activated FAK autophosphorylates Tyr 397, creating a binding site for the Src homology 2 (SH2) domain of Src or Fyn. The Src-kinase then phosphorylates a number of focal adhesion components. The major targets include paxillin, tensin and p130*cas. FAK combines with, and may activate phosphoinositide 3-OH kinase (PI 3 kinase) either directly or through the Src kinase. On the other hand, Src-family kinases also phosphorylate FAK at Tyr 925, creating a binding site for the complex of the adapter Grb-2 and SOS. Therefore, these Src-family kinases mediate interactions linking FAK to signaling pathways that modify the cytoskeleton and activate mitogen-activated protein kinase (MAPK) cascade.

In leukocytes, another tyrosine kinase related to focal adhesion, Pyk2, is more prevalent than FAK, and has been shown to associate with Src-family kinases (reviewed in ref. 166). For T lymphocytes, for example, stimulation with anti-CD3 or PHA induces rapid tyrosine phosphorylation of Pyk2, and activates its kinase activity. This stimulation also increases the association between Pyk2 and Fyn, and between Pyk2 and paxillin, an important cytoskeletal component involved in focal adhesions (167, 168). In addition to Fyn, Pyk2 co-immunoprecipitates with Lck by binding to the SH2 domain of Lck in vitro (168). Although Pyk2 can interact with both Lck and Fyn, TCR-induced tyrosine phosphorylation of Pyk2 is
selectively dependent on Fyn, but not on Lck (168). Taken together, these results indicate that Src-family kinases can act on Pyk2 or FAK to regulate cytoskeletal changes induced by integrins or by TCR stimulation.

Besides Src-family kinases, CD45 is also involved in regulating integrin-mediated leukocyte adhesion. In some studies, anti-CD45 mAbs trigger homotypic cell adhesion (a cell adhere to another cell of the same kind) through an LFA-1/ICAM-1 pathway in human PBMCs and activated T lymphocytes, or through an LFA-1/ICAM-3 pathway in thymocytes (169-172). However, in other studies, anti-CD45 antibodies were shown to inhibit the LFA-1/ICAM-1 or the LFA-1/ICAM-3 induced homotypic cell aggregation in T cells, B cells and thymocytes (173, 174). The apparent controversy is not understood, but could be due to the different anti-CD45 antibodies used in each study. Some anti-CD45 antibodies augment the tyrosine phosphatase activity of CD45 and some do not (175). More recently, an emerging role for CD45 in negatively regulating integrin-mediated leukocyte adhesion has been demonstrated. In CD45− bone marrow-derived macrophages, β2 integrin-mediated cell adhesion and spreading were more rapid than that in CD45+ macrophages. Compared to the CD45− macrophages, the kinase activity of Hck and Lyn was decreased in CD45+ macrophages, and this was because CD45 dephosphorylated the tyrosine residue located within the kinase domain of Hck or Lyn. The enhanced kinase activity of Hck and Lyn in the absence of CD45 was found to correlate with the increased adhesiveness of CD45− macrophages (176). In another example, CD45− T cells exhibited enhanced adhesion ability via the interaction of α5β1 integrin and fibronectin compared to CD45+ T cells. However, adhesion returned to normal level upon transfection of wild-type CD45 into the CD45-deficient lines. In this case, both the transmembrane domain and the tyrosine phosphatase activity of CD45 were required for the regulation of integrin-dependent adhesion, but the highly glycosylated extracellular domain was dispensable (177). Therefore, in contrast to its positive regulatory role in TCR signaling, CD45 may exert a negative regulatory function in integrin-mediated leukocyte adhesion.
Similar to integrins, Src-family kinases are also involved in CD44 signaling. Cross-linking of cell surface CD44 activates Lck and recruits ZAP-70 in Jurkat T cells (116). Additionally, both Src-family kinases (Lck and Fyn) and CD44 are found in lipid rafts (49, 117). These lipid rafts are believed to contain special lipids and proteins that are required for receptor signaling in the plasma membrane. It is currently not understood why CD44 resides in these lipid rafts. However, with the demonstration that the association of CD44 with Lck or Fyn occurs in lipid rafts (117), it becomes evident that the pool of CD44 molecules in lipid rafts may signal other cellular events, perhaps via the interaction of CD44 with Src-family kinases.

In T cells, both Lck and Fyn are physiological substrates for CD45, and their kinase activities are regulated by CD45. Therefore, it is possible that Src-family kinases and CD45 may also participate in CD44-mediated cellular functions, as is the case for the integrin-mediated leukocyte adhesion.

1.9 The link between cell adhesion molecules and the cytoskeleton

In CD44-initiated cell adhesion and cell migration, one of the most important cellular events is to assemble cytoskeletal elements required for these processes. Research on how cell surface CD44 signals to the cytoskeletal machinery in order to prepare for cell movement has not been easy because of the complex structure of cytoskeleton, as well as the lack of information on CD44 signaling pathways. From what has been learned for integrin-mediated cell adhesion and migration, there are a large number of different molecules involved in connecting integrins to the actin machinery. These molecules include kinases, phosphatases, adapter proteins or docking proteins, GAPs, GEFs, GDIs, GTPases, cytoskeletal proteins and phosphoinositides. Therefore, for CD44-triggered reorganization of actin cytoskeleton, most studies have focused on the association of CD44 with cytoskeletal proteins or with enzymes and signaling molecules that are implicated in integrin signaling.
1.9.1 Structure of the cytoskeleton

The cytoplasm of eukaryotic cells is spatially organized by a network of protein filaments known as the cytoskeleton. The cytoskeleton is required for the maintenance of cell shape and is important for mediating changes in cell morphology and motility in response to environmental signals. Three principal types of filaments constitute the network of cytoskeleton. They are microtubules, actin filaments, and intermediate filaments. Microtubules are rigid structures that usually have one end anchored in the centrosome and the other free in the cytoplasm. In many cells microtubules are highly dynamic structures that alternately grow and shrink by the addition and loss of tubulin subunits. Motor proteins move in one direction or the other along microtubules, carrying specific membrane-bounded organelles to desired locations in the cell. Actin filaments are also dynamic structures, but they normally exist in bundles or networks rather than as single filaments. A layer called the cortex, or the membrane skeleton, is formed just beneath the plasma membrane from actin filaments and a variety of actin-binding proteins. This actin-rich layer controls the shape and surface movement of most animal cells. Intermediate filaments are relatively rigid, ropelike structures that provide mechanical stability to cells and tissues. The three types of filaments are connected to one another, and their functions are coordinated.

The sophisticated structure of the cortex confers the differences in the dynamic properties of cell surface movements. Spectrin, actin, protein 4.1, adducin, tropomyosin, tropomodulin, dematin, and p55 are the principal components of the membrane skeleton. Lateral interactions among these proteins constitute the composite structure designated as the membrane skeletal network. This network is anchored to the lipid bilayer through vertical interactions mediated by linker proteins, which cross-link actin filaments to proteins in the plasma membrane (178). Examples of these linker proteins are β-spectrin, ankyrin, band 3 (or its related proteins in non-erythroid cells), protein 4.1, and ERM family proteins. The actin filaments lie underneath the plasma membrane and project into the cytoplasm, where they form
the basis of a three-dimensional actin filament network. Different actin-binding proteins can cross-link actin filaments into loose gels, bind them into stiff bundles, attach them to the plasma membrane, or forcibly move them relative to one another. Tropomyosin, for example, binds along the length of actin filaments, making them more rigid and altering their affinity for other proteins. Filamin cross-links actin filaments into a loose gel. Fimbrin and α-actinin form bundles of parallel actin filaments. Gelsolin mediates Ca\(^{2+}\)-dependent fragmentation of actin filaments, thereby causing a rapid formation of actin gels. Various forms of myosin use the energy of ATP hydrolysis to move along actin filaments, either carrying membrane-bounded organelles from one location in the cell to another or moving adjacent actin filaments against each other. It can be, therefore, concluded that the varied forms and functions of actin depend on the versatile repertoire of actin-binding proteins.

1.9.2 Cytoskeletal changes in integrin-induced cell adhesion and cell migration

In the processes of cell adhesion and cell migration, the cortex generates cell-surface movements such as the formation of filopodia, lamellipodia, membrane ruffles, and stress fibers. When cells are moving or changing shape, the leading edge can extend a thin sheetlike protrusion, known as a lamellipodium, which contains a dense meshwork of actin filaments. Many cells also extend thin, stiff protrusions called microspikes, which are about 0.1 \(\mu\)m wide and 5 to 10 \(\mu\)m long and contain a loose bundle of about 20 actin filaments oriented with their plus ends pointing towards the plasma membrane. Some long microspikes can further extend to up to 50 \(\mu\)m long, called filopodia. Membrane ruffles are lamellipodia and microspikes that detach from the substratum and sweep backward over its dorsal surface. Stress fibers are usually produced by a contractile bundle of actin filaments associated with the motor protein myosin. These varied membrane structures are under the control of Rho family members of small GTPases. Cdc42 and Rac regulate the formations of focal complexes, lamellipodia and
membrane ruffles, whereas Rho regulates the formation of focal adhesions and stress fibers (179, 180).

Binding of integrins to ECM components induces the association of integrins with the cytoskeleton. Depending on the state of cytoskeletal organization, this can lead to clustering of integrins into focal adhesions or focal complexes. Focal adhesions are large integrin aggregates found at the ends of stress fibers, whereas focal complexes are smaller integrin clusters that occur at the tip of filopodia or lamellipodia. The significance of formations of focal adhesions or focal complexes is the recruitment of actin filaments to the cytoplasmic domain of integrins. This recruitment is achieved via a complex of interacting cytoskeletal proteins, including talin, vinculin, α-actinin, paxillin, tensin and filamin. Such a complex not only couples integrins to the actomyosin contractile apparatus, which is essential for cell migration, but also provides a surface on which a number of molecules involved in integrin signaling are sequestered. For example, upon engagement of integrins and their ligands, kinases (FAK and Src-family kinases) and cytoskeletal proteins (tensin and talin) are rapidly recruited to focal adhesions or focal complexes. This leads to the subsequent recruitment of adapter or docking proteins (Cas, Crk, Grb-2 and Shc), other actin-binding proteins (paxillin, vinculin, α-actinin and filamin), and guanine nucleotide exchange factors (GEFs) for Rho family small GTPases (Vav and C3G) (reviewed in refs. 139, 163, 181). These actin binding proteins target actin filaments to focal adhesions or focal complexes for the assembly of lamellipodia, membrane ruffles, or stress fibers. GEFs, GTPase activating proteins (GAPs) or guanine nucleotide dissociation inhibitors (GDI) act as “molecular switch” to regulate the activity of Rho family GTPases to control the formation of lamellipodia, membrane ruffles and stress fiber.

Currently, more is known about how RhoA stimulates the formation of stress fibers and focal adhesions than is known about Rac- or Cdc42-induced formation of focal complex and lamellipodia. Separate lines of investigation have converged to reveal that RhoA stimulates actomyosin-based contractility and that this contractility contributes to the assembly of stress
fibers and focal adhesions (182, 183). RhoA-GTP binds to and activates several serine/threonine kinases. One of these, known as Rho-kinase, ROCKII and ROKα, phosphorylates and inhibits myosin phosphatase, resulting in elevated myosin light chain (MLC) phosphorylation (184). In turn, MLC phosphorylation promotes both myosin filament assembly and actin-activated myosin ATPase activity (182). These effects lead to the bundling of actin filaments and tension being transmitted to integrins via their associated actin filaments (known as the contractile actin bundle or the stress fibers). The clustering of integrins into focal complexes induced by Rac and Cdc42 is less well characterized. Whether myosin is involved has not been established. One downstream target of both Rac and Cdc42 is PAK, a serine/threonine kinase implicated in the development of focal complexes (185). PAK phosphorylates and inhibits the myosin light chain kinase (MLCK), an enzyme that regulates myosin activity, resulting in decreased levels of MLC phosphorylation. It is, therefore, suggested that myosin is not involved in the assembly of focal complexes. A different conclusion has been drawn, however, from work with another kinase downstream of Cdc42, myotonic dystrophy kinase related Cdc42 binding kinase (MRCK), which directly phosphorylates the MLC.

1.9.3 Evidence for the association of CD44 with the actin cytoskeleton

The cortex is the place where interactions between CD44 and cytoskeletal proteins were first identified. Murphy, Jacobson and Brown have reported separately that CD44 localizes to the cleavage furrow, filopodia and microvillar projections (37, 44, 186-188), suggesting that CD44 may associate with the membrane cytoskeleton. Moreover, as introduced in CD44 outside-in signaling (section 1.8.1), cross-linking of cell surface CD44 by anti-CD44 antibodies or using fragmented HA induces T cell spreading, and activates the small GTPase Rac, which is known to regulate the formation of lamellipodia and membrane ruffles. In some T cell lines, the CD44-mediated adhesion to HA is impaired by pre-treatment of the cells with Cytochalasin
D, a drug that inhibits the addition of actin molecules to actin filaments, leading to filament depolymerization (98, 143). These results imply that CD44 may be tethered to the actin cytoskeleton via Rac pathways, and that the integrity of actin cytoskeleton is required for CD44 to bind HA. Subsequent studies have showed that potential cytoskeletal proteins are associated with CD44 in epithelial cells, fibroblasts, T cells and in vitro. These proteins are a family of closely related cytoskeletal linker proteins: ERM, ankyrin, and protein 4.1 (48, 119-124, 189). The interaction of CD44 with ankyrin has only been reported by one research group, whereas the interaction of CD44 and protein 4.1 was observed solely in in vitro binding assays. Compared to ankyrin and protein 4.1, ERM proteins have been extensively studied over the past few years in terms of their structure, signaling pathways, and regulatory mechanisms for actin binding. The CD44-ERM interaction has been well-established in epithelial cells and fibroblasts. Therefore, here I will only introduce the CD44-ERM interaction.

1.9.4 CD44-ERM interaction

The ERM family members (Ezrin, Radixin and Moesin), as well as the related protein Merlin (neurofibromatosis 2 tumor suppressor protein), have been proposed as links between proteins of the cell surface and the actin-based membrane cytoskeleton (190). For ERM proteins, the polypeptide can be divided into three regions: an ~300 residue N-terminal FERM domain (Band 4.1, ezrin, radixin, moesin homology domains); an ~200 residue central domain that has a heptad hydrophobicity pattern characteristic of coiled-coil proteins; and an ~100 residue C-terminal tail domain. ERM proteins are concentrated at regions such as microvilli and focal adhesions where actin filaments associate with the plasma membrane (48, 191). ERM proteins interact with membrane glycoproteins via the globular FERM domain, whereas they interact with actin filaments via the charged C-terminal domain.

The interaction of CD44 and ERM proteins has been shown to occur in epithelial cells, fibroblasts, and in vitro. The CD44-ERM interaction was first identified in BHK cells where
CD44 remained associated with actin filaments after TX-100 extraction. In immunofluorescence studies, CD44 was found to co-localize with ERM proteins in L cells (48). Subsequent studies revealed that the sequence that mediates CD44-ERM interaction involves the three arginines at the juxta-membrane cytoplasmic domain of CD44 (192, 193). An *in vitro* study showed that ERM proteins directly interact with the CD44cyt at low ionic strength, but not at physiological ionic strengths (118). Deletion of the NH2 terminal half of ERM proteins improves their binding to CD44 at physiological ionic strength. These findings support a model in which under physiological conditions, ERM proteins form homodimers or homooligomers due to an intramolecular head-to-tail association with fewer interactions with CD44. Upon changes to the cellular environment such as low ionic strength, phosphorylation of the conserved C-terminal threonine on ERM proteins, or binding of PI-4,5-P2 to the N-terminus of ERM, the intramolecular association is less favored, and the CD44-ERM interaction may increase accordingly (118, 194-198).

It has been proposed that the Rho family GTPases may regulate the interaction of CD44 and ERM proteins. Rho-GDI, an inhibitor of dissociation of GDP-bound Rho, was reported to co-immunoprecipitate with the CD44-ERM complex in BHK cells (118). More recently, Rho-GDI has also been shown to directly associate with the N-terminal region of ERM proteins *in vitro*. The consequence of ERM-Rho-GDI association is the activation of the Rho subfamily members by reducing the Rho-GDI activity (199). Rho GTPase regulates the formation of lamellipodia, stress fibers and focal adhesions (200), and thus, Rho-GDI may recruit cytoplasmic Rho to the plasma membrane to be activated, as is the case for Rab-GDI (201-203). Therefore, ERM proteins as well as Rho GDI and Rho GDP/GTP exchange factor are involved in the activation of the Rho subfamily members, which then regulate the reorganization of actin filaments through the ERM system.

While the interaction of CD44 and ERM proteins is well established, there are some questions that remain to be answered in order to fully understand the association of CD44 or
ERM with the membrane cytoskeleton. First, although ERM proteins have been shown to associate with CD44, they are not necessarily co-localized with CD44 in tissues, indicating that ERM proteins may have a broader range of functions than just to associate with CD44. This view is supported by the observation that ERM proteins co-localize with CD43, ICAM-2 and ICAM-3 in some cells, as well as the fact that CD43 and ICAM-2 also interact with ERM proteins (189, 192, 204). Second, the biological significance of CD44-ERM interaction is still obscure. Mutational analysis indicates that the interaction of CD44 and ERM proteins is unlikely to be involved in regulating HA binding because a CD44 mutant lacking the ERM interacting sequence bind HA to the same extent as wild-type CD44 when expressed in T cells (113). However, it is possible that CD44-ERM interaction may influence the recognition of other CD44 ligands, or transmit signals to the cytoskeleton upon binding of CD44 to HA. Lastly, most of the CD44-ERM interaction studies have been carried out in epithelial cells and fibroblasts, and it is not known whether this interaction also plays a role in CD44-mediated leukocyte adhesion or migration.

1.10 Summary of thesis objectives

CD44 plays an important role in a number of biological processes related to cell adhesion and cell migration during embryonic development, wound healing, and tumor cell invasion. In the immune system, CD44 is involved in haemopoiesis, T lymphocyte activation, lymphocyte homing, and leukocyte extravasation at sites of inflammation. In leukocytes, CD44-mediated cell adhesion to its best-characterized ligand, HA, is a tightly regulated cellular event. It has been suggested that the extracellular domain, the transmembrane domain, as well as the cytoplasmic domain of CD44 all contribute to regulate HA recognition. When this study was initiated, there were substantial data demonstrating the regulatory mechanisms by which the extracellular domain of CD44 affects HA binding. These mechanisms include alternative splicing of CD44 variants and post-translational modifications of the CD44 molecules (i.e.
glycosylation, addition of GAG chains, sialylation and sulfation). In contrast, there were only a few studies, which had addressed the roles of the transmembrane or the cytoplasmic domain of CD44 in regulating HA binding, and some of them were contradictory to each other. In addition, the signaling pathways initiated by CD44, including those that regulate the rearrangement of the actin cytoskeleton, were also poorly understood when this study was started. My research objectives, therefore, were focused on (i) to define the roles for the transmembrane domain and the cytoplasmic domain of CD44 in regulating HA binding; and (ii) to elucidate the CD44 signaling pathways that lead to cytoskeletal changes.

My hypothesis was that by mediating molecular interactions via the transmembrane domain and/or the cytoplasmic domain of CD44, signals triggered by cell surface CD44 were transmitted into the cells, resulting in the activation and recruitment of intracellular enzymes and noncatalytic signaling molecules. Activation or recruitment of these enzymes or molecules could then lead to the association of CD44 with the actin cytoskeleton, as well as stimulate the reorganization of actin cytoskeleton (outside-in signaling), which in turn could affect the ligand binding ability of CD44 (inside-out signaling). To address this hypothesis, I used three different approaches in this study.

First, to evaluate whether the transmembrane domain or the cytoplasmic domain of CD44 has an effect on HA binding, I generated chimeric proteins containing various combinations of the transmembrane or the cytoplasmic domain of murine CD44 in conjunction with the external domain of human CD4. These chimeric proteins were transfected into lymphoid and fibroblast cells, which expressed endogenous CD44. The ability of the transfectants to bind HA were assessed, and compared to the non-transfected cells. Since the external domain of human CD4 is unable to bind HA, any change in the overall HA binding ability of the transfectants could indicate an effect for each domain in regulating HA binding. Mechanisms underlying the effect could then be further studied.
Second, to determine whether the cytoplasmic domain of CD44 interacts with other intracellular molecules, GST-CD44 fusion protein precipitation, anti-CD44 immunoprecipitation, followed by Western blotting for potential candidate interacting proteins were conducted using T lymphocyte cell lysates. In addition, the yeast two-hybrid approach was also incorporated to identify unknown proteins that interact with CD44.

Third, to investigate the signaling pathway initiated by CD44 in T cells, anti-CD44 antibodies were used to trigger signals via cell surface CD44. Cytoskeletal changes such as the formation of filopodia, lamellipodia, or cell spreading were used as the readout for CD44 outside-in signaling. Tyrosine phosphorylation events were assessed if cytoskeletal changes were observed. Downstream targets of CD44-initiated cell spreading were then investigated.

Some results from this work led to the following publications:


CHAPTER TWO  
Materials and Methods

2.1 Cell lines

The murine T lymphoma cell lines, BW5147 (CD45\(^+\) and CD45\(^-\)) (205), T28 (206), AKR, WT and TFX cells (18) were cultured at 37°C with 5% CO\(_2\) in DMEM (GIBCO BRL Life Technologies, Burlington, ON) supplemented with 10% horse serum (HS; GIBCO BRL Life Technologies), 2 mM L-glutamine (GIBCO BRL Life Technologies) and 1 mM sodium pyruvate (GIBCO BRL Life Technologies). The murine T lymphoma cells, SAKR T200\(^+\) and SAKR T200\(^-\) (207), and NIH 3T3 fibroblasts (American Type Culture Collection; ATCC; Rockville, MD) were maintained in DMEM supplied with 10% fetal calf serum (FCS; GIBCO BRL Life Technologies), 2 mM L-glutamine and 1 mM sodium pyruvate. The human myeloid cell line, KG1a (ATCC), and the human T lymphoma cell line, HSB-2 (ATCC) were maintained in RPMI (GIBCO BRL Life Technologies) with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 \(\mu\)M 2-mercaptoethanol. Transfected lymphoid cells and fibroblasts were selected in 0.5 mg/ml and 0.35 mg/ml of active Geneticin (G418; GIBCO BRL Life Technologies) respectively. BW5147 cells transfected with CD3\(\zeta\) and \(\delta\) to express surface CD3/TCR complex were grown in the presence of 3 mM of histidinol (Sigma-Aldrich Canada, Oakville, ON) to maintain plasmid expression.

2.2 Antibodies

Antibodies used for flow cytometry, immunoprecipitation and Western blotting in this study are listed in Table 2.1.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>FC(^b)</th>
<th>IP(^c)</th>
<th>WB(^d)</th>
<th>Source and references</th>
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<td>IM7</td>
<td>mCD44</td>
<td>1/10 TCS</td>
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<td>(208)</td>
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<td>CNBr-IM7</td>
<td>mCD44</td>
<td>ND</td>
<td>25 μg</td>
<td>ND</td>
<td>R. Li and P. Johnson(^e)</td>
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<td>1/5 TCS</td>
<td>ND</td>
<td>ND</td>
<td>(18)</td>
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<tr>
<td>SIM.4</td>
<td>hCD4</td>
<td>1/2 TCS</td>
<td>ND</td>
<td>ND</td>
<td>(209)</td>
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<tr>
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<td>R. Sweet(^f)</td>
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<tr>
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<td>D. Ng and P. Johnson(^h)</td>
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<tr>
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<td>ND</td>
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<td>J. Felberg and P. Johnson(^h)</td>
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<tr>
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<td>ND</td>
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<td>A. Viellette(^i)</td>
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<td>ND</td>
<td>1/5000</td>
<td>Jackson (Mississauga, ON)</td>
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<td>ND</td>
<td>1/5000</td>
<td>Jackson (Mississauga, ON)</td>
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<td>1/5000</td>
<td>Jackson (Mississauga, ON)</td>
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<td>g anti m IgG-FITC</td>
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<td>ND</td>
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<td>Protein A-HRP</td>
<td>Protein A</td>
<td>ND</td>
<td>ND</td>
<td>1/5000</td>
<td>BioRad (Mississauga, ON)</td>
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</table>

\(^a\)Species specific antibodies and antigens are as indicated: m (murine), h (human), r (rat), g (goat) and rbt (rabbit); \(^b\)amounts used for FC (flow cytometry) are expressed as a dilution factor of TCS (hybridoma tissue culture supernatant); \(^c\)amount of antibody used for IP (immunoprecipitation) from 5 x 10^6 suspension cell equivalents; \(^d\)amount used for WB (Western blotting) as a dilution factor of TCS or antisera; \(^e\)from Department of Microbiology and Immunology, University of British Columbia, Vancouver, B.C., Canada; \(^f\)from Department of Molecular Immunology, Smithkline Beecham Pharmaceuticals, King of Prussia, PA. \(^g\)from McGill University, Montreal, PQ, Canada; \(^h\)from Department of Biochemistry, University of British Columbia, Vancouver, B.C., Canada. Abbreviations: ND, not determined; Tyr, tyrosine.
2.3 Construction of plasmids for expression of CD4 and CD4/CD44 chimeras

A cDNA clone encoding human CD4 (208) was cloned into the Hind III/Xba I site of pBlue Script SK- (pBS; Stratagene, Aurora, ON), excised with Not I/Xho I, and cloned into the eukaryotic expression vector, pBCMGSneo (209). The murine CD44.1 cDNA was provided by P. Kincade (18).

The 4/44/44 chimera containing the extracellular domain of human CD4 and the transmembrane and cytoplasmic domains of murine CD44 was generated by polymerase chain reaction (PCR). Oligonucleotide (I) 5' GT GAC TCG GGA CAG GTC CTG CTG GAA TCC 3' and (II) 5' TGC CAA GAT GAT GAG CCA TGG CTG CAC CGG GGT GGA 3' were used to generate an 89 bp fragment of the human CD4 extracellular domain. (III) 5' TCC ACC CCG GTG CAG CCA TGG CTC ATC ATC TTG GCA 3' and (IV) 5' G AGA TCT AG A CTA CAC CCC AAT CT T CAT 3' were used to produce the 310 bp fragment of the mouse CD44 transmembrane and cytoplasmic domains. Oligonucleotide (I), (IV) and the two PCR fragments then served as primers and templates respectively to yield the 367 bp fragment, which was digested with Ava I/Xba I and used to replace the human CD4 transmembrane and cytoplasmic sequences in pBS to create pBS 4/44/44. The CD4/44 (4/44/44) chimera was excised by Xho I/Not I and cloned into pBCMGSneo. 4/44/44 contains residues 1-371 of the extracellular domain of human CD4 and the transmembrane and cytoplasmic domains of mouse CD44 beginning at Trp 251.

The truncated chimera (4/44/-) was generated by PCR using oligonucleotides (III) and (V) 5' CTT ACT AGT CTA CCT TCT CCT ACT GTT AAC CGC GAT GC 3' to create a 108 bp human CD4 fragment. Oligonucleotides (VI) 5' TC GCG GTT AAC AGT AGG AGG TAG ACT AGT AAG AAA AAG CTG 3' and (IV) were used as primers to produce a 241 bp CD44 fragment. A 307 bp fragment was made with primer (III) and (IV), using the above two PCR products as templates. This was digested with Nco I/Xba I and cloned into
pBS 4/44/44 to create pBS 4/44/-, which contained a stop codon after the fifth amino acid (Arg 276) of the CD44 cytoplasmic region. 4/44/- was subcloned into pBCMGSneo as described above. The 4/44*/- chimera, which has a cysteine to serine mutation at position 268 (C268S) in the transmembrane domain of CD44 was also made by PCR.

The chimera containing the extracellular region of human CD4, the transmembrane region of mouse CD44 and the cytoplasmic tail of human CD4 (4/44/4), was derived from 4/44/-. An Hpa I/Xba I fragment from pBS 4/44/- containing five amino acids from the mouse CD44 cytoplasmic domain sequence was replaced by the human CD4 cytoplasmic domain sequence (Arg 396 - Ile 433), which was made by PCR using the primers (VII) 5’ TTC TGT CAG CGG TGC CGG CAC CG 3’ and (VIII) 5’ GG CCT CTA GAC TCA AAT GGG GCT 3’, followed by Msp A1 I/Xba I digestion. The 4/44/4 chimera was then subcloned into pBCMGSneo. The chimeric protein 4/44*/4 containing the C268S mutation was made in the same way as 4/44/4. All the PCR products were sequenced by the NAPS unit (University of British Columbia, Vancouver, B.C., Canada).

2.4 Detection of cell surface proteins and HA binding ability by flow cytometry

Flow cytometry was conducted according to Li et al. (142). Briefly, 2 x 10^5 cells were incubated on ice for 20 min with 100 μl of a 1:10 dilution of IM7 tissue culture supernatant (TCS) for cell surface CD44 expression (210) and a 1:2 dilution of SIM.4 TCS for CD4/CD44 chimera expression (211). Cells were then centrifuged and washed once with phosphate buffered saline (PBS) containing 2% FCS. The cells were then incubated on ice for 20 min with 100 μl of FITC labeled goat-anti-rat IgG (IgG-FITC) or goat-anti-mouse IgG-FITC at a 1:100 dilution, or with HA-FITC (prepared as described in ref. 212) at a concentration of 2 μg/ml. Cells were then washed, resuspended in PBS with 0.5 μg/ml propidium iodide, and analyzed on a FACScan® flow cytometer (Becton Dickinson, Mississauga, ON). For IRAWB 14.4 (18) induction, 100 μl of a 1:5 dilution of tissue culture supernatant was added to the cells.
5 min before HA-FITC was added. For Cytochalasin D treatment, cells were incubated in medium with 25 μM Cytochalasin D (Calbiochem, La Jolla, CA) at 37°C for 30 min before antibody labeling.

2.5 Transfection of CD4/CD44 chimeras by electroporation and calcium phosphate precipitation

5 x 10⁶ lymphoid cells were suspended in 0.8 ml ice-cold DMEM, to which 20 μg of purified plasmid DNA was added. Cells were then electroporated using the Gene Pulser apparatus (Bio-Rad laboratories, Mississauga, ON) at 250 V and 960 μF capacitance. G418 was added to a final concentration of 0.5 mg active G418/ml after 48 h and cells were plated in 96-well tissue culture plates. Drug-resistant colonies appeared within 2 weeks. For transfection of NIH 3T3 cells, 20 μg of purified plasmid DNA in 470 μl of phosphate buffer (140 mM NaCl, 5 mM KCl, 750 μM Na₂HPO₄, 5 mM Dextrose, pH 7.05) was precipitated with 30 μl of 2M CaCl₂ at room temperature for 15 min, then added to a 50% confluent monolayer of NIH 3T3 cells in 10 cm² tissue culture dishes with 5 ml DMEM/10% FCS. After 24 h, the medium was changed and after 48 h the cells were diluted 1:4 and 0.35 mg/ml of active G418 was added. Drug-resistant single colonies formed within 3 weeks. Clones expressing chimeric molecules on the cell surface were selected by FACS analysis using anti-human CD4 mAbs.

2.6 Biotinylation of cell surface proteins

10⁷ cells were centrifuged and washed 3 times in PBS. Cell surface proteins were labeled on ice for 15 min with 0.5 mg/ml of Sulfo-N-hydroxysulfosuccinimide-Biotin (Pierce, Rockford, IL) in 250 μl labeling buffer (PBS, 1 mg/ml glucose, pH 7.5). The cells were then washed 5 times in ice-cold washing buffer (PBS, 1 mg/ml glucose, 1 mg/ml bovine serum albumin (BSA), 2 mg/ml lysine, pH 7.5).
2.7 Chemical cross-linking of cell surface proteins

Cells were washed twice in PBS and resuspended in PBS at 5 x 10^7/ml. Dithiobis(sulfo succinimidylpropionate) (DTSSP; Pierce, Rockford, IL) reconstituted at 10 mM in PBS was added to the cell suspension at various concentrations. After incubating on ice for 1 h with occasional mixing, the reaction was quenched by two 15 min incubations with 200 μl of ice-cold TN buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl).

2.8 Detection of proteins by Western blotting

In general, protein samples were mixed with 3 x reducing or non-reducing SDS sample buffer (0.375 M Tris-HCl, pH 6.8, 30% Glycerol, 6% SDS, 0.06% v/v bromophenol blue, 15% β-mercaptoethanol, no β-mercaptoethanol for the non-reducing sample buffer) and separated by SDS-PAGE. The proteins were then transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore Canada Inc., Mississauga, ON). In all cases, pre-stained molecular weight protein markers from New England Biolabs (Mississauga, ON) or from BioRad (Mississauga, ON) were run on the SDS-PAGE and transferred to the membrane. The membrane was then air-dried for 1 h and incubated with primary antibodies in TTBS buffer (20 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.1% v/v Tween 20) with 0.5% w/v BSA for 1 h. For anti-phosphotyrosine immunoblotting, instead of air-drying, the PVDF membranes were incubated directly with 5% w/v BSA in TBS buffer (TTBS without Tween 20) for 1 h. After incubation with the primary antibody, the membrane was washed 3 times with TTBS for 5 min, and further incubated with horseradish peroxidase (HRP)- conjugated secondary antibody in 0.5% w/v BSA in TTBS for another hour. The membrane was then washed 3 times in TTBS. Antibody-reactive bands were visualized using the enhanced chemiluminescence (ECL) kit (Amersham-Pharmacia Biotech, Baie d’Urfé, PQ) and Kodak BioMax MR film (InterScience, Markham, ON) according to the manufacturers’ instructions. Primary antibodies used are indicated in Table 2.1.
If reprobing of the same membrane was required, the membrane was stripped by incubating the membrane with stripping buffer (50 mM glycine, pH 2.5, 150 mM NaCl, 0.1% v/v NP40) for 30 min at room temperature, followed by 3 washes in TTBS. The membrane was air-dried for 1 h and then reprobed with another primary antibody.

2.9 Immunoprecipitation of CD4/CD44 chimeras

5-10 x 10^6 surface-biotinylated lymphoid cells or 2-4 x 10^6 fibroblasts were lysed on ice for 10 min in 1 ml TNE buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin) containing 1% Brij-96 (Sigma-Aldrich Canada), followed by centrifugation at 86,000 x g at 4°C for 30 min. Alternatively, cells were lysed in buffers containing 1% TX-100 (Fisher Scientific Ltd. Nepean, ON) or 60 mM n-octyl-β-D-glucopyranoside (OG, Calbiochem) as the detergent. The detergent-soluble material was precleared at 4°C for 1 h with 10 μl of Protein G Sepharose 4B beads (Amersham-Pharmacia Biotech, Baie d’Urfé, PQ). CD4/CD44 chimeras were immunoprecipitated for 1 h at 4°C with 30 μl OKT4 (ATCC) TCS pre-coupled to 10 μl Protein G Sepharose 4B beads. After 3 washes with lysis buffer, proteins were eluted from beads by boiling in 3 x non-reducing SDS sample buffer, separated on 7.5% SDS-PAGE, and transferred to a PVDF membrane. Endogenous CD44 and biotinylated CD4/CD44 chimeras were detected by Western blotting (described in 2.8) using the anti-CD44 mAb, KM201 (22), and streptavidin-HRP, respectively (Table 2.1).

2.10 Conjugation of IM7 to CNBr-activated Sepharose beads

The anti-CD44 mAb, IM7, was conjugated to CNBr-activated Sepharose beads (Amersham-Pharmacia Biotech) according to the manufacturer’s instruction. Briefly, prior to coupling, purified IM7 was dialysed for 2 days against the coupling buffer (0.1 M NaHCO_3, pH 8.3, 0.5 M NaCl). An appropriate amount of freeze-dried activated beads was rehydrated in 1
mM HCl, followed by 3 washes with 1mM HCl. Generally, 1 g dried beads will yield 3.5 ml
swollen gel. The swollen beads were then incubated with IM7 (4 mg antibody/ml swollen
beads) in a total volume of 10 ml and rotated overnight at 4°C. After coupling, the antibody
solution was removed. Unreacted sites on the beads were blocked by overnight incubation in
50 ml of 0.1 M Tris-HCl, pH 8.0 at 4°C. The beads were then washed with 3 cycles of
alternating pH using acetate buffer (0.1M sodium acetate, pH 4.0, 0.5 M NaCl) and Tris buffer
(0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl). The concentration of conjugated antibody was
determined by measuring the OD\textsubscript{280} absorbance of the IM7 solution prior to and after
conjugation. The final IM7 conjugated-CNBr beads were stored at 4°C in PBS containing
0.02% NaN\textsubscript{3}.

2.11 Construction of the full-length and the truncated GST-CD44\textsubscript{cyt} fusion proteins

The complete cDNA sequence of the cytoplasmic domain of murine CD44.1 (amino
acids 290-361) was generated by PCR (R. Tang and P. Johnson unpublished data), and inserted
into the Bam HI and Eco RI sites of a bacterial expression vector, pGEX 2T (Amersham-
Pharmacia Biotech). The truncated cytoplasmic cDNA sequence of murine CD44.1 (amino
acids 303-361) was excised by digesting the full-length CD44.1 cytoplasmic sequence with
BclI and Eco RI. The truncated CD44.1 was then blunted by Klenow, and inserted into the
pGEX 2T vector that had been digested with Sma I. All constructs were sequenced to confirm
that the cDNAs were in frame.

2.12 Over-expression and purification of GST-CD44\textsubscript{cyt} fusion proteins

The two versions of GST-CD44 fusion protein constructs were transformed into \textit{E.coli}.
Due to degradation, the full-length GST-CD44\textsubscript{cyt} was maintained in a protease deficient \textit{E.coli}
strain, UT5600. The truncated version, in which no significant degradation was detected, was
maintained in the XL1 Blue strain.
The GST-CD44cyt fusion proteins were purified according to the protocol provided by Amersham-Pharmacia Biotech with minor modifications. Briefly, bacteria were inoculated into 2 ml of LA medium (LB and 20 µg/ml ampicillin) and grown overnight on a 37°C shaker (180 rpm). The overnight culture was then transferred to 100-500 ml LA media. When the OD_{600} reached 0.6-0.8, the culture was induced with 0.1 mM isopropyl β-D-thiogalactoside (IPTG, GIBCO BRL Life Technologies) and transferred to a 26°C shaker (180 rpm) to grow for additional 16 h. For purification of the GST-CD44cyt fusion proteins, the bacteria were centrifuged at 4,000 x g at 4°C for 10 min, lysed at 4°C for 30 min by rotating in 10 ml TNE buffer (components defined in 2.9) containing 1% TX-100 and 1 µg/ml lysozyme. DNase I (Amersham-Pharmacia Biotech, Baie d’Urfé, PQ) was then added to the lysate at a final concentration of 1 µg/ml and the lysate was incubated at 37°C for 10 min. The lysate was then centrifuged at 4°C for 20 min at 16,000 x g, and the supernatant was incubated at 4°C for 1 h with 200 µl of a 50% slurry of Glutathione Sepharose 4B Beads (GSH beads; Amersham-Pharmacia Biotech). The bead complexed with GST-CD44 fusion proteins attached was washed twice with ice-cold washing buffer (TNE buffer and 0.1% TX-100) with a final wash rotating at 4°C for 10 min.

To elute the GST-CD44cyt fusion proteins off GSH beads, glutathione elution buffer (10 mM glutathione, 10 mM Tris-HCl, pH 8.0) was added to the bead matrix at 1 ml buffer/ml bed volume of the beads. After gently rotating for 15 min at room temperature, the beads were spun down and the supernatant was collected. This elution cycle was repeated 2 more times and the supernatants were pooled. To remove the glutathione from the elution buffer, the GST-CD44cyt solution was passed through a PD10 column (Amersham-Pharmacia Biotech, Baie d’Urfé, PQ) with PBS as the buffer. The concentration of GST-CD44cyt fusion proteins was determined by 12.5% SDS-PAGE using BSA (GIBCO BRL Life Technologies) dilutions as concentration standards.
2.13 Steady state labeling of cells with [$^{35}$S] methionine and cysteine

Lymphoid cells were washed twice with PBS and resuspended at 5 - 10 x $10^6$/ml in methionine- and cysteine-free DMEM (ICN Biomedicals Inc., St. Laurent, PQ). After starving the cells in the medium for 30 min at 37°C, the cells were resuspended in methionine- and cysteine-free DMEM supplemented with 10% dialysed FCS and 20-40 μCi/ml [$^{35}$S] methionine and cysteine (NEN DuPont Canada, Markham, ON). After a 2 h incubation at 37°C, the cells were washed in PBS for 3 times and subject to cell lysis.

2.14 GST-CD44cyt fusion protein precipitation and autoradiography

5 -10 x $10^6$ radiolabeled lymphoid cells or 2 - 4 x $10^6$ fibroblasts were lysed on ice for 10 min in 0.5 ml of ice-cold TNE buffer (component defined in 2.9) containing 1% Brij-96. The cell lysate was centrifuged at 16,000 x g at 4°C for 10 min. Detergent soluble material was precleared at 4°C for 1 h with 1 μg of GST pre-coupled to GSH beads, followed by immunoprecipitation using 1 μg of GST alone (control) or 1 μg of GST-CD44cyt fusion proteins pre-coupled to GSH beads. After a 2 h rotation at 4°C, samples were washed 3 times with lysis buffer, eluted by boiling at 100°C in 3 x reducing SDS sample buffer, and separated on a 10% or 12.5% SDS-PAGE. Following electrophoresis, gels were soaked for 30 min at room temperature in Amplify™ (Amersham-Pharmacia Biotech, Baie d’Urfé, PQ), and dried onto a 3 MM Whatman filter paper. Films were exposed at -80°C with Kodak BioMax MR film for various time to visualize protein bands.

2.15 CD44 immunoprecipitation

5 -10 x $10^6$ radiolabeled or unlabeled cells were lysed on ice for 10 min in 0.5 ml of TK buffer (10 mM Tris-HCl, pH 7.2, 140 mM KCl, 0.5 mM sodium orthovanadate, 0.2 mM sodium molybdate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.2 mM PMSF) containing 1% Brij-58 (Pierce, Rockford, IL), 1% Brij-96, 1% TX-100, or 60 mM OG. The
cell lysate was then centrifuged at 16,000 x g for 10 min at 4°C. Detergent soluble material was precleared at 4°C for 1 h with 50 µl of a 50% slurry of Sepharose CL-4B beads (Sigma-Aldrich Canada) or 30 µl of a 50% slurry of protein G beads (Amersham-Pharmacia Biotech). CD44 was then immunoprecipitated with either 50 µl of a 25% slurry of IM7-conjugated CNBr-Sepharose beads (4 mg/ml), or 100 µl of IM7 TCS precoupled to 30 µl of a 50% slurry protein G beads. 50 µl of a 50% slurry Sepharose CL-4B beads alone or 30 µl of a 50% slurry protein G beads alone was used as a control for immunoprecipitation. After rotating for 2 h at 4°C, the immunoprecipitates were washed 3 times with lysis buffer, separated on SDS-PAGE gels, and transferred to a PVDF membrane. CD44 was detected by autoradiography (as described in 2.14) or by Western blotting (as described in 2.8) using KM201 or JIWBB (Table 2.1).

2.16 Detection of Ezrin/Radixin/Moesin (ERM) co-immunoprecipitated with CD44

To detect the CD44-ERM interaction, 5 - 10 x 10^6 BW5147, HSB-2 or KG1a cells were used. CD44 was immunoprecipitated from these cells as described in 2.15 using low salt TKE lysis buffer (10 mM Tris-HCl, pH 7.2, 0 - 100 mM KCl, 2 mM EDTA, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 0.2 mM PMSF) containing 1% TX-100. ERM that co-immunoprecipitated with CD44 was detected by Western blotting (as described in 2.8) using the anti-ERM antisera (Table 2.1).

2.17 Detection of Lck/Fyn co-immunoprecipitated with CD44

CD44 was immunoprecipitated from 10^7 CD45+ or CD45− BW5147 cells as described in 2.15 using TK lysis buffer (components defined in 2.15). Lck or Fyn that co-immunoprecipitated with CD44 was detected by Western blotting (as described in 2.8) using 543B and the anti-Fyn antisera (Table 2.1).
2.18 Performing the yeast two-hybrid interaction (Figure 2.1)

All of the vectors, control plasmids, yeast strains, *E. coli* strains and the protocols for performing the yeast two hybrid interaction were provided by Dr. Ivan J. Sadowski, Department of Biochemistry, University of British Columbia, Vancouver, B.C., Canada (Table 2.2).

2.18.1 Generation of the full-length or truncated LexA-CD44cyt bait proteins

The full-length murine CD44.1 cytoplasmic domain cDNA sequence (amino acids 290-361) was excised by Bam HI and Eco RI digestions from the pGEX 3X CD44cyt plasmid (R. Tang and P. Johnson unpublished data). The fragment containing the cytoplasmic sequence of CD44.1 was filled in with Klenow, and then inserted into the pEG202 vector (Table 2.2), which had been digested by Eco RI and filled in with Klenow. The truncated CD44.1 cytoplasmic cDNA sequence (amino acids 303-361) was excised by Bel I and Eco RI digestions from pBS CD44.1 (R. Li and P. Johnson unpublished data). The fragment containing the truncated CD44.1 cytoplasmic sequence was filled in with Klenow, and then inserted into the pEG202 vector that had been digested by Eco RI and filled in with Klenow. Both constructs were sequenced.

2.18.2 Transformation of one or two plasmids into EGY48 yeast cells

3 or 4 colonies of the yeast strain EGY48 were inoculated and grown overnight in 10 ml YEPD complete medium (CM; 1% w/v Bacto yeast extract, 2% w/v Bacto peptone, 2% w/v Glucose) on a 30°C shaker (220-230 rpm). The yeast cells were then centrifuged at 3000 x g for 10 min at room temperature, and resuspended in 10 ml sterile TL solution (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM lithium acetate). After incubation at 30°C for 1 h, the cells were spun down again and resuspended in 1 ml TL. For each transformation, 100 µl of the yeast suspension in TL, 5 µg of each plasmid DNA, 6 µl of 10 mg/ml sheared salmon sperm carrier DNA (Fisher Scientific Ltd.), 14 µl of 100% DMSO, and 400 µl of TLP (10 mM
Construct bait protein plasmid and transform yeast

Characterize bait protein expression and activity

Construct cDNA Library in pJG4-5

Transform cDNA library into EGY48/pBait yeast

Select for library plasmid

Freeze and replate transformants

Select for interacting proteins

Test for specificity

Transform *E.coli*

Analyze and sequence positives

Assess protein synthesis

Assess transcriptional activity

Assess repressor activity

Figure 2.1. Flow chart for performing a yeast two-hybrid interaction trap.
Table 2.2 Plasmids used in the yeast two-hybrid experiments.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Application</th>
<th>Selectable marker</th>
</tr>
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<tbody>
<tr>
<td>pEG202</td>
<td>Bait plasmid, encodes LexA-bait fusion, and contains DNA binding domain.</td>
<td>His Amp'</td>
</tr>
<tr>
<td></td>
<td>cDNA library plasmids, contains Gal promoter, DNA activation sequence and a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemagglutinin tag for detection.</td>
<td></td>
</tr>
<tr>
<td>pJG4-5</td>
<td>Contains LexA operator- lac Z fusion gene for selection on Xgal.</td>
<td>Trp Amp'</td>
</tr>
<tr>
<td>pSH18-34</td>
<td>Positive control for reporter gene activation.</td>
<td>His Amp'</td>
</tr>
<tr>
<td></td>
<td>Negative control for reporter gene activation, and positive control for</td>
<td></td>
</tr>
<tr>
<td></td>
<td>repression of the reporter gene function.</td>
<td></td>
</tr>
<tr>
<td>pSH17-4</td>
<td>Contains LexA operator- lac Z fusion gene and the GAL1 upstream activating</td>
<td>Ura Amp'</td>
</tr>
<tr>
<td></td>
<td>sequences (UAS_GAL) upstream of two LexA operator sites. This allows yeast</td>
<td></td>
</tr>
<tr>
<td></td>
<td>to have significant β-galactosidase activity when grown on medium in which</td>
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<tr>
<td></td>
<td>Gal is the carbon source.</td>
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</tr>
<tr>
<td>pRKHM1</td>
<td>Negative control for repression of the reporter gene function.</td>
<td>His Amp'</td>
</tr>
<tr>
<td>pJK101</td>
<td></td>
<td>Ura Amp'</td>
</tr>
<tr>
<td>pRS423</td>
<td></td>
<td>His Amp'</td>
</tr>
</tbody>
</table>

Plasmids (left) used in different applications (middle) in yeast two-hybrid experiments are indicated. Selectable marker indicates the ability to grow on the selected amino acid dropout medium or on the plates containing ampicillin (Amp'). Adopted from Current Protocols in Molecular Biology, 1994. John Wiley & Sons, Inc., PP 13.14.5.
Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM lithium acetate, 44% w/v PEG 4000 (Sigma-Aldrich Canada,) were mixed and incubated at 30°C for 1 h. The transformation mix were then heated to 42°C for 10 min and spun at 10,000 x g for 10 sec. The supernatant was discarded and the yeast cells were resuspended in 200 µl sterile PBS. Finally, the yeast cells were streaked on selection plates and incubated at 30°C for 2-3 days.

2.18.3 Detection of bait proteins by Western blotting

The plasmids encoding the full-length and the truncated LexA-CD44cyt proteins were transformed into EGY48 and the transformants were maintained in Histidine dropout medium. EGY48 cells alone and EGY48 cells transformed with pEG202 (encodes for LexA) were used as controls. A 10 ml yeast culture was grown in Glu/CM-His medium (complete medium without His, 2% w/v Glucose) on a 30°C shaker (230 rpm) until the OD$_{600}$ reached 0.6-1.0. The yeast cells were then centrifuged at 2,000 x g for 10 min, and washed once with PBS. The pellet was resuspended in 100 µl of SDS sample buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 10% Glycerol, 20% β-mercaptoethanol), followed by vortexing twice for 30 sec with 150 and 425 micron glass beads (Sigma-Aldrich Canada). Samples were then boiled at 100°C for 5 min, chilled on ice for 5 min, and centrifuged at 16,000 x g for 10 min. The supernatant (yeast extracts) was diluted 20-fold with PBS for BCA assay (Pierce, Rockford, IL) to determine the protein concentration. 40 µg of protein from each sample was separated on a 12.5% SDS-PAGE gel and transferred to a PVDF membrane. The bait proteins and the LexA control were detected by Western blotting (as described in 2.8) using an anti-LexA antibody (Table 2.1).
2.18.4 Estimation of the intrinsic transcriptional activity of bait proteins by β-galactosidase filter assay

The plasmids encoding the full-length and the truncated LexA-CD44cyt, the positive control plasmid (pSH17-4, see Table 2.2), or the negative control plasmid (pRFHM, see Table 2.2), were co-transformed into EGY48 cells along with pSH18-34 (Table 2.2). The transformants were grown on Glu/CM-His, -Ura plates (complete medium without His and Ura, 2% w/v Glucose, 2% w/v Agar). Five or six yeast colonies from each co-transformation were picked and re-streaked on Glu/CM-His, -Ura plates, and grown overnight at 30°C. A clean and dry nitrocellulose membrane (Amersham-Pharmacia Biotech) was placed on top of the colonies until the membrane became wet. The membrane was then lifted from the colonies, air-dried for 5 min, chilled at −80°C for 10 min, and placed on a piece of Whatman 3 MM filter paper slightly larger than the membrane. The membrane and the filter paper were then soaked in Z buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 M sorbitol, 1 mM DTT) with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; GIBCO BRL Life Technologies) in a 30°C incubator to monitor the color change.

2.18.5 Estimation of the ability of bait proteins to localize to the nucleus and to bind the LexA promoter by repression of β-galactosidase activity

The plasmids encoding the full-length and the truncated LexA-CD44cyt, the positive control plasmid (pRFHM1, see Table 2.2), or the negative control plasmid (pRS423, see Table 2.2), were co-transformed into EGY48 cells along with pJK101 (Table 2.2). The transformants were grown on Glu/CM-His, -Ura plates. The β-galactosidase activity was assessed by the liquid assay. Briefly, a 5 ml of yeast culture containing transformants from each co-transformation was grown on a 30°C shaker (230 rpm) until the OD₆₀₀ approached to 1.0. 100 μl of the yeast culture was then transferred to an Eppendorf tube, followed by the addition of 700 μl Z buffer (components defined in 2.18.4), 50 μl chloroform and 50 μl of 0.1% SDS. The
mixture was vortexed for 30 sec and 160 µl of ONPG solution was added. The Eppendorf tubes were incubated at 30°C until a yellow color was developed. The reaction was then quenched by the addition of 400 µl of 1 M Na₂CO₃. The tubes were then centrifuged at 16,000 x g for 10 min at room temperature, and the aqueous layer was removed for an absorbance measurement at OD₄₃₀. The β-galactosidase activity was calculated according to the following formula:

\[
\text{β-galactosidase units} = 1000 \times \frac{\text{OD}_{420}}{(t \times v \times \text{OD}_{600})}
\]

\[t\] = time of incubation at 30°C (min)

\[v\] = volume of culture added to Z buffer (ml)

**2.18.6 Propagation of the pVPMT library DNA**

The pVPMT library, a cDNA library from murine CD4⁺ T lymphocytes, was kindly provided by Dr. Prasad, Albert Einstein School of Medicine, New York, NY. This cDNA library had been cloned into the pJG4-5 vector (Table 2.2) for performing the yeast two hybrid interaction. Four Vials of the unamplified pVPMT library in E. coli DH5α were inoculated into 4 L of LA medium (LB media, 20 µg/ml ampicillin) and grown on a 37°C shaker until the OD₆₀₀ reached 0.8. The bacteria were centrifuged at 4,000 x g at 4°C for 10 min, and the DNA was purified using a Qiagen DNA purification kit (Qiagen, Mississauga, ON) following the manufacturer’s instructions. The final cDNA library preparation was dissolved in 200 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The concentration of the cDNA library was determined by spectrometry at OD₂₆₀.

**2.18.7 Transformation of the cDNA library**

Three or four colonies from transformants of the bait plasmid and pSH18-34 was inoculated in 10 ml of Glu/CM-His, -Ura medium (complete medium without His and Ura, 2% w/v Glucose). After growing overnight on a 30°C shaker (230 rpm), the culture was transferred
to 500 ml of Glu/CM-His, -Ura media. Once the OD<sub>600</sub> reached 0.5, the cells were centrifuged at 1,500 x g at room temperature for 5 min, resuspended in 30 ml sterile water, centrifuged again at 1,500 x g for 5 min, and the supernatant was decanted. The cells were then resuspended in 1.5 ml TE/lithium acetate buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M lithium acetate) and aliquotted into 30 Eppendorf tubes (50 µl/tube). To each Eppendorf tube, 1 µg of library cDNA, 50 µg high-quality sheared salmon sperm carrier DNA and 300 µl of sterile 40% PEG 4000 were added. The transformation mix was inverted to mix thoroughly and incubated at 30°C for 30 min. Then 40 µl of DMSO was added to each Eppendorf tube, followed by heat shock at 42°C for 10 min. The complete contents of one tube were plated on a 24 x 24 cm plate (Fisher Scientific Ltd.) containing agar medium (complete medium without Ura, His and Trp, 2% Glucose, 2% Agar). The plates were then incubated at 30°C for 2 to 3 days. The transformation efficiency was estimated by 1/10 serial dilution.

2.18.8 Collecting primary transformant cells

The 30 plates containing transformants of the bait and the cDNA library-encoded proteins (~6 x 10<sup>4</sup> colonies/plate) were chilled at 4°C for 3 h to harden the agar. The yeast cells from the plates were pooled into a 50 ml Falcon tube using a sterile glass microscope slide. The cells were washed twice with 25 ml of sterile water, and then resuspended in 10 ml of glycerol solution (Glu/CM-Ura, -His, -Trp, plus 2% w/v glucose, 20% v/v glycerol). The cell suspension was mixed thoroughly and frozen in 1 ml aliquots at -80°C.

2.18.9 Screening for initial CD44<sub>cyt</sub>-interacting proteins

The appropriate quantity of transformed yeast cells was thawed on ice slowly, and diluted 1:10 with Gal/CM-His, -Ura, -Trp medium (complete medium without Ura, His and Trp, plus 2% Galactose, 1% Raffinose). The culture was grown on a 30°C shaker for 4 h to induce the GAL1 promoter so that the cDNA library encoded proteins were expressed. The
cells were spun down at 2,000 x g for 10 min at room temperature, and resuspended in 50 ml of Gal/CM-His, -Ura, -Trp media. The cell suspension was then incubated at 30°C on a total of 50 100 mm² plates (Fisher Scientific Ltd.) with Gal/CM-His, -Ura, -Trp, X-gal medium (complete medium without Ura, His and Trp, plus 2% Galactose, 1% Raffinose, 1 mg/ml X-gal) for the initial screening. Colonies that grew and changed color to blue on the above plates within 3 to 5 days were picked as putative positives for further characterization. A negative and a positive control for the activation of reporter genes were also incorporated in this process.

2.18.10 Gal-dependence specificity tests for the putative CD44cyt interacting proteins

Initial putative positives, as well as the positive and negative control for the activation of reporter genes, were re-patched on parallel plates using different sugars (glucose and galactose) as the carbon source. These pairs of plates include: Glu/CM-His, -Ura, -Trp, -Leu and Gal/CM-His, -Ura, -Trp, -Leu; Glu/CM-His, -Ura, -Trp, X-gal (1 mg/ml) and Gal/CM-His, -Ura, -Trp, X-gal (1 mg/ml). Colonies that only grew and changed color to blue on plates containing galactose indicated a specific interaction with the CD44cyt, and were therefore kept for DNA isolation and subsequent sequencing.

2.18.11 Isolation of pVPMT library plasmids from yeast

Putative positives that passed the Gal-dependence tests were inoculated into 10 ml Glu/CM-His, -Ura, -Trp medium and the cultures were grown overnight on a 30°C shaker (230 rpm). The yeast cells were then centrifuged at 4,000 x g for 10 min, and resuspended in 1 ml of SCE (1 M sorbitol, 0.1 M sodium citrate, 60 mM EDTA, pH 7.0). After centrifugation at 16,000 x g for 20 sec, the yeast cells were incubated at 37°C for 1 h in 200 µl SCE-ZB solution (SCE, 2,000 unit/ml NEE-154 glucluslase, 0.1% v/v 2-mercaptoethanol). DNA was precipitated using potassium acetate and isopropanol, and re-dissolved in TE.
2.18.12 Transformation of yeast DNA into *E. coli*

Plasmid DNA isolated from yeast were electroporated into *E. coli* SURE competent cells (Stratagene). Briefly, 1 μg of DNA was added to 20 μl competent cells in a cuvette (GIBCO BRL Life Technologies). The mixture was then electroporated at 415 V using a Cell Porator (GIBCO BRL Life Technologies). After electroporation, 1 ml of LB medium was added to the mix, followed by a 1 h incubation at 37°C. The bacteria were then spun down at 16,000 x g for 10 sec, streaked on LA plates, and incubated overnight at 37°C.

2.18.13 Preparation of plasmids encoding potential CD44cyt-interacting proteins for sequencing

Individual colonies that grew on LA plates after the transformation step described in 2.18.12 were inoculated into 2 ml of LA medium and the cultures were grown overnight on a 37°C shaker. DNA was prepared using Spin Columns (Qiagen) following the manufacturer’s instructions and dissolved in water. The final concentration of the DNA was determined by measuring the OD$_{260}$.

2.18.14 Identification of putative CD44cyt-interacting proteins by sequencing

Plasmid DNA prepared according to 2.18.13 was sequenced using the ABI 310 Genetic Analyzer (Perkin Elmer Instruments, Woburn, MA) using the oligo 5' CTG AGT GGA GAT GCC TCC 3' as the primer. The readable sequence was searched against the DNA and protein databases (NCBI, NIH, Bethesda, MD) for possible matches using Blast Search program (NIH).
2.18.15 *In vitro* binding assay of GST-CD44cyt fusion proteins with potential interacting proteins produced by yeast

Yeast colonies containing putative CD44cyt-interacting proteins were grown overnight on a 30°C shaker (230 rpm) in 10 ml Glu/CM-Trp (complete medium without Trp, plus 2% Glucose). The culture was then transferred to 50 ml Gal/CM-Trp (complete medium without Trp, plus 2% Galactose). After the OD<sub>550</sub> reached 0.8, the yeast cells were centrifuged at 4,000 x g for 10 min, and washed once in PBS. The cells were lysed on ice in 500 μl of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.2 mM PMSF). 100 μl of 150 and 425 micron glass beads was added and the cell lysate was then vortexed at 4°C for 45 min. After centrifugation at 16,000 x g at 4°C for 20 min, the detergent soluble material was collected.

The protein concentration was determined using the BCA assay (Pierce). 2 mg of detergent-soluble lysate was precleared at 4°C for 1 h with 50 μl of a 50% slurry of GSH beads, and then incubated at 4°C for 1 h with 2 μg of GST alone or 2 μg of the full-length GST-CD44cyt fusion protein generated as described in 2.12. These mixtures were incubated at 4°C for 45 min with 30 μl of a 50% slurry of GSH beads. The beads were washed 3 times with lysis buffer and the bound proteins were eluted by boiling at 100°C for 5 min in 3 x reducing SDS sample buffer, and then subjected to electrophoresis. The potential interacting proteins that co-precipitated with the GST-CD44cyt fusion protein were detected by Western blotting (described in 2.8) using the anti-hemagglutinin antibody (Table 2.1).

2.18.16 Construction of putative positive sequences from the cDNA library into pET 30b(+) vector

The cDNAs encoding potential CD44cyt-interacting proteins were cloned into the bacterial expression vector, pET 30 b (+) vector (from Dr. R. Fernandez, Department of Microbiology and Immunology, University of British Columbia), which contains a His tag for
detection and a tetr gene for tetracycline selection. Briefly, pJG 4-5 cDNA library plasmids were digested with Eco RI, filled in with Klenow, and further digested with Xho I. The cDNA fragments were then inserted into pET 30 b (+), which had been digested with BamH I, filled-in with Klenow, and further digested with Xho I.

2.18.17 Co-expression/co-purification of GST-CD44cyt fusion protein and His-tagged library proteins

Plasmids encoding the full-length or the truncated GST-CD44cyt fusion protein and the His-tagged potential CD44cyt-interacting proteins were co-transformed into the E.coli BL21 strain. For co-expression studies, the bacteria were grown overnight at 37°C in 2 ml of double selection medium (LB with 20 μg/ml ampicillin and 10 μg/ml tetracycline). 200 μl of the overnight culture was then inoculated into 5 ml of the selection medium and the culture was incubated until the OD₆₀₀ reached 0.6-0.8. The culture was then induced with 0.1 mM IPTG, and incubated overnight on a 26°C shaker (180 rpm). For co-purification of the GST-CD44cyt fusion protein and His-tagged proteins, the bacteria were centrifuged at 16,000 x g for 2 min at room temperature. The cells were lysed on ice in 500 μl of TN lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.2 mM PMSF) containing 0.5% TX-100 and 1 μg/ml lysozyme. The lysate was then subjected to 3 cycles of freezing and thawing carried out by freezing the cells in a dry-ice/ethanol bath for 3 min, followed by thawing them in a 37°C water bath for 3 min. DNase I was then added to the lysate at a final concentration of 1 μg/ml and the lysate was incubated at 37°C for 10 min. After centrifugation at 16,000 x g for 20 min at 4°C, the detergent soluble material was incubated at 4°C for 30 min with 20 μl of a 50% slurry of GSH beads. The precipitates were then washed 4 times with the lysis buffer, followed by a final wash rotating at 4°C for 10 min. The proteins were eluted off the GSH beads by boiling at 100°C for 5 min in 3 x reducing SDS sample buffer, subjected to electrophoresis, and transferred to a PVDF membrane. The
potential CD44cyt-interacting proteins co-purified with the GST-CD44cyt fusion protein were detected by anti-His Western blotting (described in 2.8 and Table 2.1).

2.19 Immobilization of antibodies or HA on tissue culture plates

50 μl of purified anti-CD44 mAb, KM201 (40 μg/ml), or 2% w/v BSA in PBS, were coated in one well of the 96-well tissue culture plates at 37°C for 3 h. The plates were washed twice with PBS, then blocked with 2% w/v BSA in PBS for 2 h at 37°C and washed 3 times with PBS prior to use. To immobilize HA on tissue culture plate, the sodium salt of rooster comb HA (Sigma-Aldrich Canada) was dissolved overnight at 4°C in PBS at a concentration of 5 mg/ml. 50 μl of the HA solution was then coated onto wells of a 96-well tissue culture plates at 37°C for 24 h. The plates were washed 3 times with PBS and kept at 4°C before use.

2.20 Cell adhesion and cell spreading assay

10^5 BW5147 cells were seeded on the antibody- or BSA-coated 96-well tissue culture plates at a concentration of 2 x 10^6/ml in DMEM containing 0.1% v/v FCS for various times at 37°C. 30 μl of 3 x reducing SDS sample buffer was added directly to the cells after each time point and the samples were boiled at 100°C for 5 minutes before electrophoresis. In some experiments, the cells were incubated at 37°C for 30 minutes with DMEM/0.1% FCS containing either 10 μM PP2 (Calbiochem), 25 μM Cytochalasin D (Calbiochem), or 5 mM EDTA. The cells were then transferred to the tissue culture plates coated with antibody or HA.

2.21 Isolation of the low-density lipid fraction by density gradient centrifugation on sucrose

The total detergent treated BW5147 cell lysate was separated on sucrose density gradients according to the method of Rodgers et al. (213) with modifications. Briefly, 5 x 10^7 cells were lysed in 1 ml of ice-cold TK buffer (components defined in 2.15) containing 1% v/v
Brij-58 or 1% v/v Triton X-100. The lysate was mechanically disrupted with a Dounce homogenizer for 15 strokes, then incubated on ice for 20 minutes. The lysate was diluted with 1 ml of 80% w/v sucrose in TK buffer, transferred to an ultracentrifuge tube, and overlaid with 6 ml of 30% w/v sucrose and 3.5 ml of 5% w/v sucrose in TK buffer. The samples were then ultracentrifuged at 230,000 x g at 4°C for 16 h using an SW41 swinging bucket rotor (Beckman Instruments Inc., Mississauga, ON). The gradients were collected into 8 x 1.5 ml fractions from the top of the ultracentrifuge tube. The pellet was washed twice with TK buffer and dissolved in 150 µl 2% w/v SDS, 10 mM Tris-HCl, pH 7.2. 20 µl from each fraction and 2 µl from the dissolved pellet were boiled at 100°C for 5 min in 3 x reducing SDS sample buffer prior to electrophoresis.

2.22 Immunoprecipitation of CD44 from sucrose gradients

Fractions 2 to 4 (low-density), and fractions 7 and 8 (high-density) from the sucrose gradients were pooled separately. The high-density pool was diluted 2 times with TK buffer (components defined in 2.15) to reduce the sucrose concentration before preclearing with 60 µl of a 50% slurry of Sepharose beads. Then CD44 was immunoprecipitated for 2 h at 4°C with 60 µl of a 25% slurry of IM7 conjugated CNBr-activated Sepharose beads (4 mg/ml) or 30 µl of Sepharose beads alone as the control. Immunoprecipitates were washed 3 times with lysis buffer. Proteins were eluted off the beads by boiling at 100°C for 5 min in 3 x reducing SDS sample buffer and then subjected to electrophoresis.

2.23 Immunoprecipitation of Pyk2, FAK and Paxillin from immobilized BW5147 cells

5 x 10⁶ cells were added to one well of a 6 well tissue culture plate (Falcon) that had been coated with 0.5 ml of 40 µg/ml of KM201, and allowed to flatten at 37°C for various times. The cells were then lysed on ice in the plate in the presence of 1 ml of medium with 250 µl of 5 x lysis buffer containing 5% TX-100, 50 mM Tris-HCl, pH7.2, 140 mM KCl, 10 mM
EDTA, 2.5 mM sodium orthovanadate, 1 mM sodium molybdate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin and 1 mM PMSF. The cells were then scraped off the plate using a cell scraper, collected into Eppendorf tubes, and centrifuged at 16,000 x g for 10 min at 4°C. The detergent-soluble cell lysate was incubated at 4°C for 1 h with 1 μg of anti-Pyk2, anti-FAK, or anti-Paxillin antibody. Then 20 μl of a 50% slurry of protein A agarose beads (Repligen Corporation, Needham, MA) or protein G Sepharose 4 Fast Flow beads (Amersham-Pharmacia Biotech) were added. After rotating at 4°C for 45 min, the immunoprecipitates were washed 3 times with 1 ml lysis buffer. The proteins were eluted off the beads by boiling at 100°C for 5 min in 3 x non-reducing or reducing SDS sample buffer, and then subjected to electrophoresis.

2.24 Data analysis

The Western blots were scanned at 300 dpi. Spot densitometry was carried out using NIH image software by measuring the density of proteins of interest. The measurements were repeated 3 times and the data were imported into the Microsoft Excel™ for further analysis.

To quantify the percentage of spread cells in the cell spreading assay (described in 2.20), the total number of cells and the number of spread cells were counted in 3 individual microscopic fields. The data were analyzed using Microsoft Excel™ software.
CHAPTER THREE
Chimeric CD4/CD44 Molecules Associate with CD44 on the Cell Surface via the Transmembrane Region and Reduce Hyaluronan Binding in T Cell Lines

3.1 Introduction and rationale

CD44 participates in a number of events related to leukocyte adhesion and migration including those involved in haemopoiesis, T cell activation, lymphocyte homing, and leukocyte extravasation at sites of inflammation. Most of these CD44-mediated functions are mediated by the interaction of CD44 with its ligand HA, a component of ECM. Resting leukocytes do not normally bind to HA. However, the HA binding ability of leukocytes via cell surface CD44 is markedly increased upon appropriate stimulation. This up-regulated HA binding ability allows for the “rolling” process to occur under flow conditions, which correlates with the process of leukocyte extravasation, as well as for T cell migration on HA-coated substratum. How CD44 regulates HA binding has been extensively studied, but most of the studies are focused on the extracellular domain of CD44. An early observation based on mutagenesis studies indicated that the presence of the cytoplasmic domain of CD44 (CD44cyt) was required for the optimal HA binding in a T cell line and a tumor cell line. This stresses that the CD44cyt can potentially influence HA binding through mechanisms to be identified. When this study was initiated, how CD44cyt influences HA binding was largely unknown. As introduced in 1.4.3, since the cytoplasmic domain of CD44 lacks intrinsic enzymatic activities, it is likely to interact with other intracellular molecules, as is the case for the integrin cytoplasmic domain. By interacting with intracellular molecules important for HA binding, the CD44cyt can regulate the HA binding ability of the cell. In this chapter, I have addressed whether the transmembrane domain of CD44 or the CD44cyt plays a role in HA binding by first, identifying whether they
are important for the HA binding function of the cell, and second, determining whether they mediate molecular interactions that affect HA binding.

3.2 Experimental approach

To determine whether the transmembrane domain of CD44 or the CD44cyt is important for HA binding, a series of CD4/CD44 chimeras were generated in this study by fusing the extracellular domain of human CD4 to the transmembrane and/or cytoplasmic domains of murine CD44 (Figure 3.1). These chimeras were then transfected into two murine T cell lines, BW5147 and T28, as well as a murine fibroblast line, NIH 3T3. All three of these cell lines express endogenous CD44, however, BW5147 and T28 cells bind to HA constitutively whereas NIH 3T3 cells do not bind HA unless induced by an anti-CD44 mAb, IRAWB. The overall HA binding ability of the chimera-transfected cells was compared to that of the untransfected cells so that any change in the ability of HA binding could be correlated to the individual regions of the chimeric protein. Once the region important for HA binding was identified, molecular interactions were sought by immunoprecipitating CD4/CD44 chimeras from cell lysates, followed by Western blotting probing for possible candidates that potentially interacted with these chimeras through the CD44 transmembrane domain or cytoplasmic domain.

The reasons to choose the extracellular domain of human CD4 were that first, like CD44, CD4 is a transmembrane glycoprotein. Second, the size of the external domain of human CD4 is comparable to that of the murine CD44. Third, the extracellular domain of CD4 is less conserved between human and mouse (less than 20% sequence identity), and therefore, it is unlikely to interfere with most species-specific antibodies for CD4, or with the endogenous murine CD4 molecules in these cell lines. Last, the human CD4 external domain does not bind HA, which allows for the screening of changes in the overall HA binding of chimera transfected cells.
Figure 3.1. Diagram of the CD4/CD44 chimeras. Chimeric proteins containing the extracellular domain (ext), the transmembrane domain (tm), and the cytoplasmic domain (cyt) of murine CD44 (white) and human CD4 (grey) were indicated. * represents a cysteine to serine point mutation at position 286 of murine CD44.1 sequence.
3.3 Results

3.3.1 Reduced HA binding in T lymphoma cells after transfection of the 4/44/44 chimera

The 4/44/44 chimera containing the extracellular domain of human CD4 (unable to bind HA) and the transmembrane and cytoplasmic domains of murine CD44 was first transfected into BW5147 cells. It was expected that over-expression of the transmembrane and the cytoplasmic domain of CD44 would compete with the endogenous CD44 and sequester molecules that are important for promoting HA binding ability of endogenous CD44. Flow cytometry studies showed that the 4/44/44 BW5147 transfectants bound approximately half the amount of HA-FITC observed for the untransfected cells (Figure 3.2A). Four independent clones (CB+02, CB+04, CB+06 and CB+12) bound an average of 64 ± 10% (n=1) of HA-FITC compared to that bound by the untransfected cells. This HA-FITC binding was shown to be CD44-dependent by inhibition with the CD44 antibody, KM201 (data not shown).

Transfection of BW5147 T cells with the human CD4 molecule did not alter the HA binding ability of these cells, suggesting a role for the transmembrane or cytoplasmic regions of CD44 in mediating this effect. Reduced HA-FITC binding was also observed in another T lymphoma cell line, T28, when it was transfected with the 4/44/44 chimera, but not when CD4 was transfected (Figure 3.2B). However, transfection of the 4/44/44 chimera into NIH 3T3 fibroblast cells, which do not bind HA unless induced by the CD44 antibody, IRAWB (18), did not alter this non-binding phenotype (data not shown). Thus, these results indicate that the transmembrane or the cytoplasmic domain of CD44 can regulate the constitutive HA binding function in T cells, but do not affect the IRAWB-inducible binding in NIH 3T3 cells.
Figure 3.2. Cell surface expression of endogenous CD44, transfected CD4 and chimeric 4/44/44, and HA binding ability in BW5147 and T28 lymphoma cells as determined by flow cytometry. (A) Untransfected BW5147 T cells (-) and BW5147 T cells transfected with the chimera, 4/44/44, and human CD4 are indicated on the right. Unlabeled cells or cells labeled with secondary Ab alone are shown on the bottom row (Control). CD44 was labeled with the mAb, IM7, as shown in the first panel on the left; CD4 or the chimera, 4/44/44, were labeled with an anti-CD4 mAb, SIM.4, as shown in the second panel, and the HA binding ability of each of the transfectants is shown in the third panel after labeling with 2 μg/ml of HA-FITC. (B) As in (A) except T28 cells and transfectants were analyzed. Fluorescence intensity is shown on a log scale. Figures represent one of three independent experiments.
3.3.2 Co-precipitation of endogenous CD44 with the 4/44/44 chimera

To investigate whether the chimera was reducing HA-FITC binding by interacting with endogenous CD44 or by sequestering positive regulatory components, experiments were first performed to look for the association of endogenous CD44 with the chimeric molecule. The 4/44/44 chimera was immunoprecipitated from the three cell lines (BW5147, T28 and NIH 3T3) after cell surface biotinylation and lysis of the cells in 1% Brij-96. The 1% Brij-96 cell lysate was subjected to a high speed spin at 86,000 x g for 20 min to remove any large membrane aggregates. The immunoprecipitated proteins were then separated by SDS-PAGE, transferred to a PVDF membrane and visualized using streptavidin-HRP and ECL (Figure 3.3A). Incubation of the membranes with the anti-CD44 antibody, KM201, specific for the extracellular domain of CD44, detected the presence of endogenous CD44 in the immunoprecipitates of the chimeric 4/44/44 protein, but not in anti-CD4 immunoprecipitates from untransfected or CD4 transfected cells (Figure 3.3B). Western blotting with anti-CD45 antisera did not detect the presence of CD45, a major leukocyte glycoprotein, co-precipitating with the chimera in T28 cells. This indicated that the association of endogenous CD44 with the chimera was specific (data not shown). Endogenous CD44 co-precipitated with the chimeric 4/44/44 molecule in both lymphoid cell lines and to a lesser extent in NIH 3T3 cells (Figure 3.3B and 3.4B). Calculations from densitometric scanning of the Western blots indicated that approximately 7% of the total CD44 was associated with the chimera isolated from lymphoid cells whereas less than 1% was associated with the chimera isolated from NIH 3T3 cells.

To further determine the conditions under which co-precipitation occurred, cells were lysed in three different detergents. Figure 3.4A demonstrates that while 4/44/44 chimeric molecules were immunoprecipitated in each case, endogenous CD44 only co-precipitated with the chimera when the cells were lysed in 1% Brij-96, and not when cells were lysed in 1% Triton-X-100 or 60 mM OG (Figure 3.4B).
Figure 3.3. Immunoprecipitation of transfected CD4 and 4/44/44 molecules and association with endogenous CD44 in BW5147, T28, and NIH 3T3 cells. (A) Cells were surface biotinylated and solubilized in lysis buffer containing 1%Brij-96. Double the amount of CD4 transfected cells were used to compensate for the lower levels of expression of CD4. Approximately equal amounts of CD4 and 4/44/44 molecules were immunoprecipitated (ip) using an anti-CD4 mAb, OKT4. Lane 1 of each panel is control CD4 immunoprecipitated from untransfected cells (-); lane 2, CD4 immunoprecipitated from CD4 transfected cells; lane 3, CD4 immunoprecipitated from cells transfected with the chimera 4/44/44. Western blot using straptavidin-HRP was conducted to visualize the biotinylated 4/44/44 chimera. (B) The Western blot in (A) was stripped and reprobed with an anti-CD4 mAb, KM201 (which recognizes the extracellular domain of CD44), to identify any endogenous CD44 co-precipitated with the transfected molecules. Relative molecular mass of the prestained markers is indicated on the left in kDa. Figures represent one of three independent experiments.
Figure 3.4. Immunoprecipitation of the 4/44/44 chimera and the association with endogenous CD44 in BW5147, T28, and NIH 3T3 cells under different detergent conditions. (A) 5 x 10^6 BW5147 and T28 T lymphoma cells or 2 x 10^6 NIH 3T3 fibroblast cells were surface biotinylated and solubilized in 1% Brij-96, 60 mM OG or 1% TX-100. Chimeric 4/44/44 molecules were immunoprecipitated (+) from transfected BW5147, T28 and NIH 3T3 cells using an anti-CD4 mAb, OKT4. Control immunoprecipitations (-) were performed in the absence of anti-CD4 mAb. The first lane labeled L, is unlabeled cell lysate from 5 x 10^5 lymphoma cells and 2 x 10^5 fibroblasts. Western blots using streptavidin-HRP were conducted to visualize the biotinylated 4/44/44 chimera. (B) The Western blots in (A) were stripped and reprobed with an anti-CD44 mAb, KM201, to identify any endogenous CD44 co-precipitated with the transfected molecule. Relative molecular mass of the prestained markers is indicated on the left in kDa. Figures represent one of three independent experiments.
3.3.3 The transmembrane domain of CD44 is required for the self-association of CD44

To determine whether the transmembrane or the cytoplasmic domain of CD44 was responsible for the interaction with endogenous CD44, a series of CD4/CD44 chimeras were made. A truncated chimera consisting of the extracellular domain of CD4, the transmembrane domain and only the first five amino acids of the cytoplasmic domain of CD44 (4/44/-), was generated and expressed in BW5147 T cells. As can be seen from Figure 3.5, this chimera co-precipitated endogenous CD44, localizing the region responsible for the interaction to the transmembrane domain or the first five amino acids of the cytoplasmic tail. However, a second chimera consisting of the extracellular domain of CD4, the transmembrane region of CD44 and the cytoplasmic tail of CD4 (4/44/4) also co-precipitated endogenous CD44, further localizing the region responsible for the interaction to the 21 amino acid transmembrane region of CD44 (Trp 251-Val 271).

A unique cysteine residue in the transmembrane region of mouse CD44 (Cys 286) is highly conserved between several mammalian species. Mutation of this cysteine has been shown to affect the inducible HA binding ability of CD44 when transfected into Jurkat T cells (111). Subsequent studies showed that the cysteine was involved in the dimerization of CD44 upon PMA stimulation, which induced the HA binding of Jurkat cells (50). To determine if this cysteine was important in mediating the interaction with endogenous CD44, it was mutated to a serine in both the 4/44/- chimera (4/44*-/-) and the 4/44/4 chimera (4/44*/4). As can be seen from Figure 3.5, this mutation did not abolish the association of CD44 with the chimera.

3.3.4 Correlation between expression of the transmembrane region of CD44 in the chimera, association with endogenous CD44 and reduced HA binding

To determine the effect of the expression of these different CD4/CD44 chimeras on the HA binding ability of these BW5147 T cell transfectants, FACS analysis was performed (Figure 3.6). As before, expression of the CD4/CD44 chimeras did not significantly affect the
Figure 3.5. Immunoprecipitation of various chimeric CD4/CD44 molecules and their association with endogenous CD44 in BW5147 T cells. (A) 5 x 10^6 cells were surface biotinylated and solubilized by 1% Brij-96. Different chimeric CD4/CD44 molecules or CD4 were immunoprecipitated using an anti-CD4 mAb. Double the amount of cells was used to immunoprecipitate similar amounts of protein from CD4, 4/44/4 and 4/44*/4 transfected cells which expressed approximately two fold less protein than the other chimeras. The proteins immunoprecipitated from the BW5147 transfectants are indicated on the top of the gel, untransfected cells are represented by (-). Immunoprecipitates were performed in the presence (+) or absence (-) of the anti-CD4 mAb. The first lane, L, is unlabeled cell lysate from 5 x 10^5 cells; the second lane, C, is anti-CD4 antibody alone with no cell lysate. Western blot using straptavidin-HRP was conducted to visualize the biotinylated CD4/CD44 chimeras. (B) The Western blots in (A) were stripped and reprobed with an anti-CD44 mAb, KM201, to identify endogenous CD44 co-precipitated with the transfected molecule. The prestained molecular weight markers are indicated on the left in kDa. Figures represent one of three independent experiments.
expression levels of endogenous CD44. All chimeras expressing the transmembrane region of CD44, including those with the C286S mutation, exhibited an approximately two-fold reduction in HA-FITC binding. Comparison of the mean fluorescence values for HA-FITC binding in the transfected and untransfected cells indicated that the transfected cells bound 60 ± 10% (n=15) of the HA-FITC that bound to untransfected cells. Expression of the CD4/CD44 chimeras containing the transmembrane region of CD44 thus consistently reduced the HA binding ability of endogenous CD44 in these T cells. The expression levels of the CD4/CD44 chimeras and CD4 molecules varied slightly between constructs and between cell types. The expression of similar levels of some CD4/CD44 chimeras to the control CD4 molecule indicated that the slightly lower expression levels of the CD4 molecule could not explain its inability to affect HA-FITC binding. Likewise, immunoprecipitation of equivalent amounts of CD4 to the 4/44/44 chimera, did not co-precipitate endogenous CD44. Thus the transmembrane region of CD44 was found to be required for the co-precipitation of endogenous CD44 by the chimeric CD4/CD44 molecules and this correlated with a reduction in HA-FITC binding.

3.3.5 Chemical cross-linking revealed that the 4/44/44 chimera and endogenous CD44 associated on the T cell surface

To further explore the possibility that CD4/CD44 chimeras containing the transmembrane domain of CD44 and endogenous CD44 associate on the cell surface, chemical cross-linking studies of cell surface proteins were conducted using a water-soluble chemical cross-linker, DTSSP. This cross-linker is cell membrane-impermeable, thiol-cleavable, and reactive with primary amines. Therefore, it only cross-links the extracellular domain of membrane proteins that reside within a very close proximity (12Å) to each other. Furthermore, the cross-linking product with higher molecular weight can be cleaved by reducing agents into
Figure 3.6. HA binding ability of BW5147 T cells expressing endogenous CD44, transfected CD4 and different CD4/CD44 chimeras as determined by flow cytometry. The transfected molecules expressed in BW5147 T cells are indicated on the right (see Figure 3.1 for additional details of the chimeric molecules). Labeling of untransfected cells (-) is shown and the unlabeled cells or cells labeled with secondary antibody alone (Control) are shown. CD44 was labeled with the mAb, IM7 in the first panel; CD4 or the CD4/CD44 chimeras were labeled with an anti-human CD4 mAb, SIM.4, in the second panel; and the HA binding ability of each of the transfectants is shown in the third panel after labeling with 2 µg/ml of HA-FITC. Fluorescence intensity is shown on a log_{10} scale. Figures represent one of three independent experiments.
monomeric proteins to verify the specificity of the chemical reaction. In this study, the 4/44/44 transfected BW5147 cells were treated with various concentrations of DTSSP before solubilization with 1% TX-100. Both the detergent soluble material and the pellet were analyzed. As shown in Figure 3.7A and Figure 3.7B, the higher molecular weight forms of both 4/44/44 and endogenous CD44 were detected in the non-reduced TX-100 soluble fractions at the concentrations of 10-100 µM DTSSP. After treating the samples with reducing agent, however, the higher molecular weight forms of both molecules were diminished. It was also noticed that the cross-linked product of 4/44/44 was more visible than that of the endogenous CD44. There are two possible reasons for this. First, in BW5147 cells, the 4/44/44 chimera was expressed at a higher level (~ 5 fold) than the endogenous CD44, as determined using an antiserum that recognizes the cytoplasmic domain of CD44 (R. Li unpublished data). Second, the affinity of the antibodies that recognize the cross-linked external domains of CD4 and CD44 might be different. The anti-CD4 antibody was a polyclonal antiserum, whereas the anti-CD44 antibody was a monoclonal antibody. To further test the specificity of the chemical reaction, CD45, an abundant leukocyte cell surface protein, was analyzed from cells treated with DTSSP. As can be seen in Figure 3.7C, no higher molecular weight forms of cross-linked CD45 were detected within the concentration range of DTSSP tested. It is thus unlikely that CD45 forms aggregates on the cell surface. In summary, this result, in agreement with the observations provided by chimera studies, supports the idea that CD44 forms membrane complexes, and that this association may facilitate the ability of CD44 to bind HA in T cells.
Figure 3.7. 4/44/44 chimera and endogenous CD44, but not CD45, associate on BW5147 cell surface. 5 x 10^5 of BW5147 cells transfected with the 4/44/44 chimera were chemically cross-linked by DTSSP at the various concentrations indicated. 1% TX-100 soluble fraction (S) and insoluble pellet (P) were treated with non-reducing sample buffer (non-reduced) or reducing SDS sample buffer (reduced). (A) Western blot of 4/44/44 chimera with the anti-CD4 antisera, T4-4. (B) Western blot of endogenous CD44 with the anti-CD44 mAb, KM201. (C) Western blot of endogenous CD45 with the anti-CD45 antisera, R02.2. Arrows indicate the cross-linked products of 4/44/44 and CD44. Relative molecular mass of the prestained markers is indicated on the left in kDa. Figures represent one of three independent experiments.
3.4 Discussion

3.4.1 Relationship between self-association of CD44 and the HA binding ability of T cells

This work demonstrates that the transmembrane domain of CD44 is responsible for the association of the CD4/CD44 chimeras with endogenous CD44 (Figure 3.3, 3.4 and 3.5). When such an association occurs in T cells, which constitutively bind HA, the ability of these cells to bind HA is reduced (Figure 3.2 and 3.6). Association of endogenous CD44 with the chimera also occurs in NIH 3T3 fibroblast cells, albeit to a lesser extent (Figure 3.3 and 3.4), indicating that it does not occur only in cells that constitutively bind HA. Expression of non-HA binding chimeric CD4/CD44 molecules in T cells reduces HA binding, possibly by competing for and disrupting endogenous CD44-CD44 interactions. This suggests that CD44-mediated HA binding in T cells is enhanced when endogenous CD44 is associated with itself on the cell membrane. Consequently, factors that affect interactions of the transmembrane region of CD44 may modulate the aggregation state of CD44 in the cell membrane and thus provide a means of regulating the HA binding ability of CD44 in T cells.

Previous studies have indicated that IRAWB-induced HA binding of CD44 is not observed when a monovalent Fab fragment is used (75). Likewise, substitution of the transmembrane domain of CD44 with that of CD3ζ, which forms disulfide-linked homodimers, resulted in the up-regulation of HA binding by CD44 in AKR T lymphoma cells (113), showing that artificial dimerization of CD44 can enhance HA binding. Liu et al. reported that PMA-induced HA binding in Jurkat cells was mediated by dimerization of CD44 involving the transmembrane cysteine (50). However, I found no evidence for the CD44 transmembrane region mediating disulfide-linked dimer formation, which would have been observed on the non-reducing gels and would have been abolished by mutation of the only cysteine residue present in the transmembrane region. The discrepancy here could be due to the fact that the T cell lines used in this study express endogenous CD44 and bind to HA constitutively, whereas Jurkat cells transfected with CD44 cannot bind HA. PMA stimulation, which converts Jurkat
cells to bind HA, may also trigger other cellular events different from unstimulated cells. In another example, self-association of an alternatively spliced form of CD44 (CD44v4-7) has recently been shown in a pancreatic carcinoma cell line which results in enhanced HA binding. In this case, however, glycosylation, rather than the transmembrane region, was implicated in mediating aggregate formation (141). Therefore, these results favor the "aggregation model" that self-association of CD44 molecules on the cell surface will optimize the ligand binding function of CD44 probably by increasing the binding avidity as HA is a polymeric GAG. However, the mechanisms that lead to this CD44 self-association seem to be diverse.

Although it is evident that CD4/CD44 chimeras and endogenous CD44 forms oligomers, it is also observed that the transfected chimeras are expressed at higher levels (approximately 5 fold) than that of the endogenous CD44 (R. Li unpublished data). Thus, it is a possibility that the association of chimeras with themselves or with endogenous CD44 may not necessarily occur if they are expressed at physiological levels. One way to overcome this limitation is to utilize different clones of CD4/CD44 chimeras that exhibit different levels of expression and compare the difference of associations with endogenous CD44. However, in this study, transfectants with only high expression of CD4/CD44 chimera were sorted and kept, therefore, it is impossible to make a direct comparison between clones with varied surface levels of chimeras.

3.4.2 The transmembrane domain of CD44 is responsible for the association of CD4/CD44 chimeras and the endogenous CD44

Other proteins have been shown to co-precipitate with transmembrane proteins when cells are solubilized with mild detergents such as digitonin or Brij. These include the association of Src-family kinase, Lck, with TCR, CD2, CD4, CD8, CD11a, CD43, and CD55; the association of the TCR complex with CD2, CD4, CD5 and CD8; and the association of CD63 with VLA-3 and VLA-6 integrins (214-218). In another study, CD45 was found to
associate with CD45AP in 1% Brij-96 and interestingly, the transmembrane domains of both proteins were found to be required for this association (219-221). Furthermore, mutational analysis of amino acids in the transmembrane region indicated that no specific amino acids were responsible for the interaction (222).

Glycophorin A is another cell surface molecule that is found as a dimer on the cell surface (223). Dimerization was found to be mediated by a stable, non-covalent interaction with the transmembrane region, indicating that specific associations can occur between two identical transmembrane helices (224, 225). Although this study established that the transmembrane region of CD44 is required for its self-association, it is not known whether this represents a direct interaction or whether an intermediate molecule is involved. Chemical cross-linking results indicated that the association of the 4/44/44 chimera or the endogenous CD44 occurs in the cell membrane (Figure 3.7). However, since only cell lysate was analyzed after DTSSP cross-linking, it is not clear whether the 4/44/44 chimera or the endogenous CD44 formed homodimers, heterodimers, or associated with a third unknown protein. A more definitive experiment would be to immunoprecipitate CD44 after DTSSP cross-linking in 4/44/44 transfected BW5147 cells, followed by anti-CD4 Western blotting or vice versa. However, immunoprecipitation of either CD44 or 4/44/44 chimera after DTSSP treatment was not very efficient, suggesting that the antibodies for immunoprecipitation did not recognize the cross-linked products very well.

This interaction between CD4/CD44 chimeras and the endogenous CD44 was readily observed in two T cell lines, which bind HA constitutively. This interaction also occurred to a much lower extent in NIH 3T3 fibroblast cells, which express CD44 but do not constitutively bind HA. This suggests that the transmembrane interaction may be influenced or regulated by factors, such as membrane lipid composition or cytoskeletal organization, which can vary between cell types. Interestingly, other cell adhesion molecules such as ICAM-1, LFA-3 and Mac-1 have all been shown to bind with higher affinity to their ligands (LFA-1, CD2 and C3bi).
when they are dimeric or multimeric (140, 226-228). In the case of ICAM-1, both the extracellular and transmembrane regions have been implicated in the non-covalent formation of homodimers (140). In this study, the transmembrane region of CD44 was found to be responsible for mediating the dimerization of CD44 and CD4/CD44 chimeras and that this association correlated with reduced HA binding in T cells. I also provided evidence that endogenous CD44 molecules may self-associate on the T cell surface and propose that this interaction promotes HA binding probably by increasing the binding avidity of CD44 and HA.
CHAPTER FOUR
CD44 Interacts with Lck, Fyn, Grb-2, and the Ezrin/Radixin/Moesin (ERM) Family of Cytoskeletal Proteins in Lymphoid and Myeloid cells

4.1 Introduction and rationale

The process of cell adhesion and cell migration in response to environmental stimuli involves the dynamic reorganization of the cytoskeleton. During this process, special structures such as focal adhesions and focal complexes are developed to connect the ECM to the cytoskeletal machinery via adhesion molecules on the plasma membrane. A dual function has been assigned to these cell adhesion molecules in coordinating the crosstalk between cytoskeleton and the ECM. First, they provide a foothold for cell interactions, and second, they transmit information across the cell membrane. This is best manifested in integrin-mediated cell adhesion and cell migration. As introduced in 1.8.3, upon binding of integrins to ECM components, signaling molecules and cytoskeletal proteins are recruited to the focal adhesions via the cytoplasmic domain of integrins. This results in the activation of kinases (i.e. FAK or Pyk2) and phosphorylation of important cytoskeletal or adapter/docking proteins (i.e. paxillin and vinculin), which then recruit other signaling molecules (i.e. other kinases, adapter or docking proteins, GAPs, GEFs, GDIs and small GTPases) as well as actin-binding proteins. Targeting of these signaling and cytoskeletal proteins to focal adhesions allows for the assembly of actin filaments into special membrane structures such as filopodia, lamellipodia, membrane ruffles and stress fibers, as well as for the generation of internal force for cell migration. On the other hand, dissociation of the focal adhesions from the substratum at the trailing edge of a migrating cell is as important as the formation of focal complexes at the leading edge because only the release of these contact points can allow cells to migrate to their designated destination.
As mentioned in 1.9.3, CD44 has been shown to localize to focal adhesions, uropods, filopodia and lamellipodia. Moreover, the ERM family cytoskeletal proteins were shown to interact with CD44 in BHK cells and in L cells (118). As well, triggering of CD44 by antibodies or its ligand, HA, can initiate cytoskeletal changes, such as cell spreading in lymphocytes (143-146). These results indicate that, like integrins, CD44 not only associates with the actin cytoskeleton in cellular structures related to cell adhesion and cell migration, but also signals to rearrange the cytoskeleton during the process of cell adhesion and migration. How CD44 might associate and signal to the cytoskeleton was one of my thesis objectives since the signaling cascade initiated by CD44 and the association of CD44 with cytoskeleton were poorly understood when this study was first started. It is known that similar to selectins and integrins, the cytoplasmic domain of CD44 (CD44cyt) does not have any intrinsic enzymatic activity. Thus, it is more likely that the CD44cyt is involved in mediating protein-protein interactions. Signaling molecules and cytoskeletal components that are required for the assembly of focal adhesions or focal complexes are potential downstream targets for CD44, and may also associate with the CD44cyt. In this chapter, I used two different approaches to identify potential CD44-interacting proteins in leukocytes. I also determined whether CD44 interacted with ERM proteins in T cells and myeloid cells. This was because ERM proteins are highly expressed in these cells, but it is not known whether they associate with CD44, and whether they play a role in regulating the CD44-mediated HA binding in these cells.

4.2 Experimental approaches

To determine whether the CD44cyt mediates molecular interactions, GST-CD44cyt fusion protein precipitation and CD44 immunoprecipitation were conducted using T cell lysates. To identify proteins that interact with the CD44cyt, the yeast two-hybrid interaction trap was performed.
4.2.1 Immunoprecipitation of CD44

GST fusion proteins have been successfully utilized in studying many different protein-protein interactions (229, 230). The major advantage of this approach is the convenience in manipulating the amount of a protein of interest for detecting its target proteins or characterizing an interaction. However, since this is an in vitro approach, information derived from it may not be physiological, and needs to be re-tested in vivo. In this study, radiolabeled T cell lysates were incubated with GST-CD44cyt fusion proteins to determine whether there was any protein that potentially interacted with the CD44cyt. Results were then further confirmed by CD44 immunoprecipitation using either radiolabeled cell lysates. Western blotting was also performed to identify candidate proteins, based on the relative molecular mass and their biological relevance to CD44.

4.2.2 Yeast two hybrid interaction trap

Protein-protein interactions play a critical role in most biological processes. The yeast two-hybrid system is a yeast-based genetic assay to detect protein-protein interactions in vivo. This system exploits the two domain nature of many site-specific eukaryotic transcription factors to detect interactions between two different hybrid proteins. These transcription factors consist of a site-specific DNA-binding domain that is distinct from a domain responsible for transcriptional activation. In the two-hybrid system, two fusion proteins must be generated. One is the fusion between the DNA-binding domain of a transcription factor and a test protein (bait). The other is a fusion between the activation domain of a transcription factor and another test protein (prey). Plasmids encoding these fusions are introduced together into a yeast strain that contains one or more reporter genes with upstream binding sites for the DNA-binding domain present in the first hybrid. The expression of either the hybrid of the DNA-binding domain with bait or the hybrid of the activation domain with prey fails to activate transcription.
Figure 4.1. LexA yeast two-hybrid interaction trap. (A) A hybrid consisting of a DNA-binding domain (BD) of LexA fused to Protein X is able to bind the LexA operator, but unable to activate transcription of a reporter gene (LEU or Lac Z). (B) A hybrid consisting of an activation domain (AD) fused to Prey1 is introduced into A, but fails to activate to the reporter gene because Prey1 cannot interact with Protein X, the activation domain cannot localize to the reporter gene. (C) If both hybrid are expressed in the same cell and Prey2 and Protein X interact, the activation domain is anchored to the binding site and the reporter gene is expressed.
of a reporter gene. However, if bait and prey interact, the transcriptional activator is anchored to the binding site and leads to the expression of the reporter gene (Figure 4.1).

The two-hybrid system has been used to detect interactions among proteins produced by prokaryotic organisms such as *E. coli* and by a wide range of eukaryotes including yeast, plants and mammals. In addition, protein interactions successfully detected by this system include those found normally in a variety of subcellular locations, including nucleus, cytoplasm, and mitochondria, associated with membrane and extracellular. This system has been applied to the study of a variety of cellular processes, such as cell cycle progression, signal transduction, and oncogenesis. More specifically, interactions of oncoproteins in this system include ras and raf (231-233), p53 and SV40 large T antigen (234), Rb and large T antigen (235), myc and max (236), and p53 with itself (234).

The yeast two-hybrid method has many advantages over other techniques for studying protein-protein interactions (237-239). First, the method not only allows for the identification of interacting proteins, but also results in the immediate availability of the cloned sequences for these proteins. Second, this method is very sensitive and can detect weak and transient interactions. Third, no purified target protein or antibodies to the target protein are required. Despite its numerous advantages, the major concern of using yeast two-hybrid system is the false positives. Much attention has been drawn to solve this problem in the past, and it has been found out that the best way to eliminate false positives is to include a series of controls in each step of the procedure (reviewed in ref. 240).

The two commonly used yeast two-hybrid systems are the Gal4 and the LexA system, representing *Saccharomyces cerevisiae* transcription factor Gal4 and the *E. coli* repressor LexA as site-specific DNA-binding domains. The LexA two-hybrid system was chosen for this study due to the advantages of this system. First of all, LexA is derived from a heterologous organism (241), has no known effect on the growth of yeast, and possesses no residual
transcriptional activity. In contrast, since Gal4 is an important yeast transcriptional activator, experiments using the Gal4 system must be done in Gal4⁻ yeast strains, which are frequently less healthy and more difficult to transform than the wild-type strains. Secondly, the LexA system can be used with a Gal-inducible promoter. The Gal-inducible promoter is engineered on the prey plasmid to control the selective expression of prey proteins using galactose as the carbon source, thus, allowing to gauge the specificity of the bait and prey interaction. In the Gal4 system, by contrast, this extra discrimination step is unavailable.

In this study, the CD44cyt was inserted next to the LexA protein and the LexA-CD44cyt was used as the bait. CD44cyt-interacting proteins were screened against a murine CD4⁺ T lymphocyte cDNA library, which was served as the prey. After confirming by the discrimination tests, cDNA sequences encoding the putative positive proteins were isolated from yeast, and then sequenced. cDNAs for these proteins were also cloned into a bacterial expression vector and the interaction of these proteins with the CD44cyt was further tested in vitro.
4.3 Results

4.3.1 Generation of the full-length and the truncated GST-CD44cyt fusion proteins

In order to determine whether the CD44cyt interacts with other cellular proteins, the full-length and the truncated GST-CD44cyt fusion proteins were expressed and purified from *E. coli*. cDNAs corresponding to the full-length CD44cyt and the truncated CD44cyt lacking the first 13 amino acids were cloned downstream of the GST cDNA in the pGEX-2T vector. GST-CD44cyt fusion proteins were then over-expressed in *E. coli* after IPTG induction, and purified using GSH beads. The truncated GST-CD44cyt was constructed because more than 50% of the full-length GST-CD44cyt underwent degradation during the purification procedure, likely due to the protease-sensitive arginine- and lysine-rich regions located at the beginning of the CD44cyt. Deletion of these regions significantly improved the yield and integrity of the fusion protein (Figure 4.2). Generally, after induced overnight by IPTG, the yield of the full-length GST-CD44cyt is 1-2 mg/L culture, whereas the truncated is ~10 mg/L bacterial culture.

4.3.2 Cellular proteins co-precipitate with GST-CD44cyt fusion proteins in T cell lines

TFX and BW5147 T lymphoma cells were metabolically labeled with $^{35}$S methionine and cysteine, solubilized in lysis buffer containing 1% Brij-96, and incubated with two versions of the GST-CD44cyt and with GST alone as a control. Figure 4.3 shows the profiles of potential CD44cyt-interacting proteins. It was observed that some cellular proteins, such as p55, p32 and p25, co-precipitated with the two versions of the GST-CD44cyt, but not with the GST alone control. Interestingly, compared to the truncated GST-CD44cyt, the full-length fusion protein either interacted with more proteins, as observed with p55, or mediated stronger interactions, as observed with p25. This indicated that the 13 amino acid membrane proximal region of the CD44cyt might be required for the optimal interaction of these proteins. Thus, this preliminary result confirmed the hypothesis that the CD44cyt might be involved in protein-
Figure 4.2. Purification of GST-CD44cyt fusion protein over-expressed in *E.coli*. (A) Schematic diagram of two versions of GST-CD44cyt fusion proteins, the full-length (F) and the truncated (T). Numbers correspond to the murine CD44.1 protein sequence. (B) GST-CD44cyt fusion proteins were constructed, transformed into *E.coli* XL1 blue, over-expressed after IPTG induction, and purified by GSH Sepharose beads. 1 µg of GST alone (GST), full-length GST-CD44cyt and truncated GST-CD44cyt were analyzed on 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Relative molecular mass is indicated on the left in kDa. Figure represents one of four independent experiments.
protein interactions, and provided a rationale to further identify molecules that potentially interact with the CD44cyt.

4.3.3 p32 and p25 co-immunoprecipitate with endogenous CD44 in T cell lines

To verify the observations obtained using the GST-CD44cyt fusion proteins, CD44 was immunoprecipitated by an anti-CD44 mAb, IM7, from [35S] methionine and cysteine labeled BW5147 and T28 T lymphoma cells. The CD44-null T lymphoma cell line, AKR, was used as a control for immunoprecipitation (Figure 4.4). Two prominent proteins at 32 kDa and 25 kDa were found to co-immunoprecipitate with CD44 in BW5147 and T28 cells, but not in the AKR control, suggesting that the interactions were specific for CD44. In order to map the regions of CD44 that interact with these proteins, I took advantage of two AKR transfected cell lines. TFX cells are AKR cells transfected with the wild-type CD44H. WT cells are AKR cells transfected with a truncated CD44H lacking all but the first 6 amino acids of the CD44cyt. In these two cell lines, p25 was found associated with the full-length CD44, and to a lesser extent, with the truncated CD44, whereas p32 only interacted with the full-length CD44 (Figure 4.4). This result is consistent with what was previously observed with the GST-CD44cyt fusion proteins, that both p32 and p25 could be detected in the precipitates of the full-length GST-CD44cyt, whereas only p32 was evident in the precipitates of the truncated GST-CD44cyt. Therefore, this result indicated that p32 and p25 might interact with CD44cyt in different regions. p25 is likely to associate with CD44 through the membrane proximal region of the CD44cyt, whereas p32 is likely to associate with CD44 through regions downstream of the first 6 amino acids of the CD44cyt.
GST-CD44cyt fusion protein precipitation

Figure 4.3. Intracellular proteins, p32 and p25, associate with GST-CD44cyt fusion proteins in T cells. 5 x 10^6 TFX cells, CD45^+ TCR^+ BW5147 (BW+) and CD45^- TCR^+ BW5147 (BW-) cells were labeled with [^35S] methionine and cysteine and solubilized using lysis buffer containing 1% Brij-96. Detergent soluble materials were incubated with 1 μg of GST, the full-length GST-CD44cyt (F), or the truncated GST-CD44cyt (T), which had been pre-coupled to GSH beads. Bead complexes were washed and analyzed on 10% SDS-PAGE. Proteins were visualized by autoradiography. Arrowheads indicate potential CD44 interacting proteins. Relative molecular mass is indicated on the left in KDa. Figures represent one of three independent experiments.
### CD44 Immunoprecipitation

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**Figure 4.4.** p32 and p25 co-immunoprecipitate with CD44 in T cells. 5 x 10^6 cells from CD45+ TCR+ BW5147 (BW+), CD45- TCR+ BW5147 (BW-), T28, AKR (CD44 null cells), WT (AKR transfected with CD44H Δ cyt) and TFX (AKR transfected with CD44H w.t.) were labeled with [35S] methionine and cysteine and solubilized in lysis buffer containing 1% Brij-96. Detergent soluble materials were precleared using 20 μl of protein G beads and subjected to immunoprecipitation by 20 μl of protein G beads pre-coupled to 100 μl of TCS of the anti-CD44 mAb, IM7 (+), or 20 μl of protein G beads alone (-). Immunoprecipitates were washed and analyzed on 10% SDS-PAGE. CD44 interacting proteins were visualized by autoradiography. Wild type CD44 (w.t.), tailless CD44 lacking all but the first 6 amino acids (Δ cyt), or no CD44 (-) was immunoprecipitated from each cell line and indicated at the bottom. Relative molecular mass is indicated on the left in kDa. Figure represents one of three independent experiments.
4.3.4 CD44 interacts with Lck and Fyn in BW5147 T cells

To further identify proteins that may have associated with CD44, but may not have been detected using radiolabeled cell lysate, an anti-phosphotyrosine Western blot was conducted on CD44 immunoprecipitates. CD45^+ TCR^+ and CD45^- TCR^- BW5147 cells, referred to as BW^+ and BW^- respectively, were lysed in lysis buffer containing either 1% Brij-58 or 1% TX-100, and CD44 immunoprecipitated. Anti-CD45 immunoprecipitations were also performed as a control. Anti-phosphotyrosine Western blotting revealed three tyrosine phosphorylated proteins at 56 kDa, 59 kDa and 80 kDa that co-immunoprecipitated with CD44 in BW^- cells, but not in BW^+ cells (Figure 4.5). Based on the relative molecular weight of the proteins at 55 kDa and 59 kDa, anti-Lck and anti-Fyn Western blotting were performed. This was because both Lck and Fyn are physiological substrates of CD45 (242-244), and both are dephosphorylated on tyrosines in BW^+ cells, and hyperphosphorylated on tyrosines in BW^- cells (245). Results from Figure 4.6 confirmed the prediction that Lck and Fyn were in fact associated with CD44 in both BW^+ and BW^- cells. However, more Lck and Fyn were found associated with CD44 in BW^- cells, suggesting that CD45 may play a negative role in regulating the CD44-Lck and CD44-Fyn interactions in these cells. This issue will be further addressed in Chapter Five. The isotype control for this experiment, the anti-CD45 immunoprecipitation, revealed a strong interaction of CD45 and Lck and a weak interaction of CD45 and Fyn in BW^+ cells in 1% Brij-58 treated cell lysate. No such interactions were observed in BW^- cells, indicating that the interactions of Lck and Fyn with CD44 in BW^- cells are specific to CD44, and not due to the nonspecific interactions with the beads used in the immunoprecipitation.

Since the interaction of CD44 and Lck in BW^- cells is weak and not always easy to detect, an attempt to optimize this interaction was made. Various cell lysis conditions were assessed, including the high and low salt concentration, the presence and absence of EDTA or Mg^2+, and a series of different detergents (Figure 4.7). It was observed that 1% Brij-58
Figure 4.5. Tyrosine phosphorylated proteins co-immunoprecipitate with CD44 in BW5147 cells. $10^7$ cells from CD45+ TCR+ BW5147 (BW+) and CD45- TCR+ BW5147 (BW-) were solubilized in lysis buffer containing 1% Brij-58 or 1% TX-100 respectively. Detergent soluble materials were precleared with Sepharose beads and incubated with the anti-CD44 mAb, IM7, conjugated to CNBr Sepharose beads (2), or alternatively, with the anti-CD45 mAb, I3/2, conjugated to CNBr Sepharose beads (1). No cell lysate was added to the control lanes (Ctrl). Immunoprecipitates were washed, analyzed on 10% SDS-PAGE, and transferred to a PVDF membrane. Soluble lysate from $2 \times 10^5$ cell was indicated as L. (A) anti-phosphotyrosine Western blot using mAb 4G10. (B) membrane used in (A) was stripped and reprobed with the anti-CD44 antisera JIWBB. Arrows indicated tyrosine phosphorylated proteins and CD44. Relative molecular mass is indicated on the left in KDa. Figures represent one of at least three independent experiments.
CD44 Immunoprecipitation

A. | 1% Brij-58 | 1% TX-100 |
---|---|---|
Ctrl | BW+ | BW- |
1 2 | 1 2 L | 1 2 L |

83 — 62 — 47 — 32 —

Lck

B. | 83 — 62 — 47 — 32 — |

Fyn

C. | 83 — 62 — 47 — 32 — |

CD44

Figure 4.6. Lck and Fyn co-immunoprecipitate with CD44 in BW5147 cells. $10^7$ cells from CD45$^+$ TCR$^+$ BW5147 (BW+) and CD45$^-$ TCR$^+$ BW5147 (BW-) were solubilized in lysis buffer containing 1% Brij-58 or 1% TX-100 respectively. Detergent soluble materials were precleared with Sepharose beads and incubated with the anti-CD44 mAb, IM7, conjugated to CNBr Sepharose beads (2), or alternatively, with the anti-CD45 mAb, 13/2, conjugated to CNBr Sepharose beads (1). No cell lysate was added to the control lanes (Ctrl). Immunoprecipitates were washed, analyzed on 10% SDS-PAGE, and transferred to a PVDF membrane. Soluble lysate from $2 \times 10^5$ cell was indicated as L. (A) anti-Lck Western blot using antisera 54 3B. (B) membrane used in (A) was stripped and reprobed with anti-Fyn antisera. (C) membrane used in (B) was stripped again and reprobed with the anti-CD44 antisera JIWBB. Relative molecular mass is indicated on the left in kDa. Figures represent one of at least three independent experiments.
(condition 7 in Figure 4.7) and the absence of EDTA (condition 9 in Figure 4.7) preserved more Lck associated with CD44 than other conditions. Interestingly, however, 1% Brij-58 only preserved a small proportion of cellular CD44 compared to 1% TX-100 or 1% Brij-96 (condition 2, 6 and 7 in Figure 4.7), suggesting that Brij-58 might selectively solubilize certain pools of CD44 and Lck on the cell membrane where they interact with each other.

4.3.5 CD44 interacts with Grb-2 in BW5147 cells

From previous experiments, p32 and p25 have been found to co-precipitate with GST-CD44cyt fusion proteins, as well as with endogenous CD44 in T cells. Based on the biological relevance of potential p32 and p25 to CD44-mediated cellular functions, Western blots against Annexin II (34 kDa), Grb-2 (25 kDa) and RhoA (21 kDa) were performed. Among these proteins, only Grb-2, but not Annexin II or RhoA, was found to associate with CD44 (Figure 4.8 and data not shown). BW5147 T cells were lysed in different cell lysis conditions using different detergents (1% Brij-58, or 1%TX-100, or 60 mM OG), which were known to selectively solubilize different pools of membrane proteins based on previous experiments. The interaction of CD44 and Grb-2 could be detected under all of the three conditions. But once again, 1% Brij-58 preserved this interaction better than 1% TX-100 or 60 mM OG. There was a background binding of Grb-2 to Sepharose beads in 1% Brij-58 lysis condition, however, no such background was observed when more stringent lysis conditions (1% TX-100 or 60 mM OG) were used, indicating a specific interaction between CD44 and Grb-2. Further work needs to be focused on whether the CD44-Grb-2 interaction has some impact on ligand binding of CD44, and what the molecular nature of this interaction is. Answers to these questions will be useful to delineate the biological relevance and the signaling pathway of the CD44-Grb-2 interaction.
**CD44 Immunoprecipitation**

A.

1. 1% TX-100 TE
2. 1% TX-100 TKE
3. 0.5% TX-100 TKE
4. 1% NP-40 TKE
5. 0.5% TX-TKM
6. 1% Brij-96 TKE
7. 1% Brij-58 TKE
8. 1% TX-100 TKE + 10% glycerol
9. 1% TX-100 TK
10. 1% TX-100 TK_{low} E

**Figure 4.7. Biochemical characterization of the CD44-Lck interaction in BW5147 cells.**

10⁷ BW5147 cells were solubilized in 1-10 lysis buffers as indicated. TE contains 10 mM Tris-HCl, pH 7.4, and 2 mM EDTA; TKE contains 10 mM Tris-HCl, pH 7.4, 140 mM KCl, and 2 mM EDTA; TKM contains 10 mM Tris-HCl, pH 7.4, 140 mM KCl, 5 mM Mg²⁺, and 2 mM EGTA; TK contains 10 mM Tris-HCl, pH 7.4, and 140 mM KCl; TK_{low} E contains 10 mM Tris-HCl, pH 7.4, 40 mM KCl, and 2 mM EDTA. Detergent soluble materials were precleared by Sepharose beads, followed by immunoprecipitation using the anti-CD44 mAb, IM7, conjugated to CNBr Sepharose beads. Immunoprecipitates were washed, analyzed on 10% SDS-PAGE, and transferred to a PVDF membrane. Soluble lysate from 2 x 10⁵ cells was indicated as L. (A) Anti-Lck Western blot using anti-Lck antisera 54 3B. (B) Membrane used in (A) was stripped and reprobed by the anti-CD44 antisera JIWBB. Relative molecular mass is indicated on the left in KDa. Results represent one of three independent experiments.
CD44 Immunoprecipitation

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Figure 4.8. Grb-2 co-immunoprecipitates with CD44 in BW5147 cells. 10^7 BW5147 cells were solubilized in lysis buffer containing 1% Brij-58, or 1% TX-100, or 60 mM OG. Detergent soluble material was precleared with Sepharose beads and subjected to immunoprecipitation using the anti-CD44 mAb, IM7, conjugated to CNBr Sepharose beads (A) or Sepharose beads alone (B) as a control. Equal amounts of IM7 CNBr Sepharose beads without cell lysate served as another immunoprecipitation control (C). Immunoprecipitates were washed, analyzed on 12.5% SDS-PAGE, and transferred to a PVDF membrane. Soluble lysate from 2 x 10^5 cells was indicated as L. Anti-Grb-2 Western blot (top panel) was performed using an anti-Grb-2 antisera. The same membrane was stripped and reprobed with the anti-CD44 antisera J1WBB (bottom panel). Relative molecular mass is indicated on the left in kDa. Data represent one of three independent experiments.
4.3.6 CD44 interacts with the Ezrin/Radixin/Moesin (ERM) family of cytoskeletal proteins in T cells and myeloid cells

CD44 mediates cell adhesion and cell migration, a process that requires the dynamic rearrangement of the cytoskeleton in response to environmental stimuli. However, the connection between CD44 and the cytoskeleton remains to be elucidated. ERM proteins are cytoskeletal proteins that link actin cytoskeleton to membrane proteins (196, 198). In leukocytes, ERM proteins were found to co-localize with ICAMs, CD43 and CD44 in the uropod, a specialized structure enriched in cell adhesion molecules (189, 204, 246). Additionally, ERM proteins were also found to co-immunoprecipitate with CD44 in BHK cells (48) and in vitro (118). ERM proteins, especially ezrin, became a 81 kDa tyrosine phosphorylated protein in T cells activated through TCR or in tumor cells stimulated by growth factor EGF (247, 248). The anti-phosphotyrosine Western blotting shown in Figure 4.5 indicated that an 80 kDa protein was tyrosine phosphorylated and co-immunoprecipitated with CD44 in BW− cells. Therefore, it is possible that this protein may be phosphorylated ezrin. To test this possibility, CD44 was immunoprecipitated from BW5147 and HSB-2 T lymphoma cells, as well as KG1a myeloid cells, and the ERM proteins were detected by Western blotting (Figure 4.9). All of the three members of the ERMs, ezrin, radixin and moesin, were co-immunoprecipitated with CD44 in the two T cell lines (BW5147 and HSB-2), indicating that CD44 probably interacted with the conserved region of the ERM proteins. In KG1a myeloid cells, however, only one member of the ERM proteins was detected to co-immunoprecipitate with CD44, and the reason for this preferential association is not clear. Of note, the interaction of CD44 and ERM proteins was weak in buffers containing physiological ionic strength (150 mM KCl), but was markedly increased when lower ionic strength (40 mM KCl) was used. An isotype control for immunoprecipitation using an anti-CD45 mAb revealed no ERM proteins interacting with CD45. This indicated that the association of ERM and CD44 was specific in these cells. On the other hand, anti-phosphotyrosine Western blotting proved that ERM
CD44 Immunoprecipitation

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- 83
- 62
- 47
- 175
- 83
- 62
- 175
- 83

Figure 4.9. ERM proteins co-immunoprecipitate with CD44 in BW5147, HSB-2 T cells and KG1a myeloid cells. 10^7 cells from murine BW5147 and human HSB-2 T cells and human KG1a myeloid cells were solubilized in lysis buffer containing 1% TX-100 and either 40 mM or 140 mM KCl salt concentration. Detergent soluble materials were pre-cleared with Sepharose beads and incubated with the indicated anti-CD44 mAb, IM7, or anti-CD45 mAb, I3/2, conjugated to CNBr Sepharose beads, or Sepharose beads alone as a control. Immunoprecipitates were washed, analyzed on 7.5% SDS-PAGE, and transferred to a PVDF membrane. Soluble lysate from 2 x 10^5 cells was indicated as L. Western blots against ERM (top panel), CD44 (middle panel) and CD45 (bottom panel) were conducted using anti-ERM antisera, J1WBB and R02.2 respectively. Relative molecular mass is indicated on the left in kDa. Data represent one of at least three independent experiments.
proteins were not tyrosine phosphorylated in BW cells (data not shown). Therefore, it can be concluded that the tyrosine phosphorylated protein, p80, which co-immunoprecipitated with CD44 in Figure 4.5, is another CD44 interacting protein that is distinct from ERM.

4.3.7 The CD44-ERM interaction occurs in low-ionic cell lysis conditions

As observed in Figure 4.9, CD44 interacted with ERM proteins to different extents depending on the ionic strength of the lysis buffer. Figure 4.10 shows the relationship between the CD44-ERM interaction and the ionic strength of cell lysis. CD44 was immunoprecipitated from BW5147 cells lysed in buffers containing increasing concentrations of KCl (0 – 150 mM), and the ERM proteins associated with CD44 were detected by Western blotting. It was observed that the amount of ERM proteins that co-immunoprecipitated with CD44 decreased as the salt concentration of the lysis buffer increased.

In addition to the ionic strength, a number of other lysis conditions were also studied to understand the nature of the CD44-ERM interaction. These included different ions, the presence of Mg\(^{2+}\) or EDTA, and different detergents. Figure 4.11 shows the profiles of the CD44-ERM association in BW5147 cells under 11 different lysis conditions. The interaction of CD44-ERM is not specifically dependent on potassium ions since replacement of KCl with NaCl did not alter the pattern of ERM proteins that associated with CD44. The low ionic strength plus EDTA resulted in the maximum amount of ERM proteins that co-immunoprecipitated with CD44. If EDTA was removed from the lysis buffer, the interaction was markedly reduced (condition 6 in Figure 4.11). Because EDTA chelates divalent cations such as Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\) and Zn\(^{2+}\), it is possible that the presence of these divalent cations may have a negative effect on the CD44-ERM interaction. In one lysis condition, the effect of Mg\(^{2+}\) on the CD44-ERM interaction was examined (condition 9 in Figure 4.11). 5 mM Mg\(^{2+}\) and 2 mM EGTA were used in this condition because EGTA chelates large cations like Ca\(^{2+}\), but not
**Figure 4.10.** The interaction of CD44 and ERM is low ionic strength-dependent. $10^7$ BW5147 T cells were solubilized in lysis buffer containing 1% TX-100 and indicated concentrations of KCl. Detergent soluble materials were precleared with Sepharose beads and incubated with the anti-CD44 mAb, IM7, conjugated to CNBr Sepharose beads (IP). Immunoprecipitates were washed, analyzed on 7.5% SDS-PAGE, and transferred to a PVDF membrane. Soluble lysate from $2 \times 10^5$ cells was indicated as L. Anti-ERM Western blot (top panel) was performed using anti-ERM antisera. The same membrane was stripped and reprobed with the anti-CD44 antisera J1WBB (bottom panel). Relative molecular mass is indicated on the left in kDa. Data represent one of at least three independent experiments.
CD44 Immunoprecipitation

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83 — 62 — 47 — 175 — 83 — 62 —

1. 1% TX-100 TKE (40 mM KCl)
2. 1% TX-100 TKE (150 mM KCl)
3. 1% TX-100 TNE (40 mM NaCl)
4. 1% TX-100 TNE (150 mM NaCl)
5. 0.5% TX-100 TKE (40 mM NaCl)
6. 1% TX-100 TK (40 mM KCl)
7. 1% Brij-58 TKE (40 mM KCl)
8. 1% Brij-96TKE (40 mM KCl)
9. 0.5% TX-100 TKM (40 mM KCl, 5 mM Mg^{++}, 2mM EGTA)
10. 0.5% TX-100 HNM (20 mM NaCl)
11. 0.5% TX-100 HNM (150 mM NaCl)

Figure 4.11. Biochemical characterization of CD44-ERM interaction in BW5147 cells. 10^7 BW5147 T cells were solubilized in 1-11 lysis buffers. TKE contains 10 mM Tris-HCl, pH 7.4, KCl at indicated concentrations, 2 mM EDTA; TNE contains 10 mM Tris-HCl, pH 7.4, NaCl at indicated concentrations, 2 mM EDTA; TK contains 10 mM Tris-HCl, pH 7.4, KCl at indicated concentration; TKM contains 10 mM Tris-HCl, pH 7.4, 40 mM KCl, 5 mM Mg^{++}, and 2 mM EGTA; HNM contains 20 mM Hepes, pH 7.2, 10.3% sucrose, NaCl at indicated concentrations, 5 mM Mg^{++}, 0.01% BSA. Detergent soluble material was precleared with Sepharose beads and incubated with the anti-CD44 mAb, IM7, conjugated to CNBr Sepharose beads (IP). Immunoprecipitates were washed, analyzed on 7.5% SDS-PAGE, and transferred to a PVDF membrane. Soluble lysate (L) from 2 x 10^5 cells was indicated. Western blots against ERM (top panel) and CD44 (bottom panel) were performed using an anti-ERM antisera and the anti-CD44 antisera J1WBB respectively. Arrows indicated the position of ERM proteins and CD44. Relative molecular mass is indicated on the left in KDa. Data represent one of three independent experiments.
small cations like Mg$^{2+}$. It was noticed that the CD44-ERM interaction under these conditions was abolished, suggesting that Mg$^{2+}$ might play an important role in the CD44-ERM interaction. However, the underlying mechanism needs to be determined.

### 4.3.8 Cytochalasin D disrupts the CD44-ERM interaction

If the hypothesis that ERM proteins link cell surface CD44 to actin filaments in order to coordinate CD44-mediated adhesion and migration is correct, then disruption of the ERM-actin interaction should affect the CD44-ERM interaction and the adhesion function of CD44. To test this hypothesis, prior to cell lysis, BW5147, HSB-2 T cells and KG1a myeloid cells were treated at 37°C for 30 min with 25 μM Cytochalasin D, a drug that prevents actin polymerization. CD44 was then immunoprecipitated from cells treated with or without Cytochalasin D, and the ERM proteins were detected by Western blotting (Figure 4.12A). Densitometry results indicated that Cytochalasin D treatment significantly reduced the interaction of CD44 and ERM by 51.2 ± 0.3% (n = 2), 45.1 ± 13.9% (n = 3) and 57.2 ± 5.8% (n = 2) per unit of CD44 in HSB-2, KG1a, and BW5147 cells respectively. To further explore whether the CD44-mediated adhesion was affected by Cytochalasin D treatment, the HA binding ability of these cell lines was assessed by flow cytometry. BW5147 and KG1a cells, which bind HA constitutively, lost 42.2 ± 8.7% (n = 2) and 43.7 ± 11.1% (n = 3) of their binding ability when treated by Cytochalasin D. HSB-2 T cells, however, bind HA poorly, and the effect of Cytochalasin D could not be determined (Figure 4.11B and data not shown). Thus these results suggested that in leukocytes, ERM proteins might play a role in CD44-mediated adhesion by linking the cell surface CD44 to the actin cytoskeleton. Intracellular factors that regulate the interaction of ERM proteins and actin filaments might also influence the CD44-ERM association as well as the CD44-mediated ligand binding function.
Figure 4.12. Cytochalasin D treatment decreases the interaction between CD44 and ERM in KG1a and HSB-2 cells, and decreases the HA binding ability in KG1a cells. (A) $10^7$ cells from KG1a myeloid and HSB-2 T cells were treated with (+) or without (-) 25 μM Cytochalasin D. Cells were then lysed in low salt lysis buffer containing 1% TX-100. Detergent soluble materials were precleared with Sepharose beads and subjected to immunoprecipitation using the indicated anti-CD44 mAb, IM7, conjugated to CNBr Sepharose beads or Sepharose beads alone as a control. Immunoprecipitates were washed, analyzed on 7.5% SDS-PAGE, and transferred to a PVDF membrane. Soluble lysate from 2 x $10^5$ cells was indicated as L. Western blots against ERM (top panel in A) and CD44 (bottom panel in A) were performed using an anti-ERM antisera and the anti-CD44 antisera JIWBB respectively. Relative molecular mass is indicated on the left in kDa. (B) The HA binding ability of Cytochalasin D treated (+CD) or untreated (-CD) KG1a cells was assessed by flow cytometry using FITC labeled HA. Cells alone was served as the Control. Data represent one of three independent experiments.
4.3.9 **LexA-CD44cyt fusion proteins meet the requirements of a bait protein**

Some proteins such as Lck, Fyn, Grb-2 and ERM have been successfully shown to interact with CD44 using the immunoprecipitation technique. However, some proteins such as p32 and p80, which were also detected to associate with CD44 by immunoprecipitation, could not be easily identified. Therefore, the yeast two-hybrid interaction technology was used.

The bait plasmid was generated by inserting the cDNA sequence of the full-length CD44cyt or the truncated CD44cyt (lacking the first 13 amino acids) next to the LexA sequence in the pEG202 vector. The bait plasmids were then transformed into the yeast strain, EGY48, which carries two reporter genes, \textit{LEU2} and \textit{lac Z}. After transformation, the full-length and the truncated LexA-CD44cyt bait proteins were assayed for the level of expression in yeast, the ability to localize to the nucleus and bind DNA, and the intrinsic transcriptional activity. A good bait protein should be able to be expressed in yeast cells, localize to the yeast nucleus and bind DNA, but should not activate transcription by itself. As shown in Figure 4.13A, the two versions of LexA-CD44cyt bait proteins were all expressed in yeast extracts. However, similar to the truncated GST-CD44cyt fusion protein produced in bacteria (Figure 4.2), the truncated LexA-CD44 bait was also expressed at a higher level (5 fold) than that of the full-length bait in yeast. This is probably due to the N-terminal CD44cyt, which contains arginine- and lysine-rich regions and therefore is sensitive to proteolysis. To test whether the two baits exhibit any intrinsic transcriptional activity, yeast cells containing the bait proteins were streaked on X-gal plates to monitor the activation of the \textit{Lac Z} reporter gene. Shown by the blue color change in Figure 4.13B, the two bait proteins exhibited background levels of \textit{Lac Z} gene activity comparable to the negative control, but were much lower than the positive control. The ability of the bait proteins to localize to the yeast nucleus was tested by the repression assay (Figure 4.13C). The bait plasmid was co-transformed into yeast cells with pJK101 (Table 2.2), which allows yeast to have significant \(\beta\)-galactosidase activity. The
positive and negative controls were also included by substituting the bait plasmid with pRFHM1 (can localize to the nucleus and bind the LexA operator, Table 2.2) and pRS423 (cannot localize to the nucleus and bind the LexA operator, Table 2.2) respectively. Overnight cultures were then assayed for β-galactosidase activity indicative of the Lac Z reporter gene expression. Repression of β-galactosidase activity indicates the ability of the bait protein to migrate into the yeast nucleus and to bind the LexA operator, thus preventing the access of the transcription factor (encoded by pJK101) to the LexA operator site. Both the full-length and the truncated bait proteins repressed β-galactosidase activity to a similar extent to the positive control, confirming that both of them can successfully localize to the yeast nucleus. Together, these results suggested that the two LexA-CD44cyt proteins met the requirements of being a bait protein in the yeast two-hybrid screen.

4.3.10 Yeast two-hybrid interaction screening provided preliminary evidence that several intracellular proteins tentatively interact with CD44cyt

Yeast cells harboring the bait protein were transformed with a cDNA library from murine CD4+ T lymphocytes, and grown on selection plates (Leu dropout plates or X-gal plates) to screen for potential CD44 interacting proteins. One round of screening using the full-length bait and two rounds using the truncated bait were performed. This is due to the fact that compared to the full-length bait, the truncated LexA-CD44cyt was more stable and expressed at a higher level in yeast, and therefore, was more likely to retain any weak and transient interactions. Table 4.1 illustrates the result of the 3 rounds of screening. A total of 332 clones were picked from the initial interaction plates, which contained potential CD44cyt-interacting prey proteins. These clones were then subjected to the Gal-dependence test designed to distinguish the real interactions (Figure 4.14). Since the cDNA library plasmids were constructed under the control of a GAL1 promoter, clones that only grew and changed color to blue on plates containing galactose, not glucose (which shuts off the Gal promoter and stops the
Figure 4.13. Characterization of LexA-CD44cyt bait proteins. (A) Expression of LexA bait proteins in yeast. 20 ng of yeast extract from EGY48 transformed with no plasmid (Control), pEG202 (LexA), full-length pEG202-CD44cyt (LexA-CD44cyt F), or truncated pEG202-CD44cyt (LexA-CD44cyt T) were analyzed on 12.5% SDS-PAGE, transferred to a PVDF membrane. Western blot against LexA was performed. Relative molecular mass is indicated on the left in kDa. (B) Estimation of the intrinsic transcriptional activity by β-galactosidase filter assay. Yeast transformants containing indicated proteins were streaked on X-gal plates and the color change was observed (see Materials and Methods). (C) β-galactosidase repression assay. Yeast transformants containing the indicated proteins were analyzed for the DNA binding ability by the repression of the β-galactosidase activity (see Materials and Methods). Figures represent one of three independent experiments.
transcription of library cDNAs) tended to be the true positives. After the Gal-dependence test, the number of positive clones was dropped to one third of the original number, indicating the necessity and efficacy of this test (Table 4.1). 71 yeast clones were sequenced and more than half of them were ribosomal and mitochondrial proteins, known to be “sticky” proteins. Among the rest of the sequenced clones, there were a number of clones that were isolated more than once, including H*-ATPase, SLAP, Wilm’s tumor protein, p24.6 QM protein and two unknown mouse ESTs. Interestingly, H*-ATPase and p24.6 QM proteins were identified from screens using both the full-length and the truncated bait, thus increasing the likelihood of a real CD44cyt interacting protein. In addition to the repetitive clones, some interesting single-hit clones were also identified, including amphiphysin, a cytoskeletal protein involved in vesicle trafficking, and PP1Cγ, a serine/threonine phosphatase. These two proteins might interact functionally with CD44 because CD44 is thought to mediate the association with the cytoskeleton (35), and serine phosphorylation can regulate the CD44-HA interaction as well as the CD44-mediated cell migration (114, 127).

4.3.11 In vitro binding assay of GST-CD44cyt fusion proteins and prey proteins did not confirm the interactions originally detected in yeast

To further assess whether the interactions seen in yeast cells could be reproduced in vitro, GST-CD44cyt fusion proteins were incubated with yeast extracts containing potential CD44cyt-interacting proteins. Figure 4.15 shows the result of an in vitro binding assay using H*-ATPase, amphiphysin, one unknown mouse EST, and the truncated GST-CD44cyt. A weak but specific interaction of H*-ATPase and GST-CD44cyt was observed, but it was not reproducible. Amphiphysin did not interact with CD44 in three independent binding assays (data not shown). The unknown mouse EST interacted not only with the GST-CD44cyt, but also with the GST control, suggesting the non-specific nature of the interaction.
Figure 4.14. An example of the Gal-dependence test of the putative positive clones isolated from the initial interaction plates. Putative positive clones derived from the initial interaction plates were re-patched on selection plates using either glucose or galactosidase as the carbon source in the presence of 1 mg/ml Xgal. Clones that turned blue only on the galactose plate, but not on the glucose plate, are likely to carry specific cDNA library encoded proteins that interacted with the LexA-CD44cyt bait. Arrow A indicates one putative positive clone. Arrow B indicates a false positive clone. The bait proteins used in this assay was the truncated LexA-CD44cyt fusion protein.
Table 4.1. Identification of putative positives of CD44cyt-interacting proteins from the yeast two-hybrid screen.

<table>
<thead>
<tr>
<th></th>
<th>Full-length LexA-CD44cyt</th>
<th>Truncated LexA-CD44cyt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of the clones</td>
<td>204</td>
<td>128</td>
</tr>
<tr>
<td>picked from the initial screening</td>
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<td></td>
</tr>
<tr>
<td>Total number of clones</td>
<td>33</td>
<td>85</td>
</tr>
<tr>
<td>survived the Gal-dependence tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of the clones</td>
<td>11</td>
<td>60</td>
</tr>
<tr>
<td>sequenced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of the repetitive clones from sequencing</td>
<td>4</td>
<td>7</td>
</tr>
</tbody>
</table>

**Identifications of putative positive clones after sequencing**

<table>
<thead>
<tr>
<th></th>
<th>Full-length LexA-CD44cyt</th>
<th>Truncated LexA-CD44cyt</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLAP (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H⁺-ATPase (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wilm's tumor proteins (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p24.6 QM (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHIP (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 unknown ESTs (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p24.6 QM (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP1Cy (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sigma 3A (1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Identifications of false positive clones after sequencing**

<table>
<thead>
<tr>
<th></th>
<th>Full-length LexA-CD44cyt</th>
<th>Truncated LexA-CD44cyt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal proteins (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial proteins (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribosomal proteins (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial proteins (14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β₂-microglobin (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total numbers and the identifications of clones derived from each step of the yeast two-hybrid screenings are indicated. Numbers in the parentheses stand for the number of repeat of positive clones that were identified from DNA sequencing.
One of the major problems encountered in the above binding assays using yeast extracts was the low yield of prey proteins produced in the yeast. To improve the expression levels of these proteins and to increase the sensitivity of the bait-prey interaction, co-expression and co-purification of the GST-CD44cyt and the His-tagged preys were performed in *E. coli*. Putative positive interacting proteins including H\(^{+}\)-ATPase, Amphiphysin, SHIP, p24.6 QM, PP1C\(\gamma\), SLAP, Sigma 3A, and the unknown mouse EST, were cloned into the pET 30b(+) vector, and then co-expressed with the full-length GST-CD44cyt in the *E. coli* BL21 strain. A positive control containing the GST-tagged phosphatase (PTPase) domain I (D1) of CD45 and the His-tagged PTPase domain II (D2) of CD45 was included. It was shown that the two CD45 PTPase domains interacted with each other using the same *in vitro* assay (249). After overnight IPTG induction, the GST-CD44cyt was purified by GSH beads, and the His-tagged prey proteins that co-purified with the GST-CD44cyt were detected by anti-His immunoblotting. As shown in Figure 4.16, except for SHIP and Sigma 3A, the rest of the prey proteins were expressed at reasonable levels in *E. coli*. The expression of SHIP and Sigma 3A was also undetectable in yeast (data not shown). Thus, it is possible that either the cDNAs from the library were not from the coding region for these two proteins, or the proteins were rapidly degraded once made in yeast. In contrast, the expression level of SLAP, p24.6 QM, PP1C\(\gamma\) and the unknown mouse EST, were significantly improved in *E. coli*, compared to that in yeast (data not shown). However, in spite of the improved yield of these proteins, only PP1C\(\gamma\) and the unknown mouse EST were found to co-purify with the GST-CD44cyt. The interaction of CD44 and H\(^{+}\)-ATPase, which was observed previously in yeast (Figure 4.15), was not evident in *E. coli*, suggesting that H\(^{+}\)-ATPase either did not fold properly in *E. coli*, or did not specifically interacted with CD44cyt at all. In order to further eliminate false positive interactions, two irrelevant GST fusion proteins were used as controls. These were the GST-Lck SH2 and the GST-Lck N-terminus fusion protein, both of which exhibited similar level of
A. GST fusion protein precipitation

<table>
<thead>
<tr>
<th></th>
<th>Vector</th>
<th>H(^{-})ATPase</th>
<th>Amphiphysin</th>
<th>Unknown EST</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST/CD44</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GST</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Anti-Hema Western blot

Coomassie blue stain

B. Yeast lysate

Figures 4.15. Interaction of GST-CD44cyt fusion proteins purified from *E.coli* with prey proteins from yeast extracts. Overnight yeast cultures containing indicated hemagglutinin (Hema)-tagged cDNA library proteins were solubilized in lysis buffer. (A) Pull-down assay. 2 mg of yeast extract was precleared using GSH beads, incubated with 2 μg of GST or truncated GST-CD44cyt fusion protein, followed by incubation with GSH beads. Precipitates were washed, analyzed on 7.5% SDS-PAGE, and transferred to a PVDF membrane. Anti-Hema Western blot (top panel in A) was performed to detect cDNA library proteins. Coomassie Brilliant Blue R-250 staining (bottom panel in A) of the same membrane used in the top panel to show equal amount of GST or GST-CD44cyt was loaded in each lane. (B) Expression of prey proteins. 5 μg of yeast extract from each indicated clone was analyzed on 12.5% gel and transferred to a PVDF membrane. Anti-Hema Western blot was performed to show the expression level of each protein from yeast extracts. Relative molecular mass is indicated on the left in kDa. Figures represent one of three independent experiments.
Figure 4.16. Co-expression and co-purification of the full-length GST-CD44cyt and Histagged prey proteins in *E. coli*. Plasmids encoding the full-length GST-CD44cyt and 10 indicated His-tagged prey proteins were co-transformed into *E. coli*. 2 ml of bacterial culture was induced with IPTG overnight to over express proteins. The “Control” which contains the GST-CD45 PTPase Domain 1 and the His-CD45 PTPase Domain 2 was incorporated. 30 μl of total cell lysate was analyzed by Western blot against His (top panel in A) and CD44 (top panel in B). Additionally, bacterial lysate was incubated with GSH beads. The bead complex was washed, analyzed on 12.5% SDS-PAGE, and transferred to a PVDF membrane. Western blots against His (bottom panel in A) and CD44 (bottom panel in B) were performed. Arrows indicated two potential interacting prey proteins PP1Cγ and an unknown mouse EST respectively. Relative molecular mass is indicated on the left in kDa. Data represent one of three independent experiments.
expression to that of the GST-CD44 cyt in E. coli (data not shown). PP1Cγ and the unknown mouse EST were co-expressed with different GST fusion proteins (GST-CD44 cyt, GST-Lck SH2, and GST-Lck N terminus) in E. coli. The GST fusion proteins were then precipitated by GSH beads, and the co-purified PP1Cγ or the mouse EST was detected by anti-His Western blotting. As shown in Figure 4.17, both PP1Cγ and the unknown mouse EST co-purified with all of the GST fusion proteins including the irrelevant controls. This result suggested that the interaction of PP1Cγ or the unknown mouse EST with GST-CD44 cyt was likely to be mediated by GST, but not by the CD44 cyt. In a separate experiment when GST alone was included as a control, it was confirmed that both PP1Cγ and the unknown mouse EST also co-precipitated with GST (data not shown).

While some interactions found in yeast could be reproduced in E. coli, none of them were consistently seen in yeast, E. coli, and in vitro assays. This raises questions as to whether any of these interactions will occur under physiological conditions. However, it is possible that some of the interactions may still take place physiologically. The most likely candidates are PP1Cγ and amphiphysin. This is because serine/threonine phosphorylation of CD44 was implicated in the regulation of CD44-mediated cell migration (section 1.7.3), while amphiphysin is a cytoskeletal protein, which could connect CD44 to the cytoskeleton. However, given the lack of significant and consistent data, this possibility was not pursued further.
Figure 4.17. Co-expression and co-purification of His-tagged PPIC\(\gamma\) and the unknown EST with GST-CD44\(\text{cyt}\), GST-Lck SH2 and GST-Lck N-terminus fusion proteins. Plasmids encoding the full-length GST-CD44\(\text{cyt}\), His-tagged PPIC\(\gamma\) or unknown EST were co-transformed into \textit{E}.\textit{coli}. Controls of non-relevant GST fusion proteins were incorporated using GST-Lck SH2, or GST-Lck N terminus to replace GST-CD44\(\text{cyt}\). A negative control, which contains no interaction of GST-CD44\(\text{cyt}\) and p24.6 were also included. 2 ml of bacterial culture was induced overnight. 30 \(\mu\)l of total cell was solubilized in 3 x SDS sample buffer. Additionally, the rest of the bacterial lysate was incubated with GSH beads. The bead complex was washed, analyzed on 12.5% SDS-PAGE, and transferred to a PVDF membrane. (A) Western blots against His were performed. (B) Coomassie Brilliant Blue R-250 staining of the membrane used in the bottom panel of A. Arrows indicate PPIC\(\gamma\) and unknown EST. Relative molecular mass is indicated on the left in kDa. Data represent one of three independent experiments.
4.4 Discussion

4.4.1 The interaction of CD44 with p32 and p25 in T cells

The CD44cyt is thought to mediate interactions with cellular proteins in order to communicate between the intracellular and the extracellular environments (reviewed in refs. 35, 37). In this study, by using radiolabeled cell lysates, a p32 protein and a p25 protein were shown to co-precipitate with the GST-CD44cyt as well as the endogenous CD44 in several T cell lines (Figure 4.3 and 4.4). Interestingly, the N-terminal 13 amino acids of the CD44cyt might distinguish the interaction region of p32 and p25, since the truncated GST-CD44cyt lacking the first 13 amino acids of the CD44cyt only interacted with p32, and not with p25. Consistent with this result, in WT T cells, the tailless CD44 containing only the first 6 amino acids of the CD44cyt (stop at Cys 295), failed to interact with p32, whereas in TFX cells, the wild-type CD44 interacted with both p25 and p32 (Figure 4.3 and 4.4). These results indicate that p32 may interact with a region downstream of Cys 295 on CD44. In the same WT and TFX cell lines, a difference in the ability to bind soluble HA was reported by Lesley et al. (18). The tailless CD44 transfectants failed to bind soluble HA, whereas the wild-type CD44 transfectants bound HA constitutively. Therefore, the sequence downstream of the first 6 amino acids on CD44cyt may play an important role in HA binding. Subsequent mutational studies in this cell line indicated that the presence of the first 16 amino acids of the CD44cyt was able to restore the ability to bind soluble HA, further narrowing down the sequence required for HA binding to 10 amino acids (296-306). Similar results were also obtained using melanoma cells transfected with a tailless CD44 construct. These cells bound HA much less than cells transfected with the wild-type CD44, and were unable to migrate on an HA coated substrate (127). Thus, these results suggest that the region encompassing Cys 295 to Gly 306 may contain sequences required for HA binding and the cell movement. p32, whose interaction
site overlaps with this region, may be one promising candidate for studying the correlation between the CD44-mediated protein interactions and the HA binding and/or cell movement.

In contrast to p32, p25 was found to associate with the full-length GST-CD44cyt as well as the tailless CD44 containing only the first 6 amino acids of the CD44cyt, but not with the truncated GST-CD44cyt lacking the first 13 amino acids (Figure 4.3 and 4.4). These results suggest that the interaction region of p25 is mainly located within the first 6 amino acids of the CD44cyt. It is unlikely that p25 is involved in regulating HA binding because the tailless CD44 transfectants (WT cells) bound HA poorly, yet p25 was still found to interact with the truncated CD44 in these cells (Figure 4.4). However, it remains to be determined whether p25 participates in cellular events downstream of the engagement of CD44 and HA.

4.4.2 CD44 interacts with Lck and Fyn in BW5147 cells

Anti-phosphotyrosine Western blotting revealed the presence of Lck and Fyn in CD44 immunoprecipitates in both 1% Brij-58 and 1% TX-100 lysed BW5147 cells (Figure 4.6). Of note, compared to 1% TX-100 or 1% Brij-96, 1%Brij-58 solubilized the least amount of CD44, yet maintained the most Lck that associated with CD44 (Figure 4.7). The mechanism for this is unclear, and may be due to the structure of the detergents. Brij-58 is a 20 cetyl ether (C_{16}E_{20}), whereas Brij-96 is a 10 oleyl ether (C_{18}E_{10}), and TX-100 is a t-Octylphenoxypolyethoxyethal. Thus, the length of the hydrophobic tail of Brij-58 is about twice as long as that of Brij-96 or TX-100. The size of the detergent micelles of Brij-58 may therefore be bigger than that of Brij-96 and TX-100. This may potentially affect the ability of the detergents to solubilize different membrane components. This may also provide one explanation as to why Lck was not detected by GST-CD44cyt precipitation or by CD44 immunoprecipitation using radiolabeled T cell lysate because 1% Brij-96 was used in those experiments.

Taher et al. reported that ligation of CD44 by mAb transmitted signals to Jurkat T cells, and induced an increase in the intrinsic activity of Lck as well as the tyrosine phosphorylation
of ZAP70 and other cellular proteins (116). In BW5147 cells, the interaction of CD44 and Lck did not require cross-linking of cell surface CD44, and ZAP70 was not tyrosine-phosphorylated when the interaction of CD44 and Lck was observed (Figure 4.5, 4.6 and data not shown). Ilangumaran et al. and Rozsnyay also showed that a significant proportion of Lck and Lyn from human peripheral blood T and B lymphocytes as well as endothelial cells were associated with CD44, and no cross-linking of cell surface CD44 was required (117, 250). CD44 does not contain the CXCP consensus sequence known to mediate the CD4-Lck or CD8-Lck interaction in T cells (251-253). Results from peptide competition studies, however, suggested that the cysteine residue in the transmembrane region, as well as the three arginine tandem of the juxtamembrane CD44cyt, were important for this interaction (250). Our preliminary data using recombinant GST-CD44cyt and GST-Lck proteins revealed no direct interaction of CD44cyt and Lck (D. Lefebve, R. Li and P. Johnson unpublished data). Therefore, the Src-family tyrosine kinases may interact with CD44 in a different manner than with other partners like CD4 and CD8. Whether the interaction is direct or indirect still needs to be determined.

4.4.3 CD44 interacts with Grb-2 in BW5147 cells

Grb-2 is an adapter protein that has SH2 and SH3 domains for protein-protein interactions. It can associate with proteins that contain phosphorylated tyrosines via its SH2 domain, or with proteins that contain proline-rich regions like SOS via its SH3 domain. The Grb-2/SOS complex can activate the Ras, Raf, MAP kinase pathway, and lead to new gene transcription. Therefore, Grb-2 is implicated in a number of receptor signaling pathways that result in the activation of MAP kinases. In this study, Grb-2 was shown to co-immunoprecipitate with CD44 in BW5147 cells (Figure 4.8). There is only one report showing that in CD4+ peripheral blood T cells, cross-linking of cell surface CD44 by a mAb promoted the formation of a trimeric complex of Grb-2, phospholipaseγ (PLCγ) and LAT (254). A lateral association of CD44 and CD4 was also observed in this study. However, I found no evidence
that LAT or PLCγ co-immunoprecipitated with CD44 in BW5147 cells (data not shown). Subsequent studies using immobilized anti-CD44 mAb revealed that CD44 signals triggered the tyrosine phosphorylation of only a subset of proteins compared to anti-CD3 stimulation (Figure 5.5 in Chapter Five). Therefore, future work needs to be completed to fully address the following questions: (i) what is the role of Grb-2 in the CD44 signaling pathway? (ii) how does CD44 interact with Grb-2? and (iii) what is the biological significance of the interaction between CD44 and Grb-2?

4.4.4 CD44 interacts with ERM proteins in T cells and myeloid cells

The interaction of ERM proteins and CD44 was first identified in BHK cells (48). Only the 140 kDa isoform of CD44 (CD44v9,10, which contains the alternatively spliced exon 9 and 10) was found to associate with ERM proteins. It is mysterious that the CD44H (contains no alternatively spliced exons), which is predominant in BHK cells and shares the same sequence of transmembrane and cytoplasmic domains with the CD44v9,10, failed to interact with ERM proteins. Immunofluorescence microscopy revealed that in these cells, CD44v9,10 remained associated with the actin-based cytoskeleton after TX-100 extraction whereas CD44H did not. Therefore, one explanation would be that compartmentalization of cellular CD44 may potentially influence their ability to associate ERM and the actin cytoskeleton. How this compartmentalization is achieved remains to be determined.

In BW5147, HSB-2 T cells and KG1a myeloid cells, a weak interaction between CD44 and ERM proteins was detected under regular cell lysis conditions (detergent, physiological concentrations of salt, and EDTA, Figure 4.9). This interaction remained unchanged when different detergents or different ions were used (Figure 4.11). In contrast, a dramatic increase of the interaction was observed when the ionic strength of the extraction buffer was decreased (Figure 4.9 and 4.10), suggesting that the CD44-ERM interaction is ionic strength-dependent. In agreement with this result, another in vitro study (118) also showed that the binding affinity
of the recombinant CD44cyt and ERM was quite low at physiological ionic strength, but was markedly increased at low ionic strength. Deletion of the C-terminal domain on ERM or binding of PI-4,5-P$_2$ to the N-terminus of ERM restored its association with the CD44cyt at physiological ionic strength. It was also demonstrated that the ERM proteins can form an intramolecular head-to-tail interaction, which masked the N-terminal binding site for membrane proteins, such as CD44, and the C-terminal binding site for F-actin (reviewed in refs. 194, 198). Taken together, these data suggested that the low ionic strength or the binding of PI-4,5-P$_2$ (255) might act to open the closed conformation of ERM in order to expose the N-terminal binding site for CD44. Interestingly, the association of F-actin with talin, another actin binding protein, is also ionic strength-dependent (256), suggesting that the ionic strength may affect the general conformation of certain actin-binding proteins such as talin and ERM, regulating their ability to bind actin. In addition, like ERM proteins, talin also contains functionally independent N-terminal and C-terminal domains, which interact with different proteins including FAK, actin and vinculin (257, 258). Both the N-terminal and C-terminal domains of talin can interact with each other to form a closed conformation, and to mask the binding sites for target proteins. Certain cellular events, such as protease cleavage of talin, have been demonstrated to liberate its binding sites for F-actin and FAK (181). The low ionic strength may serve as one of the factors that can potentially change the protein conformation, and therefore, render a ready-to-bind conformation to both ERM proteins and talin.

It is difficult to understand the observation that the presence of EDTA in the extraction buffer is critical to retain the CD44-ERM interactions as no interaction was detected when EDTA was removed even under the low ionic strength extraction conditions (Figure 4.11). EDTA is not present inside of the cells and it chelates divalent cations. This result implies that the presence of divalent cations, such as Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$ from the cell may negatively affect the interaction between CD44 and ERM. Our preliminary results suggest that the presence of Mg$^{2+}$ might negatively affect this interaction through an unknown mechanism.
Currently, there is no divalent cation binding sites identified on either CD44 or ERM. However, these cations may not act directly on CD44 or ERM proteins. For example, activation of some enzymes requires the action of divalent cations. These enzymes may affect the interaction between CD44 and ERM. Therefore, EDTA in the lysis buffer may inactivate these enzymes and preserved the interaction of CD44 and ERM.

Treatment of HSB-2 T cells and KG1a myeloid cells with Cytochalasin D, a drug that disrupts actin depolymerization, significantly reduced the association of CD44 and ERM proteins, and the CD44-mediated HA binding (Figure 4.12). This suggests that the CD44-ERM interaction, as well as HA binding, require the integrity of the actin cytoskeleton. Notably, the ERM-actin association also exhibits similar characteristics to that of the CD44-ERM interaction. Shuster and Herman observed that the in vitro association of ezrin and actin was Ca^{2+} sensitive, and was abolished by the Cytochalasin D treatment (259). Therefore, it can be speculated that the loss of ERM-actin interaction after the Cytochalasin D treatment may lead to the loss of the CD44-ERM interaction, and thereafter, the inability of cells to bind HA.

4.4.5 Potential role of the CD44-ERM interaction in CD44 inside-out signaling

The interaction of CD44 with ERM family cytoskeletal proteins may be involved in the inside-out CD44 signaling. This is supported by the observations from this study. Pretreatment of cells with Cytochalasin D, a drug that abolishes the ERM-actin interaction, rendered a decreased HA-binding ability to BW 5147 T cells and KG1a myeloid cells. Interestingly, concomitant with the reduced HA binding, a decrease in CD44-ERM interaction was also observed in these cells after treated with Cytochalasin D. These data, therefore, suggest that there is a link between the CD44-ERM interaction and the ability of CD44 to bind HA, and that intracellular changes, which reduce the CD44-ERM or the ERM-actin association, may also impair the CD44-mediated HA binding (inside-out signaling). Recently, the crystal structure of human moesin has just been resolved, and factors that regulate the ERM-actin
The N-terminal FERM domain of moesin interacts with the C-terminal tail with high affinity. This high affinity interaction is derived from the binding affinities of five independent parts of the C-terminal extended tail. Therefore, it can be predicted that multiple independent signals can act on different parts of the interaction surface to achieve differing levels of activation to open up the moesin molecule. These different signals possibly include the ionic strength (discussed in 4.3.7 and 4.4.4), binding of PIP₂ (118), and phosphorylation of the C-terminal Thr 558 of moesin (261, 262). In addition to the role of ERM in regulating the CD44-mediated HA binding (CD44 inside-out signaling), it cannot be forgotten that CD44 has multiple ligands and can signal bidirectionally across the cell membrane. Therefore, interactions of CD44 and cytoskeletal proteins such as ERM may play an important role in regulating the binding of CD44 to HA as well as other CD44 ligands. Alternatively, these interactions may also transmit signals to the cytoskeleton upon engagement of CD44 with its ligands (outside-in signaling).

4.4.6 Limitation of the yeast two-hybrid approach to identify the real CD44-interacting proteins

As detected by GST-CD44cyt and CD44 immunoprecipitation using radiolabeled T cell lysate, there might be different protein binding sites within the first 13 amino acids of the CD44cyt (discussed in 4.4.1). Thus, both the full-length and the truncated CD44cyt were used as the bait in the yeast two-hybrid interaction trap in order to identify different target proteins. It was also noticed that the truncated LexA-CD44 expressed at a higher level (approximately 5 fold) than the full-length bait in yeast cells (Figure 4.13A). Thus, one additional round of screening was performed using the truncated bait because a reasonable level of bait expression is thought to be important for a successful screening (263). However, data published later showed that as a matter of fact, the transmembrane domain (Trp 269 – Val 289) and membrane proximal region of the CD44cyt (Asn 290 – Leu 318) contained most of the interaction sites for
different proteins. The two positively charged arginine (292 – 294) and lysine (298 – 300) clusters at the beginning of the CD44cyt were shown to be important for the association of ERM, protein 4.1, and Lck (124, 192, 193, 250). Cysteine 286 and 295, located in the transmembrane and the juxta-membrane CD44cyt, are palmitoylated and are involved in the interaction of CD44 with ankyrin and Lck (123, 250). Another ankyrin binding site was shown to be in the region from Asn 304 to Leu 318 (120, 264).

Three rounds of screening using the two LexA-CD44cyt bait proteins revealed some interesting CD44cyt-interacting proteins. But none of them agreed with the previously identified CD44-interacting proteins, including the Src-family kinases (Lck or Fyn), ERM, protein 4.1, and ankyrin. Results from peptide competition studies indicated that the CD44-Lck interaction required the transmembrane Cys 286 and the three arginines (292-294) located at the very beginning of CD44cyt (250). Our preliminary results also showed that no direct interaction occurred between the recombinant GST-CD44cyt and GST-Lck (D. Lebfeve, R. Li and P. Johnson unpublished data), suggesting that either the interaction is indirect, or it is through regions other than the CD44cyt. Therefore, it is not surprising that no Lck or Fyn was shown to associate with the LexA-CD44cyt bait by the yeast two-hybrid screen.

ERM, ankyrin, and protein 4.1 were reported to directly associate with the CD44cyt. The interaction region of CD44 and ERM was located to Arg 292 - Lys 300. This region is present in the full-length bait, but not in the truncated bait. The reason that no interaction of ERM and the full-length CD44cyt bait was picked up by the system is uncertain. One possibility is that steric hindrance may affect the interaction as the ERM binding site on the full-length bait was only 3 amino acids downstream of the LexA protein. Alternatively, since the ERM proteins can form a head-to-tail association, a closed conformation that masks the binding site for CD44 (194, 198), it may be, therefore, difficult to detect the CD44-ERM interaction in the system. Furthermore, the cDNA library, in which the inserts ranged from 0.6 to 1.5 kb, was made using oligo-dT for the first strand of cDNA. The 5’ prime end of a cDNA
larger than 1.5 kb was therefore unlikely to be included in the cDNAs in this library. Therefore, it would be difficult to detect an interaction occurred through the N-terminus (encoded by the 5' prime end of cDNA) of a large protein (cDNA>1.5 kb). The size of ERM cDNA is 1.7 –1.8 kb, and the CD44 binding site of ERM is located at the N-terminus. Thus, this may be one explanation as to why no CD44-ERM interaction was identified by the yeast two-hybrid screen.

The ankyrin-binding site on CD44 is located to Asn 304–Leu 318, which was present in both bait proteins. However, no interaction was observed in my yeast two-hybrid experiments. This was also true for protein 4.1, which has a similar binding site to ERM on the CD44cyt. There are a number of possible reasons why the yeast two-hybrid system failed to detect the interaction of CD44 with either ankyrin or protein 4.1. These reasons include the expression level of these proteins in yeast, the 3 dimensional structure of these proteins or the CD44cyt bait when made in the yeast, the toxicity of these proteins to the yeast host, and their binding affinity to CD44, etc.

From more than 300 clones isolated from screenings using the two CD44cyt bait proteins, approximately two-thirds failed the GAL-dependence tests, which are designed to identify the specific bait-prey interaction. Among the 71 sequenced clones, more than half of them were ribosomal and mitochondrial proteins, known to be proteins with high non-specific binding (reviewed in ref. 265). Subsequent in vitro studies were focused on the repetitive clones, or the interesting single-hit clones. However, the dilemma in this study was that none of the interactions obtained from the yeast could be consistently reproduced by in vitro binding assays (Figures 4.15, 4.16 and 4.17). Thus it is difficult to estimate the reliability of the interactions that occurred in yeast. In order to fully analyze the reasons for the failure of using yeast two-hybrid screen to identify reliable CD44cyt-interacting proteins, several factors need to be taken into consideration.
First, the overall analysis and design of the bait protein are important for performing a yeast two-hybrid trap. Regions that are exposed to the surface of a protein, or contain important consensus or motifs, are likely to mediate protein-protein interactions, and therefore, need to be included in the bait protein. The fact that this study was focused on the truncated CD44cyt bait, which expressed relatively high level, but lacked the binding regions for most target proteins, may explain why no convincing CD44cyt-interacting protein was identified.

Second, post-translational modifications may play an important role in bait-prey interactions. For example, tyrosine phosphorylation is a critical factor that influences the interactions mediated by SH2 domain-containing proteins. CD44 is phosphorylated solely on serine residues. Ser 323 and Ser 325 are the only residues of the CD44cyt that are phosphorylated \textit{in vivo} (114, 127). It was shown that the interaction of CD44 and ankyrin was regulated by serine phosphorylation of CD44 (266). Moreover, wound healing experiments indicated that mutation of Ser 325 to an alanine, which reduced CD44 phosphorylation by approximately 90%, was sufficient to inhibit CD44-mediated melanoma cell migration on a HA coated substrate (127). Therefore, serine phosphorylation of CD44 might be involved in the cell migration process, which is coordinated by protein-protein interactions. In addition to serine phosphorylation, acylation of the cysteine residues on CD44 was also required by the CD44-ankyrin interaction (123). This provides another example of a CD44-mediated protein-protein interaction regulated by post-translational modifications. However, in this study, no test was performed to ensure that the CD44cyt bait proteins in yeast were serine phosphorylated to a similar level to what was seen \textit{in vivo}. Therefore, this may potentially affect interactions that are serine phosphorylation-dependent.

Third, the limitations of yeast two-hybrid system itself may also partly account for the failure to identify real CD44-interacting proteins in this study. For example, even though the yeast two-hybrid screen is suitable for detecting associations between proteins that are normally found in the nucleus, cytoplasm, mitochondria, membrane or even extracellularly, interactions
that occur in the cytoplasm or the cell membrane are more difficult to detect than those in the nucleus (reviewed in ref. 240). For CD44, potential interacting sites are located in the transmembrane domain or the juxta-membrane region of the cytoplasmic domain. This may therefore, make the interactions difficult to detect. Besides, if CD44-mediated interactions require more than one protein at the interaction site to stabilize the complex, like the focal adhesion complex formed upon engagement of integrins and their ligands, it would be difficult to identify such interactions using the yeast two-hybrid system. Other common limitations of the yeast two-hybrid system may also account for the results of this study. These limitations include masking of the interaction site on the bait protein by endogenous yeast proteins, cytotoxicity to the yeast host by prey proteins, low or unstable expression of the bait or preys, failure of the hybrid proteins to fold properly in yeast, failure of the hybrid proteins to enter the nucleus, unoptimal interacting environments in yeast nucleus, DNA-binding or activation domains that occlude the site of interaction, etc.

Finally, it cannot be ruled out that technical problems could also account for the outcome of the study. Some critical parameters in the system require thorough knowledge and extensive experience with yeast. These include the design of the bait proteins, the quality of cDNA library, the efficiency of cDNA library transformation, and the interpretation of results from the discrimination test.

In summary, the precise reason for the failure of this study using the yeast two-hybrid screen to identify convincing CD44-interacting proteins may be a combination of different reasons as discussed above. The purpose for presenting the discussion here is to provide some suggestions and general guidelines, which may be beneficial for future studies. However, since only in vitro experiments were carried out in the current study, it may still be worth determining whether these proteins can associate with or co-localize with CD44 in T cells.
CHAPTER FIVE

CD44-initiated Cell Spreading is Mediated by Src-family Kinases and Pyk2, and is Negatively Regulated by CD45

5.1 Introduction and rationale

The process of cell adhesion and cell migration in response to extracellular stimuli is a complex multistep process that involves a number of signaling molecules and cytoskeletal proteins. During this process, cell adhesion molecules play important roles in the crosstalk between the ECM and the actin cytoskeleton. Once again, this can be well demonstrated by integrin-mediated cell adhesion and cell migration (section 1.9.2). Engagement of integrins with their ligands from the ECM recruits two different types of proteins to the cytoplasmic domain of integrins. The first are signaling molecules such as tyrosine kinases (i.e. FAK, Pyk2, Src-family kinases) and the others are the cytoskeletal components such as actin-binding proteins (i.e. talin, tensin). Integrins also transmit signals to activate kinases or phosphorylate actin-binding proteins, therefore creating binding sites for other signaling molecules (i.e. Grb-2, Cas, Crk, Shc, Vav, C3G, paxillin) and cytoskeletal proteins (i.e. vinculin, α-actinin). These molecules contain modules or motifs for further protein-protein interactions and can then recruit or activate the Rho family small GTPases and other downstream key enzymes (i.e. PAK, ROCK, PKN, DOCK, myosin light chain phosphatase) for the formation of stress fibers, filopodia, lamellipodia and membrane ruffles.

CD44 is a cell adhesion molecule. It has been showed that in leukocytes, CD44 can transmit signals to cause cytoskeletal changes including formation of lamellipodia and cell spreading. However, the detailed picture of signaling events that occur during these processes is not known. From Chapter Four of this thesis and studies carried out in the meantime, other investigators and I have identified that CD44 interacts with signaling molecules (i.e. Src-family...
kinases, Grb-2, Rho-GDI) as well as cytoskeletal proteins (i.e. ERM proteins, ankyrin, protein 4.1). These interactions provide a molecular basis for CD44 to signal across the cell membrane, and ultimately lead to the rearrangement of the actin cytoskeleton. However, no study has addressed the question as to where these CD44-mediated molecular interactions take place in the CD44 outside-in signaling pathway. In this chapter, I focused on the characterization of one of these molecular interactions, the interaction of CD44 with Src-family kinases, as well as its relationship to the leukocyte transmembrane phosphatase CD45. This is because Src-family kinases, Lck and Fyn in particular, are physiological substrates for CD45 in T cells, and their kinase activities are also regulated by CD45. Therefore, here I aimed to understand how Lck, Fyn and CD45 collaborate to regulate the CD44 outside-in signaling which leads to the cytoskeletal reorganization.

5.2 Experimental approach

In this study, immobilized anti-CD44 mAb or HA were used to trigger signals via CD44. The cytoskeletal changes, such as cell spreading or formation of filopodia or lamellipodia, were used as readout for CD44 antibody induced outside-in signaling. To assess the role of CD45 in CD44-initiated cytoskeletal rearrangement, cell lines with or without CD45 were used to compare the CD44-induced cytoskeletal changes. To study the role of Src-family protein tyrosine kinases in regulating CD44-mediated cytoskeletal rearrangement, tyrosine phosphorylation events were examined upon anti-CD44 antibody stimulation. A Src-family kinase-specific inhibitor, PP2, was also used to determine the effect of Src-family kinases in mediating tyrosine phosphorylation as well as cytoskeletal changes triggered by the CD44 mAb. The interaction of CD44 and Lck was also analyzed on fractions isolated from sucrose gradients to determine how this interaction occurred on the cell membrane. Finally, downstream targets in the pathway of CD44 outside-in signaling were also sought based on tyrosine phosphorylation events occurring as a result of CD44 stimulation.
5.3 Results

5.3.1 CD45 negatively regulates CD44-mediated cell spreading in TCR+ T cells

CD45+ and CD45− TCR/CD3+ BW5147 T lymphoma cells (205), hereafter called BW+ and BW− cells, both adhered to anti-CD44 coated plates. However, only BW− T cells underwent a dramatic morphological change, flattening and spreading lengthwise in response to immobilized anti-CD44 mAb, KM201 (Figure 5.1A). This elongated flattening was observed with an immobilized antibody concentration ranging from 40 -250 µg/ml, with a higher percentage of cells flattening at the higher concentrations. Differences in cell spreading were also observed between the BW+ and BW− cells lacking TCR/CD3 expression and between the CD45+ and CD45− SAKR T cell lines, although the differences were less pronounced in the SAKR cells (Figure 5.2). The cell flattening and spreading was observed after 30 min but increased over a period of 2 h, and was still observed after 16 h (Figure 5.1B). The cell spreading was abolished by pretreatment of cells with 25 µM Cytochalasin D for 30 min or in the presence of 5 mM EDTA (Figure 5.3). This indicated that cell spreading involved the reorganization of the actin cytoskeleton as well as divalent cations or enzymes whose activity requires cations. The anti-CD44 antibody, KM201, binds to the hyaluronan-binding site of CD44, yet adhesion of either cell line to immobilized hyaluronan did not cause cell spreading under any condition tested (Figure 5.4A). This was also reported previously (144).

One of the main functions of CD45 in T cells is to regulate the Src-family tyrosine kinases, Lck and Fyn. To determine if Src-family kinases played a role in CD44-triggered cell spreading, the BW− T cells were pre-treated for 30 min with 10 µM PP2, a Src-family kinase inhibitor (267). PP2 did not prevent cell adhesion of either BW+ or BW− cells to immobilized KM201 anti-CD44 antibody, but it did prevent the cell spreading observed in the CD45− BW T cells (Figure 5.3). This indicated that CD44-mediated spreading in BW− T cells is mediated by Src-family kinases.
Figure 5.1. Immobilized anti-CD44 antibody induces cell spreading in CD45 deficient BW5147 T cells. (A) $10^5$ CD45$^+$ and CD45$^-$ BW cells (BW+ and BW- respectively) were immobilized for 2 h at 37°C on either BSA or anti-CD44 mAb, KM201, coated tissue culture plates. Data represent one of at least three independent experiments. Bar = 10 μm. (B) Graph of percentage of cells showing morphological change over time. ■, CD45$^+$ BW cells added to dishes coated with immobilized KM201; ▲, CD45$^-$ BW cells added to dishes coated with immobilized KM201; □, CD45$^+$ BW cells added to dishes coated with immobilized BSA; Δ, CD45$^-$ BW cells added to dishes coated with immobilized BSA. % morphologic change indicates the percentage of the number of cells showing elongated cell spreading morphology over the total number of cells in one microscopic field.
Figure 5.2. Immobilized anti-CD44 antibody induces cell spreading in CD45 deficient SAKR T cells. (A) $10^5$ CD45$^+$ and CD45$^-$ SAKR cells were immobilized for 2 h at 37°C on either BSA or anti-CD44 mAb, KM201, coated tissue culture dishes. Results represent one of at least three independent experiments. Bar = 10 μm.
Figure 5.3. Different factors abolish the anti-CD44 antibody induced cell spreading in CD45 deficient BW5147 T cells. 10^5 CD45- BW5147 were pretreated with 10 μM of PP2, 25 μM of Cytochalasin D, or 5 mM EDTA. Cells were then transferred to anti-CD44 mAb, KM201, coated tissue culture dishes for 2 h at 37°C. Results represent one of at least three independent experiments. Bar = 10 μm.
5.3.2 **CD45 negatively regulates TCR\(^+\) BW5147 T cell adhesion to soluble HA**

One of the best-characterized ligands of CD44 is HA. To study whether CD45 also regulates HA binding in BW5147 cells, fluorescently labeled soluble HA was used to assess the binding ability of BW\(^+\) and BW\(^-\) cells by FACS analysis (Figure 5.4B). The surface level of CD44 in BW\(^+\) and BW\(^-\) cells was similar (BW\(^-\):BW\(^+\) = 1.06 ± 0.29, n = 7), and both cell types constitutively bound HA. However, in the absence of CD45, the HA binding ability increased 3-5 fold (BW\(^-\):BW\(^+\) = 4.48 ± 1.75, n=7), indicating that CD45 negatively affected the ability of BW5147 cells to bind soluble HA. This result is in agreement with previous results showing that CD45 also negatively regulates the CD44 antibody-triggered cell spreading in BW\(^+\) cells.

To study whether HA induces cell spreading like the CD44 mAb, KM201, and whether CD45 is also involved in this process, BW\(^+\) and BW\(^-\) cells were immobilized on HA coated tissue culture plates. Both BW\(^+\) and BW\(^-\) cells adhered to the immobilized HA, however, the cells maintained a normal morphology and no cell flattening or spreading was observed in either cell type (Figure 5.4A). Therefore, unlike the CD44 antibody, immobilized HA did not induce cell spreading in BW5147 cells, suggesting that cellular events triggered by the anti-CD44 antibody may not exactly mimic those triggered by HA, or the HA used in this experiment may not mimic the physiological HA.

5.3.3 **Induction of tyrosine phosphorylation by CD44-triggered cell spreading occurs in CD45\(^-\) BW T cells and is inhibited by a Src-family kinase inhibitor**

To further examine the role of Src-family kinases in CD44-mediated signaling events in BW\(^+\) or BW\(^-\) T cells, I examined whether adhesion to immobilized CD44 mAb (KM201) involved the induction of tyrosine phosphorylation. Figure 5.5 showed that CD44 cross-linking induced tyrosine phosphorylated proteins in the BW\(^-\) cells, but not in the BW\(^+\) cells. There are at least two prominent tyrosine phosphorylated proteins of 120-130 kDa that are induced upon
Figure 5.4. CD45 negatively regulates the ability of BW5147 cells to bind soluble HA, but has no effect on cell spreading on immobilized HA. (A) $10^5$ CD45$^+$ and CD45$^-$ BW5147 cells (BW+ and BW- respectively) were immobilized on tissue culture dishes without coating (PBS) or coated with 5 mg/ml HA for 2 h at 37°C. Results represent one of three independent experiments. Bar = 10 μm. (B) $10^5$ cells were analyzed by FACS for the soluble HA binding ability and cell surface CD44 level using 2 μg/ml HA-FITC and the anti-CD44 mAb, IM7, respectively. The fluorescence intensity values for BW+ cells were normalized to 1, and the fluorescence intensity values for BW- cell were adjusted according to that of BW+ cells. Data presented in the graph were derived from 7 independent experiments.
binding to immobilized CD44 mAb, as well as a less defined band at ~80 kDa, although the p80 was not consistently observed. The time course of the induction of this tyrosine phosphorylation was similar to that observed for the morphological changes, becoming noticeable after 30 min and being sustained up to 16 hours (data not shown). The induction of tyrosine phosphorylation correlated with the induction of cell spreading, which occurs in the BW" cells. It was noted that after 4 h, induction of some tyrosine phosphorylation was sometimes observed in CD45" BW T cells in wells coated with BSA (Figure 5.5). However, these tyrosine phosphorylated bands were distinct from those observed upon incubation with immobilized CD44 antibody, and did not result in cell spreading (Figure 5.1A). The addition of PP2 inhibited both the spreading and the induction of tyrosine phosphorylation induced by immobilized anti-CD44 antibody (Figure 5.3 and 5.5). Therefore, CD44 signaling resulted in cytoskeletal changes and cell spreading in the BW" cells. This event was initiated by Src-family kinases, and resulted in the sustained tyrosine phosphorylation of cellular proteins.

5.3.4 Tyrosine phosphorylation of Pyk2 triggered by the immobilized CD44 antibody

Several proteins of approximately 120-130 kDa are known to become tyrosine phosphorylated after the activation of Lck and Fyn. These include, p130Cas, p130 SLAP (fyb), FAK, and Pyk2. Both FAK and Pyk2 have been implicated in mediating integrin-triggered cell adhesion and cell spreading by linking the integrin-mediated signal to the cytoskeleton (reviewed in refs. 163, 166). Moreover, FAK and Pyk2 can be inducibly tyrosine phosphorylated in response to TCR ligation and can associate with Lck (268). It was therefore determined whether or not these kinases became phosphorylated upon immobilization of the BW T cells by anti-CD44 antibody. In the BW" cells, Pyk2 became strongly tyrosine phosphorylated after 30 min, and was sustained over the measurement period of 2 h,
Figure 5.5. Immobilized anti-CD44 antibody triggers tyrosine phosphorylation in CD45 deficient BW5147 cells. (A) Anti-phosphotyrosine Western blot (PY) of CD45+ and CD45− BW5147 cell lysates (BW+ and BW- respectively) prepared from 10⁵ cells immobilized on anti-CD44 antibody, KM201 (CD44 mAb) coated tissue culture plates after the indicated timepoints. (B) Anti-phosphotyrosine Western blot of BW− cells added to BSA coated tissue culture plates (BW-/BSA) or to BW− cells pretreated for 30 min with 10μM PP2 (BW-/CD44 mAb/PP2) and then added to anti-CD44 mAb coated tissue culture plates. Anti-actin Western blot after stripping the same membranes is shown below each blot to indicate loading amounts. Relative molecular mass is indicated on the left in kDa. Arrows show migration of inducibly tyrosine phosphorylated proteins p120 and p80. Results represent one of three independent experiments.
concomitant with the time course of the observed morphological changes (Figure 5.6). Low levels of tyrosine phosphorylation of Pyk2 were seen in the CD45+ BW T cells, suggesting that the induction of Pyk2 phosphorylation was attenuated in the presence of CD45. Low levels of FAK phosphorylation were observed only in the BW~ cells, suggesting that Pyk2 was the preferred kinase phosphorylated after CD44 ligation, although it maybe, however, that FAK was expressed at low levels in these cells (Figure 5.6B).

5.3.5 Paxillin is not tyrosine phosphorylated following cell spreading induced by CD44 antibody

Paxillin is phosphorylated on tyrosine residues by FAK or Pyk2, and interacts with talin, α-actinin and vinculin, as well as recruiting Crk to the focal adhesion upon engagement of integrins with their ligands (266). Since immobilized CD44 antibody induced tyrosine phosphorylation of Pyk2 and FAK in BW~ cells, it is possible that paxillin is also involved in this process. To test this possibility, paxillin was immunoprecipitated from BW+ and BW~ cells immobilized by CD44 antibody, and the tyrosine phosphorylation state of paxillin was examined by Western blotting (Figure 5.7). For the tested time periods of 30 min and 1 h, the BW~ cells underwent significant cell spreading concomitant with the tyrosine phosphorylation of Pyk2 and FAK shown in the lysate controls in Figure 5.7. However, during these periods, no detectable levels of tyrosine phosphorylated paxillin were observed within the region of 50-80 kDa, as reported by others (267). This implies that the signaling events mediated through CD44 may differ from those mediated through integrins.

5.3.6 CD45 negatively regulates the association of CD44 with Lck

To further investigate the link between CD45, CD44 signaling and Src-family kinases, CD44 was immunoprecipitated from both BW+ and BW~ T cells, and its association with the
Figure 5.6. Immobilized anti-CD44 antibody induces tyrosine phosphorylation of Pyk2, and to a lesser extent FAK, in CD45- BW5147 cells. 5 x 10^6 CD45+ and CD45- BW5147 cells (BW+ and BW-) were immobilized on anti-CD44 antibody coated tissue culture plates for 0, 30 min and 2 h. Cells were then lysed on the plate with lysis buffer containing 1% TX-100. Pyk2 and FAK were immunoprecipitated from soluble lysate, analyzed on SDS-PAGE, and transferred to a PVDF membrane. (A) Pyk2 immunoprecipitation (IP). Anti-phosphotyrosine Western blot (PY) is presented on the top panel and anti-Pyk2 Western on the bottom panel. (B) FAK immunoprecipitation. Anti-phosphotyrosine Western blot is presented on the top panel and anti-FAK Western on the bottom panel. The heavily tyrosine phosphorylated band at ~55kDa is the heavy chain of the immobilized anti-CD44 mAb, KM201. L is soluble cell lysate from 2 x 10^5 cells. Arrows indicate migration of Pyk2 (A) or FAK (B). Relative molecular mass is indicated on the left in kDa. Results represent one of three independent experiments.
**Paxillin Immunoprecipitation**

### A. Anti-PY blot

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**Figure 5.7. Paxillin is not inducibly tyrosine phosphorylated in anti-CD44 antibody triggered BW5147 cell spreading.** 5 x 10^6 CD45+ and CD45− BW cells (BW+ and BW-) were immobilized on anti-CD44 antibody coated tissue culture plates for 0, 30 min and 1 h. Cells were then lysed on the plate with lysis buffer containing 1% TX-100. Paxillin (Pax) was immunoprecipitated (IP) from detergent soluble lysate, analyzed on SDS-PAGE, and transferred to a PVDF membrane. (A) Anti-phosphotyrosine (PY) Western blot. (B) Anti-paxillin Western blot on membranes used in (A) after stripping. The heavily tyrosine phosphorylated band at ~55kDa is the antibody heavy chain of the immobilized anti-CD44 mAb, KM201. C, the immunoprecipitation control with no anti-paxillin antibody; IP, paxillin immunoprecipitation; Lysate, detergent soluble materials from 2 x 10^5 cells. Arrows indicate migration of paxillin. Relative molecular mass is indicated on the left in kDa. Results represent one of three independent experiments.
Src-family kinases was assessed. As described previously (4.3.4 and Figure 4.7), Lck co-immunoprecipitated with CD44 under a variety of cell lysis conditions. However, compared to the BW* cells, approximately 2 fold more Lck was co-immunoprecipitated with CD44 in BW− cells, after cell lysis in either 1% Brij-58 or 1% Triton-X-100 (Figure 5.8 and 4.6A). Similar results were also found for another T cell Src-family kinase, Fyn, which was also co-immunoprecipitated with CD44 (Figure 4.6B). This suggested that CD45 negatively regulates the association of Lck and Fyn with CD44. The fact that more Lck and Fyn associated with CD44 in the CD45− BW cells than the CD45+ BW cells provides one possible explanation as to why CD44 signaling in the CD45− BW cells resulted in the induction of tyrosine phosphorylation.

5.3.7 Distribution of Lck to the low-density sucrose fraction is increased in CD45− BW T cells

A subset of both CD44 and Lck molecule have been shown to translocate to the low-density fraction after cell lysis and sucrose density gradient centrifugation (49, 117). This has been equated with the localization of these proteins to a low-density membrane microdomain of cells. This lipid fraction, typically enriched in glycolipids, sphingomyelin and cholesterol, is not well solubilized by detergents such as Brij and Triton, and is thought to exist on the membrane of unstimulated cells as microdomains or lipid rafts (reviewed in ref. 156). In the BW cells, sucrose gradients were performed on both 1% Brij 58 and 1% Triton X-100 treated cells, and the distribution of CD44, Lck, Csk and CD45 was determined (Figure 5.9 and Table 5.1). In BW* and BW− cells, low levels of CD44 (~4%) were present in the low-density fraction in Triton treated cells, whereas considerably more was present (~50%) after cell lysis in 1% Brij-58. Slightly more Lck (40-60%) was found in the low-density fraction after cell lysis in 1% Brij 58, compared to 25-40% after lysis in 1% Triton X-100. In contrast, very low
CD44 Immunoprecipitation

A. Anti-CD44 blot

<table>
<thead>
<tr>
<th></th>
<th>1% Brij-58</th>
<th>1% TX-100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW+</td>
<td>BW-</td>
</tr>
<tr>
<td>C</td>
<td>IP</td>
<td>L</td>
</tr>
<tr>
<td>175</td>
<td></td>
<td></td>
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<tr>
<td>83</td>
<td></td>
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<tr>
<td>62</td>
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B. Anti-Lck blot

<table>
<thead>
<tr>
<th></th>
<th>1% Brij-58</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>BW+</td>
</tr>
<tr>
<td>62</td>
<td></td>
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<td>47</td>
<td></td>
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</tbody>
</table>

Figure 5.8. More Lck co-immunoprecipitates with CD44 in CD45 deficient BW5147 cells. 10⁷ CD45⁺ BW cells (BW⁺) and 10⁷ CD45⁻ BW cells (BW⁻) were solubilized in lysis buffer containing either 1% Brij-58 or 1% TX-100. CD44 was immunoprecipitated from the soluble lysate. (A) Anti-CD44 Western blot with anti-CD44 antisera J1WBB. C, control immunoprecipitate with Sepharose beads alone; IP, CD44 immunoprecipitate with anti-CD44 antibody (IM7) conjugated with CNBr Sepharose beads; L, soluble lysate from 2 x 10⁵ cells. (B) Anti-Lck Western blot on the same membrane in (A) after stripping. Arrows indicate migration of CD44 or Lck. Relative molecular mass is indicated on the left in kDa. Data represent one of three independent experiments.
levels of CD45 or Csk (~1%) were detected in this fraction when the cells were lysed with either detergent. Comparison between the BW⁺ and BW⁻ cells indicated that there was consistently a higher percentage of Lck (~1.5 - 2 fold increase) present in the low-density fraction in BW⁻ cells, after lysis with either detergent (Table 5.1). This raised the possibility that CD45 may negatively regulate the localization of Lck to this low-density fraction. As more Lck was associated with this fraction in BW⁻ cells, it is possible that this creates a larger pool of Lck, which is available to associate with CD44.

5.3.8 CD44 and Lck association occurs in the low-density fraction

To determine if the association of Lck with CD44 occurred in the low-density fraction in BW⁺ and BW⁻ cells, CD44 was immunoprecipitated from both the low-density (fractions 2 to 4, Figure 5.9) and high-density (fractions 7 and 8, Figure 5.9) regions of the sucrose gradient. Figure 5.10 demonstrates that Lck associated with CD44 in the low-density fraction isolated from both the BW⁺ and BW⁻ cells, but that approximately 2 fold more Lck co-precipitates with CD44 in BW⁻ cells. Thus in the BW⁻ cells, more Lck is present in the low-density fraction and more Lck associates with CD44 isolated from this fraction.
Figure 5.9. Distribution of Lck, CD44, CD45 and Csk on sucrose-density gradients. 5 x 10^7 CD45⁺ BW cells (BW⁺) and CD45⁻ BW cells (BW⁻) were solubilized in lysis buffer containing either (A) 1% Brij-58, or (B) 1% TX-100 and kept on ice for 20 min. Total cell lysate was added to centrifuge tube and overlaid with sucrose then centrifuged for 16 h at 4°C. 20 µl of each 1.5 ml fraction from the top (low-density) to the bottom (high-density) of the centrifuge tube, indicated as fraction 1 (low) to 8 (high), as well as the pellet, indicated as P, were analyzed on SDS-PAGE, then transferred to PVDF membranes. Western blotting was performed on separate membranes using antibodies against Lck, CD44, CD45 and Csk. Results represent one of three independent experiments.
Table 5.1. Distribution of Lck, CD44, CD45 and Csk on sucrose density gradients.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Cell line</th>
<th>1% Brij58</th>
<th>1% TX-100</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Low density</td>
<td>High density</td>
</tr>
<tr>
<td>Lck</td>
<td>BW⁺</td>
<td>39 ± 5% (n=4)</td>
<td>60 ± 8% (n=4)</td>
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<tr>
<td></td>
<td>BW⁻</td>
<td>58 ± 7% (n=4)</td>
<td>37 ± 8% (n=4)</td>
</tr>
<tr>
<td>CD44</td>
<td>BW⁺</td>
<td>50 ± 9% (n=4)</td>
<td>49 ± 8% (n=4)</td>
</tr>
<tr>
<td></td>
<td>BW⁻</td>
<td>46 ± 3% (n=4)</td>
<td>48 ± 6% (n=4)</td>
</tr>
<tr>
<td>CD45</td>
<td>BW⁺</td>
<td>&lt;1 ± 2% (n=4)</td>
<td>99 ± 4% (n=4)</td>
</tr>
<tr>
<td></td>
<td>BW⁻</td>
<td>1 ± 0.1% (n=2)</td>
<td>99 ± 0.1% (n=2)</td>
</tr>
<tr>
<td>Csk</td>
<td>BW⁺</td>
<td>1 ± 1% (n=2)</td>
<td>98 ± 0.2% (n=2)</td>
</tr>
</tbody>
</table>

Low and high density represent sucrose density fractions 2 to 4, and 7 and 8 respectively after cell lysis in either 1% Brij-58 or 1% TX-100, as indicated. Numbers represent the density percentage of the molecule in that fraction ± the standard deviation over the total number of density from fraction 1 to 8 and the pellet (see Materials and Methods). n= the number of times the experiment was performed.
CD44 Immunoprecipitation

A. Anti-CD44 blot

<table>
<thead>
<tr>
<th></th>
<th>BW+</th>
<th>BW-</th>
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<tbody>
<tr>
<td>C</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>+</td>
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<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Density</td>
<td>Anti-CD44 mAb</td>
<td>Lysate</td>
</tr>
</tbody>
</table>

B. Anti-Lck blot

Figure 5.10. CD44-Lck interaction occurs in the low-density of sucrose-density gradient centrifugation. CD45^+ and CD45^- BW5147 cells (BW+ and BW- cells) were solubilized in lysis buffer containing 1% Brij-58 and then centrifuged on a sucrose gradient for 16 h at 4°C. Low-density fractions (fractions 2 to 4, see Fig.5.9) and high-density fractions (fractions 7 and 8, see Fig.5.9) were pooled and CD44 was immunoprecipitated by anti-CD44 antibody as described in Materials and Methods. (A) Anti-CD44 Western blot. C, antibody alone control; and L, soluble lysate from 2 x 10^5 cells. (B) Anti-Lck Western blot on the same membrane of (A) after stripping. Arrows indicate migration of CD44 and Lck. Relative molecular mass is indicated on the left in kDa. Data represent one of three independent experiments.
5.4 Discussion

5.4.1 CD44-mediated spreading and adhesion to HA in CD45 T Cells

Engagement of CD44 with immobilized antibody resulted in significant morphological changes and polarized spreading in the BW T cells (Figure 5.1). At a higher concentration of CD44 antibody (250 μg/ml), the BW cells did begin to spread, but exhibited a non-polarized, circular spreading (N. Wong and P. Johnson unpublished data). This type of spreading of CD45 T cells on high concentrations of CD44 antibody has also been observed previously and dendrite formation has been reported in activated B lymphocytes (144, 145). Investigation of the morphological change of another two CD45-deficient T cell lines, TCR/CD3 BW5147 and SAKR (Figure 5.2), on immobilized CD44 antibody further support the observation that T cells lacking CD45 have an enhanced ability to flatten and spread in response to anti-CD44 antibody.

As noted by others, cell spreading did not occur in response to immobilized HA, a physiological ligand for CD44 (Figure 5.4A and ref. 144). This may be due to the low-affinity interaction of CD44 and HA, in which case the K_d ranges from 5 to > 150 μM by affinity capillary electrophoresis using CD44-Ig fusion protein (269). The low affinity of the CD44-HA interaction may set up a high threshold for intracellular changes such as cell spreading to occur. Alternatively, whether or not the immobilized HA mimics the physiological HA molecule is uncertain. It has been reported that only HA fragments, and not the high molecular weight HA, can initiate tyrosine phosphorylation and activate PKC in endothelial cells (270). In activated macrophages, HA fragments also induce the secretion of cytokines and chemokines, up-regulate integrin functions, and induce nitric-oxide synthase via an NF-κB-dependent mechanism (152-154). These data indicate that CD44 can signal to the cell in response to binding a physiological ligand. Both cross-linking of CD44 and HA fragments can induce integrin-mediated adhesion in colon carcinoma cells (149), suggesting that in these
cells, CD44 stimulation by mAb may mimic the interaction of CD44 with fragmented HA. In this study, only the high molecular weight HA was immobilized on tissue culture plates for the assessment of morphological change in BW5147 cells. Therefore, it is not known whether BW5147 cells can be induced to spread by the fragmented HA. In addition to HA, CD44 can also bind to osteopontin, serglycin, itself, fibronectin, and some types of collagen. Therefore, it is conceivable that CD44 cross-linking may mimic the binding of one of these ligands.

5.4.2 Role of the Src-family kinases and CD45 in CD44-mediated cell spreading

Cell spreading induced by anti-CD44 antibody occurs in BW T cells, where the Src-family kinases are predominantly phosphorylated at the negative regulatory site (245, 271). This indicates that first, Src-family kinases play an important role in CD44-mediated signaling in CD45 T cells, and second, CD45 has a negative regulatory effect on CD44-associated Src-family kinases. PP2 inhibited BW cell spreading (Figure 5.3), further indicating that Src-family kinases are required for the CD44 signaling leading to the cytoskeletal changes. In addition, this result, together with the observations made from integrin studies, provide additional data supporting an emerging role for CD45 in negatively regulating leukocyte adhesion and spreading. In macrophages derived from the CD45 bone marrow, β2 integrin-mediated cell adhesion and spreading was more rapid than the CD45+ macrophages, but was not sustainable. The Src-family kinases, Hck and Lyn, were also hyperphosphorylated at the negative regulatory site (176). These data imply that the hyperphosphorylated form of Src-family kinases, which are more prevalent in CD45 cells, enhances both integrin and CD44 signaling to the cytoskeleton.
5.4.3 Comparison of CD44-induced signaling and cell spreading with integrin-mediated signaling and adhesion

The activation of Src-family kinases by CD44 antibody results in the phosphorylation of Pyk2, a member of the focal adhesion family of tyrosine kinases, expressed in the brain and haemopoietic system (166). Although FAK is considered the primary kinase mediating integrin adhesion events (163), it was phosphorylated to a lesser extent in the CD44 signaling pathway, suggesting a possible divergence between CD44 and integrin signaling pathways. Paxillin, a common downstream target of FAK and Pyk2, was not consistently tyrosine phosphorylated upon CD44 antibody stimulation, suggesting that paxillin phosphorylation is not essential for CD44-mediated cell spreading to occur (Figure 5.7). Paxillin phosphorylation is thought to be a key event in integrin mediated signaling leading to cell spreading (272), indicating another possible divergence between the CD44 and integrin signaling pathways. Despite these potential differences, many similarities exist in the signaling pathways induced by the integrins and CD44. Most noticeable is the involvement of Src-family kinases and the negative regulatory effect of CD45 on leukocyte adhesion and spreading.

5.4.4 The role of Pyk2 and FAK in CD44-mediated cell spreading

Anti-CD44 antibody induces cell spreading and tyrosine phosphorylation of Pyk2, and to a lesser extent, FAK, in BW5147 cells (Figure 5.1 and 5.5A). Pretreatment of cells with PP2 abolishes tyrosine phosphorylation of Pyk2 and FAK (Figure 5.5B), indicating that Pyk2/FAK phosphorylation is downstream of Src-family kinase activation upon CD44 engagement. The cell spreading process in BW5147 cells requires Pyk2/FAK, and the reorganization of the actin cytoskeleton. This is because pre-treatment of cells with Cytochalasin D completely prevented the spreading phenotype and the tyrosine phosphorylation of Pyk2 and FAK (Figure 5.3 and R. Li unpublished data). Therefore, there is a link between the activation of Pyk2/FAK and the rearrangement of actin cytoskeleton in CD44-mediated cell spreading. More interestingly, pre-
treatment of cells with EDTA also abolished BW\textsuperscript{-} cell spreading (Figure 5.3). This result suggests that the process of CD44-induced cell spreading may require the influx of cations. Alternatively, kinases whose activity is dependent of divalent cations may also be required in the spreading process. Among these kinase, Pyk2 is a good candidate because its activation depends on Ca\textsuperscript{2+} (273, 274). This is supported by our preliminary results showing that the tyrosine phosphorylation of p120 proteins was inhibited upon treatment of cells with EDTA (R. Li and N. Wong unpublished data). In BW5147 cells, tyrosine phosphorylation of FAK induced by CD44 stimulation is less pronounced than Pyk2 (Figure 5.6). This could be due to the fact that a low level of FAK is expressed in these cells, or that Pyk2 is the preferred substrate for Src-family kinases. Given that FAK is expressed in almost all tissues, whereas Pyk2 is expressed mainly in the central nerve system and in cells and tissues derived from haematopoietic lineages, it is reasonable to argue that in leukocytes, Pyk2 and FAK may function differently. Therefore, the detailed signaling events need to be identified before the role of Pyk2/FAK in CD44 signaling is fully appreciated.

5.4.5 CD44-Lck association in lipid domains

Localization of proteins to the low-density fraction of sucrose gradients has been associated with protein localization in the cell to specific lipid domains or rafts in the cell membrane (156). In BW\textsuperscript{-} cells, CD44-mediated spreading requires the activation of Src-family kinases. As CD44 associates with Src-family kinases in the low-density sucrose fraction, this implies that Src-family kinases present in this fraction can be activated in the absence of CD45. Since CD44-mediated Src-family activation is severely attenuated in BW\textsuperscript{+} cells, this suggests that CD45 can exert a negative regulatory effect on this pool of Lck. However, to do this directly, CD45 would have to access to Lck in this fraction. Others and I have shown that very little CD45 is present in the low-density fraction (Figure 5.9 and 213, 275). Therefore, CD45 may access transiently to these membrane microdomains, may access to these microdomains
from the periphery, may be solubilized from these domains upon detergent lysis, or may act indirectly to down regulate Lck activity. In this report, lysis in 1% Brij-58 or 1% TX-100 resulted in different amounts of CD44 or Lck being present in the low-density fraction (Figure 5.8 and 5.9), indicating that different detergents can differentially solubilize CD44 and Lck. This suggests that caution should be exercised when extrapolating the presence or absence of a protein in the low-density fraction to its presence or absence in lipid microdomains or rafts in the cell membrane. As suggested by others (276), the low-density fraction may contain more than one type of lipid vesicles or membrane domains. However, despite the difference in CD44 distribution after cell lysis with the two detergents, the CD44-Lck association was consistently found to occur only in the low-density fraction and to occur to a greater extent in CD45\(^{-}\) T cells (Figure 5.10).

5.4.6 Regulation of CD45 on the distribution of Lck in lipid domains

The negative effect of CD45 on CD44-mediated signaling contrasts with its overall positive effect on TCR/CD3 mediated signaling. More Lck was associated with CD44 in BW\(^{-}\) T cells, suggesting that CD45 regulates the distribution and association of Lck in the cell. One simplistic view is that tyrosine phosphorylation of Lck at 505 promotes its location to lipid domains associated with this low-density fraction, and that dephosphorylation at Tyr 505 by CD45 results in reduced association with this fraction. This would create differences in the phosphorylation state of Lck in the high- and low-density sucrose fractions. In CD45\(^{+}\) Jurkat cells, Rodgers and Rose noted that the Lck present in the low-density sucrose fraction was 3 fold more tyrosine phosphorylated (at Tyr 505) and a third less active than the Lck present in the high-density sucrose fractions, thus supporting this view (213). The Lck located to the low-density fraction may thus represent an inactive, but activatable form of Lck. The fact that CD45 is not present in this fraction and that Lck can be activated after immobilization of BW\(^{-}\)
cells on CD44 antibody suggests that CD44-initiated Lck activation in this fraction occurs in a CD45-independent manner.
CHAPTER SIX

Summary and Perspectives

6.1 Summary of results

CD44 is a cell adhesion molecule implicated in a variety of biological processes such as embryonic development, wound healing, angiogenesis and tumor metastasis. In the immune system, CD44 also plays an important role in leukocyte adhesion during haemopoiesis, T lymphocyte activation, lymphocyte homing and leukocyte extravasation at sites of inflammation. Most of these CD44-mediated cellular events involve the interaction of CD44 with one of its major ligands, HA. The HA binding ability of CD44 in leukocytes is strictly regulated in a developmental stage- and activation state-specific manner such that not all CD44 bearing leukocytes will normally bind to HA. When this study was initiated, the mechanisms underlying the regulation of CD44-HA interaction, especially those mediated by the transmembrane domain or the cytoplasmic domain of CD44, were largely unknown. Therefore, in this study, I aimed to answer two questions. First, whether the transmembrane domain or the cytoplasmic domain of CD44 regulates HA binding. Second, whether the cytoplasmic domain of CD44 mediates molecular interactions with intracellular proteins as a potential means for signaling as well as for the association with cytoskeleton. Results from this study indicated that the transmembrane domain of CD44 mediated a non-covalent association of CD44 molecules on the cell membrane. This self-association of CD44 facilitated the HA binding in T cells. On the other hand, the cytoplasmic domain of CD44 mediated molecular interactions with intracellular proteins including Lck, Fyn, Grb-2 and ERM cytoskeletal proteins. The interaction of CD44 with Src-family kinases was responsible for the cell spreading induced by an anti-CD44 mAb, as well as for the tyrosine phosphorylation of Pyk2 and FAK that was concomitant with cell spreading. The interaction of CD44 and Lck occurred in the lipid rafts, and this interaction was negatively affected by CD45. The action of CD45
was likely to influence the distribution of Lck on the cell membrane, and then, regulate the CD44-Lck interaction, the tyrosine phosphorylation of Pyk2 and FAK, and the cell spreading upon activation of CD44 by an anti-CD44 mAb.

6.2 Receptor clustering acts as one regulatory mechanism for CD44 to increase the binding avidity to HA, a model

For most cell adhesion molecules, such as selectins, integrins, Ig superfamily molecules and CD44, the affinity of ligand binding is fairly low in unstimulated cells (1, 140, 269, 277). However, upon stimulation by different factors, the affinity of ligand binding can be up-regulated (inside-out signaling). For CD44, the enhanced HA binding ability can be achieved by increasing the cell surface expression, expressing new isoforms, generating post-translational modifications, or associating with the actin cytoskeleton. Most of these events can fit into a model, which introduces the concept that high affinity binding of HA by CD44 can be regulated by receptor clustering.

First of all, HA is a long chain GAG composed of repeating disaccharide units, which may provide a biochemical explanation as to why high affinity binding of HA requires the clustering of CD44. Increase in levels of expression of CD44 after cellular stimulation provides a larger pool of CD44 on the cell membrane. These CD44 molecules may then associate laterally with one another to increase the multivalency for HA binding. Data presented in the current study confirmed the presence of this lateral interaction of CD44 on the plasma membrane of T cells that constitutively bind to HA. In this case, the transmembrane domain of CD44 mediates this interaction. Of note, the lateral interaction of cell surface CD44 also occurred in a fibroblast line, which did not constitutively bind HA, but could be induced to bind HA by IRAWB mAb. However, the percentage of self-association of CD44 in fibroblasts was much lower (7 times less) compared to the constitutively HA binding cells, indicating a correlation between the self-association of CD44 and the HA binding. Besides, generation of
new isoforms, such as CD44v, may provide additional sites for post-translational modifications including glycosylation, sulfation, sialylation and addition of GAG side chains. These modifications may change the overall charge of the external domain of CD44 or mediate intermolecular interactions on the membrane, which may facilitate receptor clustering of CD44 (141, 278). Moreover, the association of CD44 with cytoskeleton may also contribute to HA binding by mediating receptor clustering. Co-localizations of CD44 with ERM family cytoskeletal proteins and actin filaments to the uropods support this view (204). Additionally, results from this study indicated that disruption of the CD44-ERM-actin interaction also reduced HA binding in a myeloid cell line. This implies that tethering of CD44 to the actin cytoskeleton by ERM proteins can facilitate HA binding. It would be useful to determine in the future whether the CD44-ERM interaction mediates receptor clustering or conformational changes of CD44, which may then optimize HA binding.

Interestingly, receptor clustering is not only observed as a regulatory mechanism for CD44-mediated ligand binding, it has also been described for other cell adhesion molecules such as integrins and Ig superfamily molecules. For example, the binding affinity of LFA-3 and Mac-1 to their ligands (CD2 and C3bi) was markedly enhanced when they are dimeric or multimeric (226, 227). It is also suggested that the receptor clustering of integrins is mainly mediated by the interaction of integrin cytoplasmic domain with actin cytoskeleton (139, 279). For ICAM-1, its native structure is a dimer (228). Both the extracellular domain and the transmembrane domain were implicated in the non-covalent formation of homodimers, and this facilitated the binding of ICAM-1 to LFA-1 (140). Therefore, it is conceivable that the receptor clustering may act as a general mechanism for cell adhesion molecules to increase the binding affinity to their ligands.
6.3 The signaling cascade that connects CD44 to the actin cytoskeleton

The outside-in signaling cascade initiated by CD44 has just started to be understood. In the current study, activation of CD44 by an immobilized anti-CD44 mAb induced a cell spreading phenotype, which requires the dynamic reorganization of actin cytoskeleton. In addition, other researchers and I have also shown that CD44 interacts with signaling molecules, such as the Src-family kinases and Grb-2, and with cytoskeletal proteins such as ERM, ankyrin and protein 4.1. These interactions provide a molecular basis for CD44 to signal to the cytoskeleton. Notably, the Src-family kinases and two focal adhesion kinases, Pyk2 and FAK, were implicated in both CD44- and integrin-mediated signaling, suggesting that there may be some common or converged signaling events downstream of these two types of cell adhesion molecules. This is not surprising because both CD44 and integrins can initiate cell spreading, which may be operated by the same cellular signaling machinery. It is thus very useful to compare the integrin outside-in signaling pathways to those mediated by CD44 in order to fully understand the signaling events triggered by CD44 (Figure 6.1).

Activation of both integrins and CD44 by either ligands or mAbs leads to the phosphorylation of FAK and Pyk2 and the recruitment of Src-family kinases. For integrins, activation of FAK autophosphorylates Tyr 397, therefore, creating a binding site for the SH2 domain of Src or Fyn (163, 280). In contrast to integrins, for the activation of CD44 by an anti-CD44 mAb in Chapter Five, the kinase activity of the Src-family kinases seemed to be required before Pyk2 or FAK is activated. This was because pre-treatment of cells with the Src-family kinase-specific inhibitor, PP2, abrogated the tyrosine phosphorylation of Pyk2 and FAK, as well as the process of cell spreading induced by the anti-CD44 mAb. Besides, the tyrosine phosphorylation of Pyk2 and FAK was also abolished by pretreating the cells with Cytochalasin D (R. Li unpublished data). Similar to this finding, Ganju et al. also described that the tyrosine phosphorylation of Pyk2 after TCR stimulation was reduced by the pretreatment of cells with Cytochalasin D (167). These results suggest that either the
Figure 6.1. CD44- and integrin-mediated adhesions activates Rho family GTPases. Signaling pathways via CD44 (blue arrows) and integrins (grey arrows) leading to the activation of Rho family GTPases are indicated. Question marks indicate proposed pathways that need to be verified.
disruption of actin filaments by Cytochalasin D, or other cellular events induced by the
treatment could act as a feedback to down-regulate the activity of FAK and Pyk2.

The downstream signaling events that occur after Pyk2 and FAK activation by CD44
have been further investigated in our lab (281). One of the downstream targets of Pyk2 and
FAK, p130Cas (Cas), was shown to be heavily tyrosine phosphorylated by CD44 activation. Cas
family members (Cas, HEF-1 and Efs) are docking proteins implicated in integrin receptor
signaling to the actin cytoskeleton. They can associate with FAK and Pyk2 via SH3 domain
interactions and can be tyrosine phosphorylated by either FAK or Pyk2 or by cooperative
interactions between Src family kinases and FAK or Pyk2. Elevated Cas expression promotes
cell migration on ECM (282). In contrast, mutations that prevent Cas tyrosine phosphorylation
block the migratory response (283). In addition, cells from Cas knockout mice exhibit
decreased focal adhesions and stress fibers, suggestive of effects on Rho activity (284, 285). In
addition, Cas also binds Crk, an SH2/SH3-containing adapter protein that interacts with C3G, a
GEF for Ras and Rap1 (286). Cell migration is promoted by expression of either Cas or Crk,
whereas cytokine-stimulated migration is inhibited in cells expressing mutations in Cas or Crk
that inhibit their interaction. Significantly, migration in response to Cas or Crk is prevented by
dominant-negative Rac, suggesting that the Cas-Crk complex leads to Rac activation (283). A
likely protein involved in Cas-Crk signaling to Rac is DOCK180 which bound to Crk in
response to integrin-mediated adhesion (287, 288), and which also binds to Rac (289, 290).
Although it does not appear to be a conventional Rac GEF itself, DOCK180 has been reported
to enhance nucleotide exchange, and when overexpressed in cells promotes elevated Rac-GTP
levels (287). In the case of CD44 outside-in signaling, several lines of evidence indicate that
stimulation of cell surface CD44 by an anti-CD44 mAb or by fragmented HA also activates
Rac (157, 158). In agreement with these findings, data presented here suggested that signals
from CD44 may activate Pyk2/FAK, recruit Cas, Crk, and then DOCK180, and lead to the
activation of Rac or Cdc42 which can promote the assembly of lamellipodia, membrane ruffles and stress fibers, as manifested by the morphology of cell spreading.

6.4 Opposing roles for CD45 and Src-family kinases in TCR signaling and CD44-mediated cell spreading

Phosphorylation of FAK and Pyk2 has been reported after immobilization of T cell lines by anti-CD3 antibody (268). However, in contrast to TCR/CD3 stimulation, cell spreading induced by the immobilized anti-CD44 antibody did not induce the same tyrosine phosphorylated proteins. For example, no significant tyrosine phosphorylation was observed between 20-40 kDa, where both LAT (36 kDa) and CD3ζ (21 kDa) migrate. Thus, CD44 signaling may induce the tyrosine phosphorylation of only a subset of proteins that become tyrosine phosphorylated upon TCR/CD3 ligation. One key difference between TCR/CD3 signaling and CD44-mediated signaling in BW T cells, is that efficient TCR/CD3 signaling occurs in BW⁺ cells (245), whereas CD44-mediated signaling leading to cell spreading occurs in BW⁻ cells. Paradoxically, the T cell Src-family kinases are required for the initiation of both signals. This leads us to propose that there are at least two pools or states of Lck, and possibly Fyn, in T cells that have distinct functions, and that the distribution between these two pools is regulated by CD45. One pool is dephosphorylated by CD45 at the negative regulatory site and primed for participation in the TCR signaling event. The other pool is phosphorylated at Tyr 505, associated with CD44 and can be activated in the absence of CD45. The second pool of Lck is rapidly inactivated by CD45 as CD44-mediated signaling is severely attenuated in the CD45⁺ BW cells. TCR signaling is severely attenuated in BW⁻ T cells, indicating a positive role for CD45 in regulating Lck activity in TCR signaling. In contrast, CD44 signaling leading to cell spreading is enhanced in BW⁻ cells, suggesting a negative role for CD45 in regulating the Src-family kinase activity involved in cell adhesion signaling and cell spreading events. Thus CD45 and Src-family kinases appear to have a dual role in T cells, one in promoting
antigen-induced T cell activation and another in preventing cell spreading in response to
signaling through the cell adhesion molecule CD44. Therefore, one function of CD45 is as an
anti-adhesion molecule in leukocytes that acts to prevent unwanted firm cell adhesion and cell
spreading in response to the binding of cell adhesion molecules such as CD44. CD45 may
achieve this by down-regulating the activity of Src-family kinases associated with CD44.

6.5 Coordination of cell adhesion molecules in the immune system

Why are there so many different types of cell adhesion molecules carrying overlapping
functions in vertebrates? For example, in the immune system, both selectins and CD44 were
found to mediate the initial "rolling" process upon leukocyte extravasation. Both integrins and
CD44 were identified to enhance the signals via TCR/CD3 during T lymphocyte activation.
Cadherins, integrins, CD44 and Ig superfamily molecules were all involved in the cellular
interactions within the bone marrow during haemopoiesis (section 1.3). One explanation for
the overlapping functions of cell adhesion molecules is that this functional redundancy may be
evolutionarily significant since they can back up each other for some important functions under
certain pathological conditions. This idea is supported by results from diseases or knockout
studies. Patients who carry a point mutation on the β2 chain of integrins develop a syndrome
known as the leukocyte adhesion deficiency (LAD). These patients exhibit reduced leukocyte
adhesion due to the malfunction of LFA-1, and are vulnerable to bacterial infections. However,
the T lymphocyte activation, which is known to require the interaction of LFA-1 and ICAM-1,
is normal in these patients. This suggests that other molecules such as CD44 may at least
partially compensate for the loss of LFA-1. In a second example, the CD44 knockout mice
developed normally except for a minor deficit in the egress of myeloid progenitor cells from
the bone marrow (84). In this case, other cell adhesion molecules or HA binding proteins were
proposed to back up the functions of CD44 in these mice.

In addition to the partial overlapping functions of the cell adhesion molecules, the
coordination of their functions is also intriguing. This can be best exemplified by the process
of leukocyte transmigration across the vascular endothelial wall in response to a local inflammatory signal. This process has been divided into four separate and sequential steps: (i) the “rolling” step, in which transient and reversible primary adhesion occurs through the selectins and CD44; (ii) the “activation” step, in which the rapid activation of integrin occurs within seconds; (iii) the “arrest” step, in which stable adhesions are formed under shear forces as integrins bind to their receptors with high affinity; (iv) the “diapedesis” or migration step, during which leukocytes migrate through the endothelial layer as cell adhesion molecules (i.e. CD44 and integrins) signal changes in the cytoskeleton in preparation for extravasation (Figure 6.2 and reviewed in refs. 2, 8, 291, 292). During this multistep process, one type of cell adhesion molecules communicates with another type of molecules to ensure that signals are fully transmitted into the cells for the preparation of cytoskeletal reorganization. The crosstalk between different types of cell adhesion molecules is not well understood at present. But some in vitro evidence suggested that signals triggered by an anti-CD44 antibody or fragmented HA enhanced the cell surface expression of LFA-1, as well as the LFA-1-mediated tumor cell adhesion to an endothelial cell line, and thereafter the transendothelial migration (148, 149).

Another example of the coordination of different types of cell adhesion molecules in the immune system is the process of integrin- and CD44-mediated migration of pro-T cells to the thymus. Bone marrow pro-thymocytes express CD44, but only a few percent of cells within the normal thymus express significant quantities of CD44. Thymocyte populations enriched in thymus-homing progenitor were also enriched in CD44hi cells. Treatment of these cells with an anti-CD44 mAb inhibited the activity of thymus homing, suggesting that CD44 may play a role in the migration of pro-T cells to the thymus (293). Consistent with this finding, a recent in vitro study using mouse fetal liver cells (FL) labeled with Green Fluorescent protein (GFP) identified that pretreatment of the thymus with an anti-CD44 antibody blocked FL migration into the thymus, but did not inhibit FL adhesion to the thymus lobe. On the contrary, treatment with an anti-integrin α4 antibody inhibited FL adhesion to the thymus. Therefore, a differential
Figure 6.2. Diagram of leukocyte adhesion to activated endothelium in a capillary at an inflammatory site. The molecules implicated at each stage are indicated above. The bacterium represents the cause of the inflammatory response. HA is associated with the activated endothelium. Picture not to scale. Adapted from (11).
regulation of CD44 and integrin in the process of thymus homing can be proposed in which integrins is required for the initial adhesion of FL to the thymus lobe, while CD44 is required for the migration of the cells into the thymus (294).

The partial overlapping function and the coordination of cell adhesion molecules are beneficial to cells since they can control the adhesion and migration events via multiple mechanisms. On the other hand, this functional redundancy also makes it difficult to study the functions of individual cell adhesion molecules. However, to understand the molecular biology of each cell adhesion molecule is as essential as to appreciate the general biology of cell adhesion and cell migration. In this thesis, I have provided evidence on the molecular interactions and signaling events mediated by one type of the cell adhesion molecules, CD44, in the immune system. It is hoped that information derived from this study can increase our understanding of the CD44-mediated cellular events that occur elsewhere in vertebrates in addition to the immune system.
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