COMMENTARY

The role of the cytoplasmic domain in regulating CD44 function

Clare M. Isacke

Department of Biology, Imperial College of Science, Technology and Medicine, Prince Consort Road, London SW7 2BB, UK

INTRODUCTION

Over the past 10 years numerous laboratories working on CD44 have presented evidence that this abundant cell surface glycoprotein can act as a cell adhesion receptor, mediating both cell:cell and cell:matrix interactions. In the latter case the principal extracellular matrix ligand for CD44 is the glycosaminoglycan, hyaluronan (Aruffo et al., 1990; Culley et al., 1990; Lesley et al., 1990), although interaction with fibronectin, collagen I and laminin may also have a function in vivo (Faassen et al., 1992; Jalkanen and Jalkanen, 1992). The ability of CD44 to mediate cell:cell interactions (Belitsos et al., 1990; St. John et al., 1990; Bruynzeel et al., 1993) does not appear to result from a homotypic CD44:CD44 interaction but rather by an association with an unidentified heterotypic ligand. Currently, much effort has been expended in understanding how the specificity of these interactions is accomplished, given that CD44 is expressed on such a broad spectrum of cell types, both in the adult (Flanagan et al., 1989; Picker et al., 1989; Kennel et al., 1993) and in the embryo (Wheatley et al., 1993). The data suggest that regulation results from a number of different mechanisms, which include alternative splicing, variable glycosylation and modulation by the cytoplasmic domain. The first two mechanisms and discussions as to the functional role of CD44 have been covered extensively in three recent reviews (Herrlich et al., 1993; Knudson and Knudson, 1993; Lesley et al., 1993) and will only be briefly mentioned here. This Commentary will concentrate on the regulation of CD44 by its cytoplasmic domain.

STRUCTURE OF CD44

CD44 is a type I transmembrane glycoprotein comprising a 248 amino acid extracellular domain, a single spanning 23 amino acid transmembrane domain and a 70 amino acid intracellular domain (Stamenkovic et al., 1993). The extracellular domain can be modified by alternative splicing (reviewed by Herrlich et al., 1993; Lesley et al., 1993), whereby different combinations of 10 variant exons can be inserted into a single membrane-proximal site in the core CD44 (CD44H) molecule. Perhaps the most exciting result with CD44 in recent years has been the demonstration that alternatively spliced isoforms containing variant exon 6 have the capacity to confer a metastatic potential on non-metastatic cells (Günthert et al., 1991). Metastatic cells have an enhanced ability to migrate through basal lamina and extracellular matrix, enter and exit the circu-

lation and lodge in new tissue sites. All of these events are associated with changes in cell adhesion and motility but the mechanism by which the expression of variant exon 6 by CD44 alters such cell behaviour is not understood, or why this variant exon confers metastatic potential on cells when other variant exons do not. The extracellular domain of CD44 is subject to both N- and O-linked glycosylation and the variable addition of chondroitin sulphate side-chains. In addition, the alternatively spliced exons can be glycosylated and at least one variant isoform has been shown to contain heparan sulphate. Evidence has been provided that the addition of glycosaminoglycan side-chains is required for binding to non-hyaluronan extracellular matrix ligands such as fibronectin, collagen I and laminin, and this interaction appears to be spatially distinct from the interaction of CD44 with hyaluronan. Again, it is not known how the variation in CD44 glycosylation is specified.

The 23 amino acid transmembrane domain is encoded by exon 17 and is essentially invariant between the six CD44 species examined to date (Fig. 1). The 70 amino acid cytoplasmic domain shows an 80-90% homology between species where the less well conserved amino acids lie in two main regions (Ala309-Gly317 and Glu335-Phe343). The first three amino acids of the cytoplasmic tail (Arg292-Arg294) are encoded by exon 17 and the remaining 67 amino acids by exon 19 (Screaton et al., 1992). Goldstein et al. (1989) have reported the existence of a shortened CD44 isoform truncated at amino acid 294, which is generated by a 3′ untranslated region in exon 18 (Screaton et al., 1992). This short-tail isoform is detectable by the polymerase chain reaction but at a 100- to 200-fold lower abundance than the long-tail counterpart. Furthermore, it is unclear whether this short-tail isoform has a functional role in vivo (Goldstein and Butcher, 1990).

In the cytoplasmic domain of CD44 there are five conserved serine residues (with the exception of a threonine at amino acid 337 in the rat) that are candidates for a role in the observed serine phosphorylation of the human protein (Isacke et al., 1986; Carter and Wayner, 1988). Ser291 lies within a protein kinase C consensus site and Ser316 lies within a protein kinase A consensus site (Fig. 1). Surprisingly, mutagenesis of human CD44 has revealed that neither of these serine residues is phosphorylated and instead mutation of either Ser323 or Ser325 abrogates phosphorylation of the transfected human CD44 protein in Cos cells (Neame and Isacke, 1992). The presence of acidic amino acids in close proximity to these two serine residues is more typical of a casein kinase-2 consensus site.

Key words: CD44, hyaluronan receptor, cytoskeleton
though the ability of this kinase to phosphorylate CD44 either in vivo or in vitro has yet to be established.

An examination of the transmembrane and intracellular domain sequences does not reveal any known homologies with other proteins. Lokeshwar and Bourguignon (1992) have pointed out a similarity between CD44 and consensus sequences for the G-protein family. This homology is very limited and relies on the first of the less well conserved regions in the cytoplasmic tail. However, these authors have reported that CD44 can bind and hydrolyse GTP and they argue that this function may regulate the association with the cytoskeleton.

Fig. 1. Comparison of the amino acid sequence of the transmembrane and cytoplasmic domains of CD44. Amino acid numbers refer to those of the human sequence (Stamenkovic et al., 1989) where the initiating methionine is amino acid 1. The published sequences are as follows: human (Stamenkovic et al., 1989); baboon (Idzera et al., 1989); horse (Tavernor et al., 1993); rat (Gunthert et al., 1991); mouse (Nottenberg et al., 1989); hamster (Aruffo et al., 1990). Underlined sequences represent the transmembrane domain. In this diagram the boundary between the transmembrane and cytoplasmic domains is shown as between Ser291 and Arg292. Some predictions have placed the boundary between Val289 and Asn290. x represents a non-conserved amino acid change; + represents a conserved amino acid change.

LOCALIZATION OF CD44 AT THE PLASMA MEMBRANE

Early studies identified CD44 as a long-lived resident plasma membrane protein found on both the dorsal and ventral aspects of adherent cultured cells with what appeared to be a concentration on the microvillar projections, in areas of membrane ruffling and in dividing cells (Murphy et al., 1983; Jacobson et al., 1984a,b; see Fig. 2). Immunoelectron microscopy of fibroblasts reveals a relatively even distribution of CD44 on the plasma membrane including the membraneous projections but excluding the protein from clathrin-coated pits (Fig. 2B and C). This exclusion was first reported in 1980 by Bretschner and colleagues using a ferritin-conjugated second layer antibody to detect CD44 (H63 antigen in their studies) on 3T3 fibroblasts. In this experiment the distribution of CD44 within the coated pits was found to be approximately 100- to 200-fold less abundant than on the remainder of the cell surface. In sparse cultures of epithelial cells, it is noticeable that there is heavy distribution of CD44 on the microvillar projections and in areas of cell:cell contact (Fig. 2D), the latter phenomenon is not observed in fibroblastic cells (Jacobson et al., 1984a; Fig. 2A).

As epithelial cells polarize, the localization of CD44 to cell:cell contact areas increases such that, when a functionally polarized monolayer has formed, CD44 is only found on the lateral plasma membrane, and concomitantly there is a loss of protein from the apical microvillar surface (Fig. 2E; Neame and Isacke, 1993). In these cells no difference can be detected in the distribution of CD44H and alternatively spliced epithelial CD44 isoforms.

ASSOCIATION WITH THE CYTOSKELETON

Over the past 10 years there have been numerous reports that CD44 is associated with the cytoskeleton. The majority of these studies employed a detergent extraction protocol whereby cells are treated with non-ionic detergents such as Triton X-100 (TX-100) or Nonidet P-40. These experiments demonstrated that 30-80% of the CD44 population is associated with the detergent-insoluble cytoskeletal fraction in NIH3T3 fibroblasts (Carty et al., 1984), fibroblasts (Lacy and Underhill, 1987; Carter and Wayner, 1988; Neame and Isacke, 1993) and resident macrophages (Camp et al., 1991). Further evidence for such an association came from the mobility and antibody crosslinking studies of Jacobson et al. (1984a,b), who observed that CD44 exhibited a restricted lateral diffusion in fibroblasts, and that in motile cells the CD44 in the leading edge was more rapidly redistributed than protein in the trailing edge. Jacobson and colleagues proposed a model whereby CD44 associates with the cytoskeleton in a dynamic manner, with this association being weaker at the leading edge of the cell. This model is consistent with the observation that there is always a significant proportion of CD44 within the fibroblasts that is detergent soluble. Despite these numerous reports, the identity of a cytoskeletal protein associated with CD44 has been elusive. Biochemical analyses demonstrated that the amount of hyaluronan binding activity associated with the detergent-insoluble cytoskeletal fraction was proportional to the amount of actin retained, and that disruption of the actin filaments with cytochalasin B or DNase I resulted in increased detergent sol-
ubility while stabilization of the actin with phalloidin resulted in increased detergent insolubility (Lacy and Underhill, 1987; Camp et al., 1991). Others have taken the approach of assaying for CD44 function in cytochalasin-B-treated cells and this has revealed that CD44-mediated cell:cell interactions are abrogated if the cells are treated with cytochalasin B (Belitsos et al., 1990; Bruynzeel et al., 1993; Murakami et al., 1994) whereas CD44-mediated binding of hyaluronan is not (Murakami et al., 1994). Thus, although the role of actin in controlling CD44 function remains unclear, these studies provide some evidence that CD44-mediated cell:cell interactions and cell:matrix interactions are independently regulated. Immunofluorescence examination of detergent-extracted cells has failed to show co-localization with actin filaments, intermediate filaments or focal contacts; rather, the detergent-insoluble CD44 is found in a reticular network (Carter and Wayner, 1988; Neame and Isacke, 1993), suggesting an association with the submembraneous cortical cytoskeleton. These observations are consistent with those of Kalomiris and Bourguignon (1988) and Bourguignon et al. (1993), who have observed a co-localization of CD44 and ankyrin after antibody- or hyaluronan-induced CD44 capping and have demonstrated that CD44 can bind to ankyrin in vitro. Together, these experiments suggest a preliminary model for CD44 in the plasma membrane as shown in Fig. 3A.

Studies on the association of CD44 with the cytoskeleton have been further complicated by the demonstration that the ability of CD44 to remain associated with the cytoskeletal fraction after detergent extraction is dependent upon cell type. This was first observed in studies by Camp et al. (1991), who showed that in resident macrophages there was a detergent-insoluble CD44 fraction, but in elicited macrophages all of the CD44 was detergent-soluble. Similarly, a detergent-insoluble CD44 population was observed in human and murine fibroblasts but not in MDCK epithelial cells (Neame and Isacke, 1993). These experiments have now been extended to include a wide variety of cultured cell types from a number of different species and it is found that the ability of CD44 to be retained in the cells following detergent extraction is a property of fibroblastic (both normal and virally transformed) but not of epithelioid or lymphoma cells (Neame and Isacke, unpublished data). These studies lend weight to the suggestion proposed by

---

**Fig. 2.** Localization of CD44 at the plasma membrane. Cells were subject to immunofluorescence or immunoelectron microscopy as described by Neame and Isacke (1993) and Isacke et al. (1990). (A) AG1523 human diploid fibroblasts; (D) sparse MDCK cells expressing human CD44H grown on coverslips; (E) confluent polarized MDCK cells expressing human CD44H grown on Transwell filters; cells were fixed, permeabilized and incubated with anti-human CD44 mAb E1/2 followed by rhodamine-conjugated anti-mouse Ig. Cells in A were visualized by conventional microscopy, cells in D and E were visualized by confocal microscopy where *xz* sections were taken in 0.1 µm steps through the cells at 90° to the plane of the coverslip or filter. Bar, 5 µm. (B and C) AG1523 human fibroblasts were fixed, stained with mAb E1/2 followed by anti-mouse Ig conjugated to 8 nm gold particles, sectioned and visualized in the electron microscope. Bar, 0.1 µm.
Camp et al. (1991) that an association of CD44 with the cytoskeleton, or at least its ability to be retained in the detergent-insoluble cytoskeletal fraction, may influence or be influenced by cell mobility.

MODULATION OF CD44 ACTIVITY BY THE CYTOPLASMIC DOMAIN

In common with other adhesion proteins, it would be expected that the cytoplasmic domain would have an important role in modulating CD44 function in terms of ligand binding, cell adhesion and cell motility. To examine this, several laboratories have taken the direct approach of creating mutations in the cytoplasmic domain and comparing mutant protein to wild-type CD44 in transfected cells. Such studies have revealed that removal of the cytoplasmic tail: (a) abolishes the ability of transfected AKR1 cells or phorbol ester-stimulated Jurkat T cells to bind soluble hyaluronan; (b) reduces the ability of transfected AKR1 cells or RPM-MC melanoma cells to bind to a hyaluronan-coated substratum; and (c) abolishes the hyaluronan-mediated migration of melanoma cells (Lesley et al., 1992; Thomas et al., 1992; Liao et al., 1993). Further mutation of the cytoplasmic tail has revealed that efficient hyaluronan binding requires cytoplasmic amino acids 310-330 (Liao et al., 1993). Detailed studies of the ability of lymphoid cells and lymphoma cell lines to bind hyaluronan have demonstrated that CD44 expressing cells can exist in three states: (1) cells that express CD44 but do not bind hyaluronan and cannot be induced to do so; (2) cells that express CD44, do not bind hyaluronan but can be rapidly induced to do so. In vitro, this activation can be accomplished by phorbol ester treatment or by incubation with an activating antibody; and (3) cells that express CD44 and constitutively bind hyaluronan (reviewed by Lesley et al., 1993). The hyaluronan binding capacity of AKR1 cells transfected with the tailless CD44 molecule can be restored after treatment with activating antibody (Lesley et al., 1992), suggesting that the cytoplasmic domain can regulate the switching of CD44 between hyaluronan binding state (2) and state (3). In addition, a number of laboratories have found that a human epithelial CD44 isoform containing variant exons 8, 9 and 10 has a reduced ability to bind hyaluronan (Stamenkovic et al., 1991; Thomas et al., 1992; Liao et al., 1993) and, where examined, this binding can again be restored by
addition of an activating antibody (Liao et al., 1993), indicating that the insertion of alternatively spliced exons may also regulate the CD44 binding state. However, these studies have been done using a single CD44 isoform and contradictory results have been obtained using four different murine alternatively spliced CD44 isoforms where all the isoforms constitutively bind hyaluronan (He et al., 1992). The role of the cytoplasmic domain in this process could be to regulate either the presentation and/or conformational state of CD44 at the cell surface or the interaction with intracellular components to mediate cell signalling and/or motility subsequent to ligand binding.

To address these possibilities, the cell surface localization and interaction with the cytoskeleton of truncated CD44 molecules has been examined. In polarized epithelial cells, CD44 is tightly restricted to the lateral plasma membrane. Removal of the cytoplasmic tail does not prevent presentation of the molecule at the plasma membrane but does result in an altered localization, in that the tailless CD44 is expressed on the apical rather than the lateral cell surface. Furthermore, a population of tailless protein is found in small vesicles within the cell, and in these cells removal of the cytoplasmic domain reduces the stability of the protein. This suggests that the pool of CD44 observed within the cell represents tailless CD44 that will subsequently be degraded in the lysosome (Neame and Isacke, 1993). Thus in these cells the cytoplasmic tail obviously has an important role in the presentation and stability of CD44 at the correct plasma membrane domain. This function of the tail is not dependent upon phosphorylation of the protein, as mutation of Ser323 and/or Ser325 does not alter the half-life or distribution of CD44 in epithelial cells (Neame and Isacke, 1992). In fibroblasts, sparse epithelial cultures or lymphoma cells, the difference in distribution between the wild-type and tailless proteins is less obvious; however, closer examination reveals that the tailless protein does not localize as strongly to the microvillar projections as has been observed for the wild-type protein (see Fig. 2A; Isacke et al., unpublished observations). Further studies are required to determine whether there is a common mechanism that mediates: (a) the localization in both fibroblasts and epithelial cells; (b) the stability of the protein; and (c) efficient ligand binding. What is clear is that any potential cytoplasmic amino acid sequence required for these functions is spatially separate from sequences required to retain the protein in the detergent-insoluble fibroblast cytoskeletal fraction, because when the CD44 cytoplasmic domain is removed there is no change in the TX-100 detergent extraction profile and no change in the distribution of the detergent-insoluble protein (Neame and Isacke, 1993). Recently, Perschl and colleagues have generated chimeric CD44 molecules whereby the CD44 transmembrane domain is substituted for by the transmembrane domain of either the T cell receptor ζ chain or CD45. Unlike the wild-type and tailless proteins, when these chimeric CD44 molecules are expressed in fibroblasts they are completely TX-100-soluble. This demonstrates a role for the transmembrane domain rather than cytoplasmic domain in retaining CD44 in the cytoskeletal fraction (Perschl et al., unpublished data). One explanation for these experiments is that there is a linker protein that interacts with the transmembrane domain of CD44, mediating an indirect association of CD44 with the cytoskeleton. The absence of the linker molecule in epithelial cells and leukocytes would explain why CD44 is detergent-soluble in these cells. Alternatively, as demonstrated by Brown and Rose (1992), TX-100 insolubility can result from protein:lipid interactions and therefore it remains a possibility that differences in lipid components between the different cell types could determine whether there is a detergent-insoluble interaction between the CD44 transmembrane domain and the plasma membrane. These different models for how CD44 might associate with the cytoskeleton are illustrated in Fig. 3. Whatever the mechanism that retains CD44 in a TX-100-insoluble fraction might be, this association is not required for CD44 to bind hyaluronan as in transfected AKR1 cells the CD44 population is entirely TX-100-soluble (Neame and Isacke; and Lesley et al., unpublished observations) and yet these cells bind soluble hyaluronan and to hyaluronan-coated dishes with high efficiency (Lesley et al., 1992).

Little is known about how CD44 might mediate hyaluronan-independent cell:cell interactions. Studies have been concentrated on the ability of CD44 to cause aggregation of non-adherent cells and as yet CD44 cytoplasmic domain mutants have not been tested in such assays. Practical difficulties are encountered in analysing the role of CD44 in the interaction of epithelial cells due to the high levels of endogenous expression in all cultured epithelial lines examined to date. Thus it remains to be determined whether a common mechanism regulates CD44-mediated cell:cell and cell:matrix interactions.

**COMPARISON WITH OTHER ADHESION PROTEINS**

Current models concerning the mechanisms that regulate the interaction of adhesion proteins with their ligands involves the concepts of inside-out and outside-in signalling. In inside-out signalling, intracellular events such as the stimulation of protein kinase C signalling pathways can result in an altered affinity for ligand. Conversely, ligand binding initiates outside-in signalling that results in changes in gene expression, cell proliferation and cytoskeletal reorganization. In the case of the integrins there is compelling evidence that both modes of signalling can be mediated by the cytoplasmic domains (reviewed by Sastry and Horwitz, 1993). CD44 shares some properties with the integrin receptors, in that ligand binding can be modulated by the activation of protein kinase C pathways or mimicked by the use of activating antibodies directed against the extracellular domain. In both cases, deletion or mutation of the intracellular domain alters ligand binding affinities, which may be due to the requirement for the cytoplasmic domain in the correct conformational presentation of the molecule at the cell surface. With respect to outside-in signalling, it is clear that many adhesion proteins interact directly with intracellular components (reviewed by Tsukita et al., 1992; Sastry and Horwitz, 1993; Stappert and Kemler, 1993). The best characterized of these is the association of the cytoplasmic domain of cadherin glycoproteins with the intracellular catenins, which in turn are associated with the actin cytoskeleton. Removal of the cadherin cytoplasmic tail abolishes the ability of this molecule to mediate homotypic cell:cell binding despite the fact that it is still presented on the cell surface. Pertinent to the topics under discussion here, it is interesting that in a similar manner to CD44, approximately 50% of wild-type E-cadherin is found in the detergent-insoluble cytoskeletal fraction. In
contrast to CD44, removal of the E-cadherin cytoplasmic tail results in complete detergent solubility (Nagafuchi and Takeichi, 1988). As previously discussed, there is increasing evidence that, unlike the cadherins, the integrins and members of the immunoglobulin superfamily, the cytoplasmic domain of CD44 does not directly interact with the underlying cytoskeleton. Interestingly, this phenomenon is not restricted to the CD44 protein, as recent detergent extraction studies on syndecan-1 have demonstrated that the ability of this protein to associate with the cytoskeletal fraction is not affected by removal of the cytoplasmic tail. In contrast to CD44, the retention of syndecan-1 in the TX-100-insoluble fraction is dependent upon the heparan sulphate side-chains, suggesting an involvement of the extracellular domain in the association with the cytoskeletal fraction (Miettinen and Jalkanen, 1994).

CONCLUSIONS

This review has focused on the role that the cytoplasmic domain plays in regulating CD44. To date, the best-characterized function of CD44 is its ability to act as a hyaluronan receptor. Binding of hyaluronan requires the presence of at least part of the cytoplasmic domain and therefore this region of the protein must have a role in inside-out signalling. However, it has yet to be determined how this signalling is regulated and whether the same mechanisms are employed to regulate the binding of CD44 to non-hyaluronan extracellular matrix ligands and in CD44-mediated cell:cell interactions. One possibility is that the cytoplasmic domain is required for the presentation of the molecule during transport through the endoplasmic reticulum and the Golgi apparatus, and that regulation of these events could result in the observed variation in CD44 glycosylation. By contrast, there is increasing evidence that at least one aspect of outside-in signalling, namely an association with the cytoskeletal fraction, is independent of the cytoplasmic domain and instead involves the highly conserved transmembrane domain. It has yet to be determined whether other outside-in signalling events such as the recently reported CD44-mediated increases in intracellular calcium (Bourguignon et al., 1993) and cAMP (Rothman et al., 1993) result from a direct or indirect interaction with intracellular components. Finally, the observation that in different cell types there is variation in CD44 glycosylation, splicing, TX-100 solubility, plasma membrane localization and hyaluronan binding capacity suggests that this protein may be subject to different regulatory mechanisms depending on its required function in particular situations.

I thank Astrid Perschl and colleagues, Heini Miettinen and Markku Jalkanen and members of my laboratory for allowing me to discuss their unpublished data; and Robert Hyman, Jayne Lesley, Markku Jalkanen, David Simmons, Stephen Neame, Humma Sheik and Susan Wheatley for their comments on the manuscript. The immunoelectron micrographs shown in Fig. 2 were kindly provided by Colin R. Hopkins, University College London. This work was funded by grants from the Cancer Research Campaign and from the Medical Research Council.

REFERENCES


Jacobson, K., O’Dell, D., Hoffi eld, B., Murphy, T. L. and August, J. T. 2358 C. M. Isacke
CD44 cytoplasmic domain


Neame, S. J. and Isacke, C. M. (1992). Phosphorylation of CD44 in vivo requires both Ser323 and Ser 325, but does not regulate membrane localization or cytoskeletal interaction in epithelial cells. EMBO J. 11, 4733-4738.


