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MECHANISMS REGULATING THE BINDING ACTIVITY OF CD44 TO HYALURONIC ACID

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1. ABSTRACT

CD44 is a cell surface glycoprotein present on many cell types. Many CD44 isoforms have been identified. All CD44 isoforms utilize identical transmembrane and cytoplasmic domains. The hematopoietic form of CD44 (CD44H) is the major CD44 protein present on normal human lymphocytes and monocytes. One of the ligands for CD44 is hyaluronic acid (HA), a polymer consisting of repeat units of disaccharide; N-acetyl-D-glucosamine and N-acetyl-D-glucuronic acid. Since HA is present ubiquitously in extracellular matrix and in circulation, promiscuous binding of HA to CD44 may have undesirable affect. Similar to other adhesion molecules, binding of HA to cell surface CD44 requires regulation. In this review, we summarized our studies using a human lymphoma cell line, Jurkat. We found that binding of $CD44^{+}$ Jurkat transfectants to HA requires cellular activation. Cellular activation induces the reorganization of the cytoskeleton proteins. Reorganization of cytoskeletal proteins results in clustering of CD44 on the cell surface. Clustering of CD44 on the cell surface is a prerequisite for the homodimerization of CD44. Our studies on Jurkat transfectants and results from other investigators suggest that interactions between CD44 and HA is a dynamic process and requires the participation of different cellular components; depending of the nature of the cell type and/or the nature of the activation signals.

2. INTRODUCTION

Cell-cell and cell-extracellular matrix (ECM) interactions are important in normal cellular physiology, signal transduction and tumorigenesis (1-5). The study of cell surface molecules that are involved in cell-cell and/or cell-ECM interactions is currently an area of great interest. Many families of molecules that are important in these interactions are cell surface adhesion molecules. Some of the best characterized adhesion molecule families are integrins, selectins and CD44. All these molecules have been reported to play important roles in cell-cell and cell-ECM interactions.

CD44 is a family of glycoproteins present on many cell types (6-10). CD44 has been implicated in a variety of physiological and pathological processes, including embryogenesis, leukocyte circulation and activation, and tumor metastasis (6-10). One of the ligands for CD44 is HA (6-10). However, it is clear that not all CD44 bearing cells can bind HA. The exact physiologic requirements for binding of HA are not known. Since HA is ubiquitous in the extracellular matrix and the circulation, promiscuous binding of HA to CD44 may have undesirable effects. Therefore, like other adhesion molecules, binding of HA to cell surface CD44 requires cellular regulation. The accumulated evidence suggests that the regulation of the binding of CD44 to HA is a complex and dynamic process. There are three states of binding of CD44 to HA defined as constitutive active, activatable and inactivatable. Constitutive binding has been documented on some tumor cell lines. The reasons that some CD44 proteins are present in an inactivatable state are not known. This state can be viewed in two ways: either the conditions for activating HA binding CD44 have not been discovered or that the CD44 on these cells functions as a receptor for ligands other than HA. The activatable state is the most interesting one, because it is linked to signal transduction and cellular activation.

3. INTERACTIONS BETWEEN CELL SURFACE CD44 ON JURKAT T CELLS AND SOLUBLE HYALURONIC ACID

3.1. Binding of CD44 to HA require cellular activation in Jurkat T cells

In order to probe the mechanisms by which the stimulants of cellular activation can regulate the binding of CD44 to HA, Jurkat cells transfected with the human CD44H gene was used as a model system to study interactions between CD44 and HA. Consistent with a previous report (11), we reported earlier that Jurkat T cells transfected with a human CD44H gene do not bind F-HA (12). The ability of PMA and anti-CD3 mAb to induce binding of high levels of F-HA suggests that the functions of CD44 are integrally connected to signal transducing pathways. It is interesting to note that binding of HA to CD44 on Jurkat T cells can be regulated by other signal transduction pathways as well. Ionomycin, a calcium mobilizer, induced HA binding. Forskolin, a reagent known to increase cAMP levels, also induced binding of HA

(13,14). Forskolin D, an inactive analog of forskalin did not induce binding. More importantly, PMA-induced binding in the CD44 Jurkat transfectant was significantly inhibited in the present of forskolin, indicating that protein kinase C and protein kinase A pathways can cross-talk to each other, as has been described in other systems (15-16).

We reported earlier that in CD44 Jurkat transfectants, PMA-induced binding was significantly inhibited by colchicine, cytochalasin D and taxol (14). Therefore, binding of high levels of F-HA requires the participation of multiple cytoskeletal proteins. Stimulation of human lymphocytes with PMA or anti-CD3 causes reorganization of cytoskeletal proteins (17,18). Cross-linking of CD3 with anti-CD3 mAb increased the association of CD44 on T lymphocytes with cytoskeletal proteins (19). Therefore, reorganization of cytoskeletal proteins and clustering of CD44 may be a prerequisite to CD44 binding of F-HA.

Some anti-CD44 mAbs induce binding of HA by cross-linking CD44 on cell surface (11,20). It was assumed that these mAbs simply pulled CD44 molecules together and/or changed the conformation of CD44. This assumption was further tested in $CD44^+$ Jurkat transfectants (14). Cytochalasin D pretreatment significantly inhibited anti-CD44 mAb F10-44-2-induced binding. Pretreatment with colchicine or taxol did not affect F10-44-2-induced binding. These results suggest that simply cross-linking CD44 with the anti-CD44 mAb is insufficient to induce binding; reorganization of actin filament is required. In contrast, PMA-induced binding was inhibited by all three cytoskeletal protein inhibitors. The levels of binding induced with PMA are consistently higher (M.F.I. > 100) than that induced with antibody (M.F.I.<50). It is possible that in order to bind high levels of F-HA, both actin filaments and microtubules may be required. Taken together with previous evidence, it can be speculated that CD44 molecules interact with the actin filament network but not the microtubule network when the cells are not stimulated. This is consistent with previous observations that CD44 interacts with ankyrin and ERM family of proteins (21,22). The mechanisms by which the cytochalasin D pretreatment can inhibit F10-44-2 induced HA binding are not known. Cytochalasin D may alter the distribution of CD44 molecules on the cell surface so that the mAb can not pull CD44 molecules into a large aggregate; which is required for the binding of HA.

3.2. The transmembrane domain of CD44 is essential for binding of HA

The most conserved region of the entire CD44 protein is the transmembrane domain. The transmembrane sequence is 100% conserved from murine CD44 to human CD44. Furthermore, all potential CD44 isoforms use the same transmembrane and cytoplasmic domain, indicating that these two regions of the molecule must be important in the functions of CD44.

We reported earlier that PMA stimulates the binding of CD44 to HA by inducing covalent homodimerization of CD44 on the cell surface (23). Covalent dimerization involves a cysteine (Cys286) in the transmembrane domain of CD44, and is essential for binding high levels of HA. CD44.C286A Jurkat cell is a CD44 transfectant bearing a CD44 protein in which the transmembrane Cys has been replaced with an Ala. Several lines of evidence suggest that the failure of the CD44.C286A transfectant to bind HA is directly related to the inability of the CD44 molecules to dimerize after cellular activation. Western blotting of CD44 proteins from wild type CD44 and CD44.C286A mutant transfectants failed to reveal any obvious change in the molecular size of these two proteins. Furthermore, immunoprecipitation followed by two dimensional gel electrophoresis failed to reveal any significant changes in the pI of the CD44.C286A protein. Therefore, post-translational events like N-linked and O-linked glycosylation were not grossly disrupted in CD44.C286A mutants. Alteration of the cysteine residue in the transmembrane domain may also influence the stability of cell surface CD44, which may be critical for binding of F-HA. We ruled out this possibility by performing metabolic labeling and pulse-chase experiments. Replacing the Cys 286 residue did not alter the half life of CD44.C286A molecule on the cell surface.

The exact molecular mechanisms of CD44 dimerization are not known. PMA treatment not only induced the dimerization of CD44, but also significantly up-regulated the expression of the CD44 protein. Potentially, PMA may alter the post-translational modification of CD44. For example, PMA has been reported to stimulate the phosphorylation of the CD44 molecule (24). Furthermore, after PMA treatment, a smaller CD44 protein can be detected in CD44 Jurkat transfectants suggesting that PMA may also alter the glycosylation pattern of CD44. This point is germane to our studies, because inhibition of N-linked glycosylation either by tunicamycin or neuraminidase increased binding of CD44 to HA (25-27). Most of the negative charge on cell surface proteins is due to the presence of terminal sialic acid residues. A decrease in N-linked glycosylation with a corresponding reduction in net negative charge on CD44 may permit CD44 to "pack" tighter to each other, and may be a pre-requisite for CD44 clustering and/or dimerization. Glycosylation of ICAM-1 has been reported to regulate adhesion of LFA-1 and Mac-1 (28), and may play a role in the oligomerization of ICAM-1 (29).

Whether increased expression of CD44 or post-translational modification of CD44 or both are essential for CD44 dimerization is not known. It is also not known why some transmembrane proteins can constitutively dimerize, while dimerization of CD44 requires cellular activation. Dimerization of CD44 on the cell surface enables the cells to bind soluble HA, however, the consequences of this binding are not known. Binding of HA to cell surface CD44 has been reported to result in signal transduction and cytokine production in a murine T cell hybridoma (30). Covalent dimerization of CD44 should enable CD44 to remain in the "activated" state for an extended period of time, and may have significance in the physiology of lymphocytes.

Earlier studies by Underhill and Toole have suggested that clustering of CD44 on the surface is important for HA binding (31-35). Clustering of CD44 may place CD44 proteins physically close to each other. However, clustering of CD44 by itself is insufficient to cause binding. The length of time during which individual components remain clustered may be more critical in receptor function (36). Therefore, aggregation may not

270 280 290 300

WL IILASLLALA LILAV **C** IAVN SRRR **C** GQKKK LVINSGNG---DMKIG V.

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Figure 1. The putative transmembrane domain and cytoplasmic domain of human CD44.

suffice if the individual receptors move in and out of the clustered state too rapidly. Covalent dimerization of receptors would prevent aggregated receptors from dispersing. High levels of binding require dimerization of CD44. Furthermore, dimerization of CD44 should facilitate multiple interactions between CD44 and HA, and therefore, increase the overall affinity of CD44 for HA.

Our results are in good accordance with other earlier findings. The zeta chain of the CD3 constitutively dimerizes via a disulfide bond in the transmembrane region (37). Artificial replacement of the transmembrane domain of murine CD44 with the transmembrane domain of zeta chain of CD3 resulted in the dimerization of CD44 protein, and binding of HA (38). Our findings may also help resolve a previous paradoxical finding on the binding of HA by a chimeric, genetically engineered, soluble human CD44-Ig fusion protein (10). Since binding of HA requires the cytoplasmic domain of CD44 and cellular activation, it has been difficult to reconcile binding of HA by a soluble human CD44-Ig protein which lacks the authentic CD44 cytoplasmic domain. It turns out that the majority of the soluble CD44 proteins existed in dimeric form (10). A similar ligand binding chimeric soluble ICAM-1-Ig fusion protein has also been shown to exist in dimeric form (29). Therefore, the capacity of chimeric receptor-Ig molecules to bind to their ligand may be due to the dimerization of the chimeric Ig molecule.

The cysteine residues in the transmembrane domain and cytoplasmic domain of CD44 are known to be covalently modified with palmitate (39,40). Palmitoylation of isolated murine CD44 protein from the thymoma, BW5147, has been reported to be important for CD44 binding to ankyrin *in vitro* (39). Interactions between CD44 and ankyrin have also been reported to be essential for binding of F-HA (39). However, since the CD44C295A Jurkat transfectant can bind F-HA when stimulated with anti-CD3 or PMA, palmitoylation of CD44 at cysteine 295 is not essential for binding of F-HA. It should be noted that BW5147 can constitutively bind low levels F-HA, while binding in CD44 Jurkat transfectants requires cellular activation. Therefore, constitutive binding of low levels of F-HA and activation induced binding of high levels of F-HA may have different cellular requirements.

The finding that the cysteine residue in the transmembrane domain of CD44 is involved in the dimerization of CD44 raises an interesting question regarding the relationship between CD44 palmitoylation and CD44 dimerization. Palmitoylation of the cysteine residue in the transmembrane domain may regulate the availability of the cysteine residue for receptor dimerization and activation. In addition, lipid modification of proteins promotes protein-lipid interactions (41,42). Palmitoylation of CD44 may favor interaction between CD44 and lipid and, thus, prevent interaction between CD44 protein molecules. This hypothesis will be explored in future experiments. Cysteine 286 and cysteine 295 are the only potential sites for palmitoylation in CD44. It is not known which residue(s) is(are) palmitoylated. The mutants described in this thesis will help resolve this question.

Binding of HA can be induced by a unique group of anti-CD44 mAb (11,20). F10-44-2 induced binding in all five wild type CD44 Jurkat transfectants and in CD44.C295A transfectants, but not CD44.C286A. The levels of binding induced by anti-CD44 mAb were lower than the levels of binding induced with anti-CD3 mAb. Therefore, aggregation of CD44 on the cell surface may enable the cell to bind low levels of HA, but cellular activation is essential for binding higher levels of HA. The reason that F10-44-2 could not induce binding in CD44.C286A was not because CD44.C286A bound less F10-44-2 antibody. Both the wild type CD44 transfectant and CD44.C286A transfectants express comparable levels of F44-10-2 epitopes. Therefore, antibody-induced binding is more complex than simply binding of antibody to the cell surface and cross-linking of CD44 molecules on the cell surface. This interpretation was further supported by our finding that antibody-induced binding was also inhibited by cytochalasin D and required the presence of the cytoplasmic domain of CD44.

3.3. Interactions between the cytoplasmic domain of CD44 and cytoskeletal proteins are also important in binding of CD44 to HA

In human melanoma cells, transfected human CD44H proteins bound HA constitutively. Truncation of the last 64 amino acid of the cytoplasmic domain in human CD44H did not have much affect on its binding to HA (43). In the murine T lymphoma cells, AKR 1, transfected murine CD44H proteins can bind HA constitutively. Truncation of the last 66, but not the last 56 amino acids of the cytoplasmic domain abolished binding of HA (38). This suggested that the small region between 56 to 66 is required for CD44 binding function. However, the internal deletion of this region did not affect the binding ability of CD44 to HA. Further studies revealed that no specific region of the cytoplasmic domain of CD44 is responsible for the binding activity of CD44. This suggested that multiple elements in the cytoplasmic domain may be required simultaneously for binding. We created two truncated mutants lacking either the last 23 or 57 amino acids of the cytoplasmic domain of CD44. In accord with the previous reports, these deletions did not interfere with the expression of CD44 on the cell surface. The mutant deletion-23, but not deletion-57, could be induced by PMA and anti-CD3 mAb to bind HA (14).

The CD44 protein also contain two clusters of basic residues (RRR and KKK) in the membrane-proximal region of the cytoplasmic domain (figure. 1). These two motifs are also 100% conserved between murine and human CD44. More recently, we used site specific mutagenesis to replace these residues with alanine. Jurkat transfectants bearing CD44 proteins lacking either the RRR or KKK motifs were unable to bind high levels of HA (Liu, et. al., in preparation). These results provide evidence that these two basic motifs are also important in the interactions between CD44 and the cytoskeletal proteins. However, the natures of the cytoplasmic proteins which interact with the cytoplasmic domain of CD44 remained to be determined.

4. PERSPECTIVE

Activation of CD44+ Jurkat cells is required for binding high levels of HA. Cellular activation results in the up-regulation of CD44 expression, reorganization of the cytoskeletal proteins, clustering of CD44, covalent dimerization of CD44 and binding of HA. Activation-induced covalent disulfide dimerization of receptors represents a novel signal transduction mechanism for regulating receptor-ligand interactions. Some of the proposed mechanisms may be applicable to other receptor molecules with cysteine residues in the transmembrane domain and multivalent ligands. While we have provided a general picture for activation induced binding of CD44 to HA, many specific questions remained unanswered. We shall briefly summarize some of the questions that need to be addressed in the future.

Up-regulation of CD44 may be essential for dimerization of CD44. The need for up-regulation of CD44 expression may reflect a simply quantitative requirement for CD44 dimerization. A certain threshold level of CD44 expression must be reached in order for CD44 to form clusters and to dimerize. A high CD44 density on the cell surface may enhance the probability of CD44-CD44 interaction. PMA may induce dimerization by simply up-regulating CD44 protein, to a level favorable for CD44-CD44 interaction. A more quantitative study of the relationship between the number of CD44 molecules expressed on the cell surface and CD44 dimerization is needed to address this issue.

Alternatively, up-regulation of CD44 expression may reflect a qualitative alteration in CD44. Newly synthesized CD44 proteins may be more prone to dimerization than pre-existing CD44. The newly synthesized CD44 protein in an activated cell may be post-translationally modified differently from the existing CD44. These differences may allow newly synthesized CD44 protein to dimerize more readily. At least three potential post-translational modifications may be different between pre-existing CD44 and newly synthesized CD44 in activated cells; glycosylation in the extracellular domain, palmitoylation in the transmembrane domain or phosphorylation in the cytoplasmic domain. Additional experiments are needed to verify whether newly synthesized CD44 in activated cells is preferentially dimerized and whether newly synthesized CD44s are biochemically different from pre-existing CD44. It is also possible that both quantitative and qualitative changes in CD44 expression are required for CD44 dimerization on the cell surface.

The molecular mechanism responsible for CD44 dimerization is not known. As with other proteins, disulfide bonding occurs subsequent to protein-protein interactions mediated by other non-covalent interactions. Mutational analysis of the zeta chain of the CD3 complex revealed that an aspartic acid in the transmembrane domain is critical for the spontaneous dimerization of CD3 (44). Since the aspartic acid can be replaced with a lysine residue, it is a charged residue within the a helix that is essential for dimerization. Whether interactions between charged residues within the transmembrane domain of CD44 is required for CD44 dimerization is not known.

The putative cytoplasmic domain of human CD44 is comprised of 72 amino acids (a.a). After activation, CD44 transfectants lacking the last 23 a.a. (CD44.C23) are able to aggregate, dimerize and bind HA. In contrast, CD44 transfectants lacking the last 57 a.a. (CD44.C57) neither aggregate nor dimerizes. The motif important in the clustering and dimerization of CD44 is located within the region between amino acid 305 to 338. (S-G-N-G-A-V-E-D-R-K-P-S-G-L-N-G E-A-**S**-K-**S**-Q-E-M-V-H-L-V-N-K-P-S-E). Within this sequence there are 5 serines. In murine CD44, Ser 323 and Ser 325 (typed in bold) are constitutively phosphorylated. PMA increased the portion of CD44 molecules that were phosphorylated (24). This region of CD44 is also important in the binding of murine CD44 to ankyrin. Phosphorylation of murine CD44 enhanced the interaction between CD44 and ankyrin (45). Whether phosphorylation of the cytoplasmic domain of CD44 is critical for the clustering or dimerization of CD44 remain to be investigated.

In addition to hyaluronic acid, CD44 also binds to other ligands including collagen (46), fibronectin (47), serglycin (48) and osteopontin (49). It has been reported that monovalent ligand occupancy of the integrin receptor induces different cellular responses than multivalent aggregation of the integrin receptor (50). Therefore, the requirements for interaction between CD44 and its ligands may also depend on the nature of the ligand. In the case of polymeric hyaluronic acid, multiple binding sites may be essential. On the other hand, binding of monovalent ligands like osteopontin may not require clustering or dimerization of CD44. Therefore, activation-induced dimerization of CD44 adds an additional flexibility to the capacity of CD44 to interact with multiple ligands. However, the mechanisms by which the interactions between CD44 and its ligands other than HA are regulated have not been studied. The system developed in this thesis work will be employed to study the interaction between CD44 and other ligands.

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