Journal of Cell Science 108, 3127-3135 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

CD44 exhibits a cell type dependent interaction with Triton X-100 insoluble, lipid rich, plasma membrane domains

Stephen J. Neame*, Caroline R. Uff, Humma Sheikh, Susan C. Wheatley and Clare M. Isacke†

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

*Present address: Eisai London Research Laboratories Ltd, Bernard Katz Building, University College London, Gower Street, London WC1E 6BT, UK
†Author for correspondence (e-mail: c.isacke@ic.ac.uk)

SUMMARY

CD44 is an abundant, widely expressed transmembrane glycoprotein which can act as a receptor for the extracellular matrix glycosaminoglycan, hyaluronan. Biochemical and morphological studies have demonstrated that in fibroblasts a significant proportion of the CD44 population is resistant to Triton X-100 extraction and that the detergent insoluble protein is co-localized with components of the cortical cytoskeleton. Surprisingly, this distribution is not abrogated upon deletion of the CD44 cytoplasmic tail indicating that mechanisms other than a direct interaction with the cytoskeleton can regulate CD44. In this manuscript, the mechanisms underlying this detergent-insoluble association are further investigated. There was no evidence that the Triton X-100 insolubility of CD44 resulted from homotypic aggregation, an association with hyaluronan or from a direct, or indirect, association with the cytoskeleton. Instead, evidence is presented that the detergent insolubility of fibroblast CD44 at 4°C results from an association of the CD44 transmembrane domain with Triton X-100 resistant, lipid rich, plasma membrane domains. The proportion of the CD44 found in these Triton X-100 insoluble structures is dependent upon cell type and cannot be altered by changing cell motility or extracellular matrix associations. These studies provide evidence for a novel mechanism regulating this adhesion protein in the plasma membrane.

Key words: CD44, detergent solubility, transmembrane domain, plasma membrane

INTRODUCTION

Currently there is intense interest in the mechanisms by which adhesion proteins are involved in both outside-in signalling, in which interactions of the adhesion protein with the extracellular milieu influence intracellular events, and inside-out signalling in which intracellular events leads to modulation of the adhesion protein extracellular activity. CD44 is a hyaluronan receptor which has been implicated to play a role in a variety of physiological processes including cell adhesion, cell migration, developmental tissue morphogenesis and metastatic spread (reviewed by Lesley et al., 1993; Herrlich et al., 1993; Knudson and Knudson, 1993). Recent studies on the regulation of CD44 have begun to elucidate how this single gene is involved in such a diverse range of cellular events.

Structural modification of CD44 occurs at the level of alternative splicing whereby up to 10 variant exons can be inserted in multiple combinations into a single site in the extracellular domain (reviewed by Lesley et al., 1993; Milde et al., 1994b). It has yet to be determined how alternative splicing modifies CD44 function, although at least in some cases, the expression of these additional exons confers a metastatic phenotype to non-metastatic cells (Günthert et al., 1991; Rudy et al., 1993). Alternative splicing is cell type dependent in that the majority of lymphoid and fibroblast lines constitutively express the 80-100 kDa haematopoietic form of CD44 (CD44H) which does not contain any alternatively spliced exons while many epithelial cells, tumours and activated lymphocytes express one or more higher molecular mass CD44 isoforms (Arch et al., 1992; Koopman et al., 1993; Mackay et al., 1994; Jackson et al., 1995). Both the core CD44 protein and the alternatively spliced exons are also subject to N- and O-linked glycosylation and the variable addition of glycosaminoglycan side chains (Camp et al., 1991; Jalkanen et al., 1988; Faassen et al., 1992; Haggerty et al., 1992; Jackson et al., 1995). Again, the extent of this glycosylation is cell type dependent (Camp et al., 1991) and these modifications can regulate function, for example by increasing the affinity for non-hyaluronan ligands such as collagen and fibronectin (Faassen et al., 1992; Jalkanen and Jalkanen, 1992) and heparin-binding growth factors (Bennett et al., 1995). It has yet to be determined whether such variation has a role to play in the observed cell type dependence of the CD44H isoform to act as a hyaluronan receptor (Lesley et al., 1990; Liao et al., 1993; Lesley et al., 1993; Murakami et al., 1994).

The highly conserved 71 amino acid CD44 cytoplasmic domain (reviewed by Isacke, 1994; Milde et al., 1994a) has also been implicated in the regulation of CD44 function. For example, truncated tailless molecules have a reduced or abolished ability to bind hyaluronan (Lesley et al., 1992;

Thomas et al., 1992; Liao et al., 1993; Lokeshwar et al., 1994; Perschl et al., 1995b). Early data suggested that between 30-70% of the CD44 was associated with the intracellular cytoskeleton. Such conclusions were drawn from classic non-ionic detergent extraction assays and from membrane mobility studies (Jacobson et al., 1984a,b; Tarone et al., 1984; Lacy and Underhill, 1987; Carter and Wayner, 1988; Camp et al., 1991). However, attempts to identify a cytoskeletal component which interacts with the cytoplasmic tail and modulates ligand binding have produced contradictory data with reports of an association of CD44 with actin filaments (Lacy and Underhill, 1987), ankyrin (Kalomiris and Bourguignon, 1988; Lokeshwar and Bourguignon, 1991; Lokeshwar et al., 1994) and members of the ERM (ezrin, radixin, moesin) family (Tsukita et al., 1994). Finally, evidence for a mechanism(s) other than, or in addition to, a direct interaction with the cytoskeleton which regulates CD44 function comes from further mutation analysis and detergent solubility studies. Perschl and colleagues (1995b) have generated a series of CD44 cytoplasmic deletion constructs and demonstrated that efficient hyaluronan binding requires a minimal 14 amino acid cytoplasmic domain but not a specific cytoplasmic domain sequence. Truncation of the CD44 cytoplasmic domain does not increase the amount of CD44 extracted into a Triton X-100 soluble phase nor alter the cellular distribution of the Triton X-100 insoluble protein (Neame and Isacke, 1993). This manuscript details further investigations into the nature of this cytoplasmic tail-independent detergent insolubility of CD44.

MATERIALS AND METHODS

Reagents

The human specific anti-CD44 mAb, E1/2, the species cross reactive anti-CD44 mAb, IM7, and the anti-annexin II rabbit polyclonal antiserum have been described previously (Isacke et al., 1986; Trowbridge et al., 1982; Cooper and Hunter, 1983). mAb D1/93 raised against human CD44 (Isacke et al., 1986) cross reacts with monkey CD44. HRP-conjugated anti-mouse and anti-rat Ig antisera were purchased from Jackson Immunoresearch. Rhodamine-conjugated rabbit anti-mouse and FITC-conjugated rabbit anti-rat Ig antisera were purchased from Dako laboratories and Vector Laboratories, respectively. Cytochalasin B and nocodazole (Sigma) were made up as 10 mg/ml solutions in DMSO. Heparan sulphate, chondroitin sulphate and phalloidin (Sigma) and *Streptococcus* hyaluronan (Calbiochem) were made up at 5 mg/ml in water. Purified murine recombinant scatter factor was a generous gift from E. Gherardi (Department Medicine, Addenbrooke's Hospital, Cambridge). To obtain MRC-5 cell conditioned medium, cells were cultured to approximately 70% confluence in DME plus 10% FCS, the medium was removed and replaced with DME alone and incubated for 3 days. The medium was then collected and filtered through a 0.2 μm filter. Conditioned medium refers to MRC-5 medium mixed 1:1 with DME and supplemented with 10% FCS.

Cell culture

All cell lines were maintained in DME supplemented with 10% FCS. The generation of wild-type (WT) and tailless (T–) human CD44 and the expression of these proteins in MDCK and Swiss 3T3 cells has been previously described (Neame and Isacke, 1992, 1993). To culture cells on hyaluronan coated substratum, plates were coated for 2 hours at 37°C with 5 mg/ml hyaluronan, washed three times with DME and then cells added immediately. For culture in semi-solid medium, cells were resuspended in pre-swollen methylcellulose (27,4410-0, Aldrich) at a final density of 10^5 cells/ml and plated into 10 cm Petri dishes. After incubation for 24 or 48 hours, the methylcellulose was diluted in 50 ml of PBS, the cells recovered by centrifugation at 1,000 ***g*** for 15 minutes and then subjected to detergent extraction.

To induce CD44 patching, clonal lines of Swiss 3T3 expressing WT or T– human CD44 were cultured on glass coverslips for 24 hours. Cells were then washed in ice-cold DME buffer (DME containing 10 mM HEPES, 0.1% BSA, 10% FCS) and incubated on ice in DME buffer containing a 1:100 dilution of mAb E1/2 ascites preparation for 45 minutes. Cells were then washed for 15 minutes and incubated on ice with DME buffer containing 10 μg/ml rhodamine-conjugated anti-mouse Ig. Cells were washed in DME buffer and then incubated in DME buffer for 30 minutes on ice or at 37°C. Cells were then fixed in 3% paraformaldehyde, stained with mAb IM7 hybridoma supernatant diluted 1:3 in DME buffer supplemented with 10% rabbit serum and 10% mouse serum followed by FITC-conjugated anti-rat Ig diluted to 10 μg/ml in DME buffer supplemented with 10% rabbit serum and viewed using a Nikon Optiphot microscope in conjunction with a Bio-Rad MRC600 confocal laser scanning unit which allows simultaneous collection of the FITC and rhodamine images.

Detergent extraction of cells

Unless otherwise stated, cells were washed with ice-cold Tris saline (15 mM Tris-HCl, pH 7.5, 120 mM NaCl) and 200 or 400 μl of extraction buffer (150 mM NaCl, 15 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$) containing 0.4% or 0.5% Triton X-100 was added. The cells were scraped into Eppendorf tubes and kept on ice for 15 minutes before centrifugation at 15,000 ***g*** in an Eppendorf microfuge to separate detergent soluble from detergent insoluble material. The detergent insoluble pellet was resuspended in an equal volume of extraction buffer, both samples sonicated for 10 seconds at 18 amplitude microns, 40 or 80 μl of 6× non-reducing sample buffer was added and the samples heated to 90°C for 3 minutes. Aliquots (5 μl) of each sample were resolved on 10% mini-SDS-polyacrylamide gels and then blotted onto nitrocellulose membrane (Hybond C extra; Amersham). Detection of CD44 was as described previously (Neame and Isacke, 1993) and the blots developed using the enhanced chemiluminescence (ECL) kit (Amersham) and exposure to X-ray film (Fuji XR) at room temperature.

RESULTS

Mechanism to retain CD44 in a fibroblast Triton X-100 insoluble fraction

We have previously demonstrated that removal of the CD44 cytoplasmic tail does not increase the amount of this protein released into the Triton X-100 soluble fraction (Neame and Isacke, 1993; see Fig. 3) leading to the suggestion that any association of CD44 with the detergent-insoluble cytoskeleton must be indirect. One possibility is that since in these experiments wild-type (WT) and tailless (T–) human CD44 are expressed in rodent fibroblasts which already contain endogenous CD44, the inability of T– CD44 to be released into the detergent soluble phase could result from an association with endogenous WT protein which in turn is directly associated with the cytoskeleton. To address this, clonal cell lines of Swiss 3T3 cells expressing transfected WT or T– human CD44 were induced to patch by the addition of a human specific anti-CD44 mAb followed by a polyclonal second layer Ab. When the cells are kept at 4°C, patching is inhibited and there is a complete co-localization of transfected and endogenous CD44

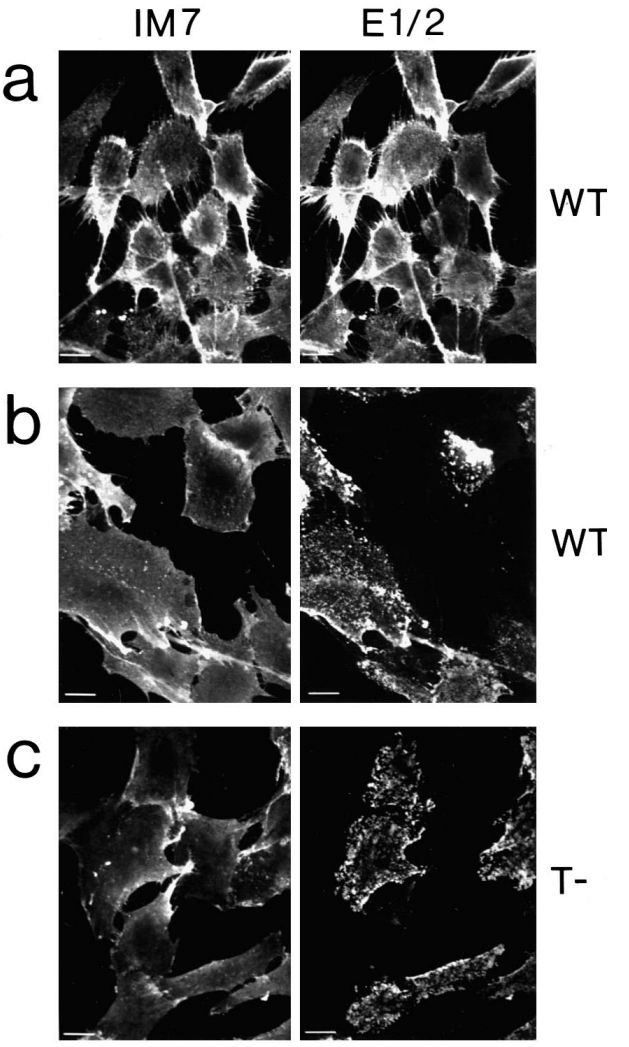

Fig. 1. Transfected CD44 does not associate with endogenous CD44. Swiss 3T3 cells expressing WT (a,b) or T– (c) human CD44 were incubated with mAb E1/2 and a rhodamine-conjugated anti-mouse Ig. Cells were then incubated at 4°C (a) or 37°C (b,c) for 30 minutes, fixed and stained with mAb IM7 and a FITC-conjugated anti-rat Ig. Cells were viewed in the confocal microscope. Bars, 20 μm.

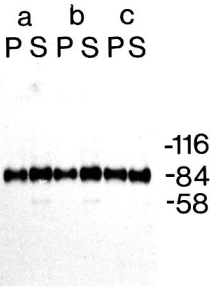

Fig. 2. Retention of CD44 in the fibroblast detergent insoluble fraction is not dependent on an association of CD44 with hyaluronan. Semi-confluent cultures of F0184 fibroblasts were treated for 30 minutes with 0 (a), 750 (b) or 5,000 U (c) bovine testicular hyaluronidase (Calbiochem). Cells were then rinsed with PBS and extracted with 0.5% Triton X-100 for 15 minutes on ice. The distribution of CD44 between the detergent insoluble pellet (P) and soluble supernatant (S) was assessed by immunoblotting using mAb E1/2 followed by HRP-anti-mouse Ig. Blots were developed using the ECL reagent and exposed to film for 20 seconds. Molecular size markers are in kDa.

(Fig. 1a). When parallel cultures are incubated at 37°C, the majority of transfected WT or T– human CD44 is found patched on the cell surface whereas there is little movement of the endogenous murine CD44 (Fig. 1b and c). In these cultures the minor amount of overlap in antibody staining is not sufficient to account for the approximately 50% of transfected human CD44 which is resistant to Triton X-100 extraction. Parallel experiments were conducted using human CD44 transfected BW5147 T lymphoma cells. BW5147 cells express high levels of endogenous murine CD44 of which >95% is soluble in 0.5% Triton X-100 (see Fig. 8). In these cells there is no evidence for co-localization of either WT or T– transfected CD44 with endogenous CD44 after incubation at 37°C (data not shown). These experiments indicate that CD44 does not form multimeric homotypic complexes in the membrane.

A second explanation for the retention of CD44 in the detergent-insoluble fibroblast fraction is through the binding of extracellular matrix components by the CD44 extracellular domain. It is well established that the CD44 ligand, hyaluronan, is synthesised and deposited by fibroblasts in culture (Chen et al., 1989; Heldin et al., 1989) raising the possibility that aggregation of CD44 with hyaluronan gives rise to detergent-insoluble CD44:hyaluronan complexes. In order to address this, cultures of human fibroblasts were treated with hyaluronidase which removes all cell associated hyaluronan as detected with a bovine proteoglycan probe (Green et al., 1988; data not shown). Prior removal of this major CD44 ligand (Fig. 2) or inclusion of hyaluronan in the extraction buffer (Fig. 4) had no significant effect on Triton X-100 solubility of CD44. Recently it has been demonstrated that the detergent insolubility of another transmembrane glycoprotein, syndecan-1, is mediated by its heparan sulphate side chains and that inclusion of heparan sulphate in the detergent extraction buffer increases the Triton X-100 solubility (Miettinen and Jalkanen, 1994). In similar experiments, no increased CD44 solubility was detected when either heparan sulphate or chondroitin sulphate were included in the detergent extraction buffer (Fig. 4).

If fibroblast CD44 is associated with the cytoskeleton, whether directly or indirectly via a linker protein, disruption of the cytoskeleton might be expected to increase the detergent solubility. Lacy and Underhill (1987) demonstrated that cytochalasin B treatment reduced the levels of hyaluronan bound by detergent extracted cells. It was suggested that this was due to a disruption of an actin:CD44 interaction leading to increased levels of detergent soluble CD44. Treatment of fibroblasts with this drug causes the cells to radically alter their morphology such that the cytoplasm retracts around the nucleus leaving branched processes radiating from this central mass (data not shown; Morris and Tannenbaum, 1980). When cytochalasin B treated and untreated cells are extracted with Triton X-100 no change in the detergent solubility of endogenous CD44 or of WT and T– transfected CD44 is observed (Fig. 3). Similar results have recently been reported using cytochalasin D (Perschl et al., 1995a). As CD44 was not examined directly in the original studies (Lacy and Underhill, 1987) this discrepancy in results may well be due to the fact that the redistribution of CD44 after cytochalasin treatment reduces its hyaluronan binding capacity rather than its detergent solubility. Stabilization of the actin network by culturing F1084 fibroblasts (Fig. 4), Swiss 3T3 fibroblasts or HT-29 cells (data not shown) in the presence of phalloidin

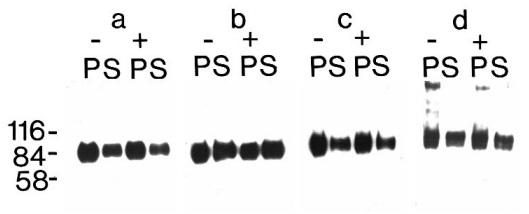

Fig. 3. Disruption of the actin cytoskeleton does not alter the detergent extraction profile of CD44. F0184 human fibroblasts (a), Swiss 3T3 cells expressing WT CD44 (b), T– CD44 (c) and Swiss 3T3 cells transfected with the pSRα vector alone (d) were cultured for 24 hours and then for a further 3 hours with 10 μl/ml DMSO (–) or 10 μg/ml cytochalasin B (+). Cells were then extracted with 0.4% Triton X-100 for 15 minutes on ice and the distribution of CD44 between the detergent insoluble cell pellet (P) and soluble supernatant (S) was assayed by immunoblotting using mAb E1/2 followed by an HRP-conjugated anti-mouse Ig (a to c) or mAb IM7 followed by an HRP-conjugated anti-rat Ig (d). Blots were developed using the ECL reagent and exposed to X-ray film for 10 minutes. Molecular size markers are in kDa.

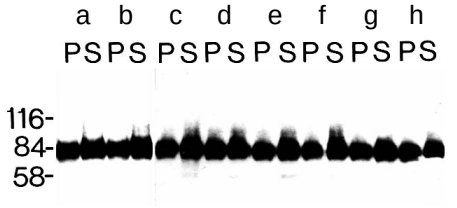

Fig. 4. Solubilization of CD44 from fibroblasts under altered culture and extraction conditions. Semi-confluent dishes of F1084 fibroblasts cultured for 48 hours were then left untreated (a,d-h) or treated for 3 hours with 25 μM phalloidin (b) or 20 μM nocodazole (c). Cells were then extracted for 15 minutes on ice in extraction buffer containing 0.5% Triton X-100 containing no additional additives (a-c), 25 μM phalloidin (d), 20 μM nocodazole (e), 0.5 mg/ml hyaluronan (f), 0.5 mg/ml chondroitin sulphate (g) or 0.5 mg/ml heparan sulphate (h). The distribution of CD44 between the detergent insoluble cell pellet (P) and soluble supernatant (S) was assayed by immunoblotting with mAb IM7 followed by an HRP-conjugated anti-rat Ig. Blots were developed using the ECL reagent and exposed to X-ray film for 1 minute. Molecular size markers are in kDa.

again had no effect on the solubility of CD44, nor did nocodazole-induced disruption of microtubules (Fig. 4).

Together these studies indicate that the ability of CD44 to be retained in the detergent insoluble cytoskeletal fraction of fibroblasts at 4°C does not result from: (a) a direct interaction of the cytoplasmic domain with intracellular components; (b) the formation of multimeric homotypic complexes in the plasma membrane; (c) an association of the extracellular domain with its major characterized ligand, hyaluronan; or (d) an indirect interaction with microfilaments or microtubules.

Finally, to determine whether the highly conserved transmembrane domain has a role in regulating CD44 solubility, F1084 or Swiss 3T3 fibroblasts were subjected to a number of different extraction conditions (Fig. 5). As previously reported (Neame and Isacke, 1993), extraction with Triton X-100 at 4°C results in approximately 50% of the CD44 being detergent soluble and 50% being retained with the insoluble cell matrix.

Table 1. The association of CD44 with Triton X-100 detergent insoluble material is cell type dependent

	Cell line	Species	TX-100 soluble	TX-100 insoluble	Cell type
A	Swiss 3T3	m	++	++	Fibroblast
	NIH-3T3	m	++	++	Fibroblast
	SM-3T3	m	++	++	v-Fms transformed fibroblast
	P19-DMSO*	m	++	++	Differentiated embryonal carcinoma
	NRK	r	++	++	Fibroblast
	PrC-NRK	r	++	++	v-Src transformed fibroblast
	Flow 2000	h	++	++	Diploid fibroblast
	F1084	h	++	++	Diploid fibroblast
	AG1523	h	++	++	Diploid fibroblast
	MRC-5	h	+++	+	Diploid fibroblast
	HT1080	h	+++	+	Fibrosarcoma
	MG63	h	+++	+	Osteosarcoma
	HeLa	h	+++	+	Epitheloid cervical carcinoma
B	HC11	m	++++	–	Mammary epithelial
	410.4	m	++++	–	Mammary epithelial
	C57MG	m	++++	–	Mammary epithelial
	HEp2	h	++++	–	Epidermoid laryngeal carcinoma
	HT-29	h	++++	–	Colonic adenocarcinoma
	A431	h	++++	–	Epidermoid carcinoma
	MDCK	c	++++	–	Madin-Darby canine kidney epithelia
	MDBK	b	++++	–	Madin-Darby bovine kidney epithelia
	COS-1	mo	++++	–	SV40 T antigen expressing fibroblast
C	BW5147	m	++++	–	T lymphoma
	AKR1-tf	m	++++	–	T lymphoma expressing human CD44
D	P19	m	–	–	Embryonal carcinoma
	AKR1	m	–	–	T lymphoma
	HepG2	h	–	–	Hepatoma

Semi-confluent cell cultures were extracted on ice for 15 minutes in buffer containing 0.5% Triton X-100 and the proportion of CD44 in the detergent insoluble and soluble fractions assessed by immunoblotting. Rat (r) cells were probed with an anti-rat CD44 mAb, OX50, and monkey (mo) cells were probed with mAb D1/93 (Isacke et al., 1986) followed by an HRP-anti-mouse Ig. Human (h), mouse (m), bovine (b) and canine (c) cells were probed with mAb IM7 followed by an HRP-anti-rat Ig. Blots were developed with the ECL reagent and exposed to X-ray film for 20-120 seconds. ++++, greater than 90%; +++, 60-90%; ++, 40-60%; +, 10-40%; –, less than 10%. *P19 cells were induced to differentiate by the addition of DMSO. Undifferentiated P19 embryonal carcinoma cells do not express CD44. After differentiation in the presence of DMSO, CD44 positive fibroblastic cells can be identified (Wheatley and Isacke, 1995).

Under these conditions greater than 95% of the transferrin receptor is extracted indicating essentially complete solubilization of plasma and endosomal membranes. No change in CD44 solubility is observed when higher concentrations of sodium chloride are present in the extraction buffer, if Ca^{2+} and Mg^{2+} are removed or if Triton X-114 substitutes for Triton X-100. By contrast, treatment of the cells with either Triton X-100 or Triton X-114 at 37°C or with *n*-octylglucoside at 4°C results in greater than 95% of the CD44 being sequestered into the detergent soluble pool. Comparable results were obtained with Swiss 3T3 fibroblasts (Fig. 5b) and with transfected WT and T– CD44 (data not shown). The behaviour of CD44 in these different detergent extraction conditions is reminiscent of

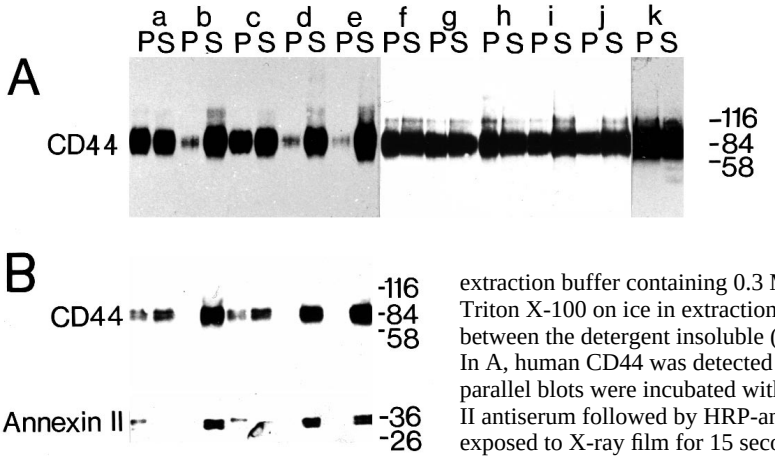

Fig. 5. Solubility of fibroblast CD44 is dependent upon detergent and temperature but not salt concentration or the presence of Ca^{2+}: 1×10^6 F1084 fibroblasts (A) or Swiss 3T3 fibroblasts (B) were cultured in 6 cm plates for 48 hours. Cells were subjected to detergent extraction for 20 minutes under the following conditions; 0.5% Triton X-100 on ice (a), 0.5% Triton X-100 at 37°C (b), 0.5% Triton X-114 on ice (c), 0.5% Triton X-114 at 37°C (d), 60 mM *n*-octylglucoside on ice (e), 0.5% Triton X-100 on ice in extraction buffer containing 0.3 M (f), 0.6 M (g), 1.2 M (h), 1.8 M (i) or 2.4 M (j) NaCl, 0.5% Triton X-100 on ice in extraction buffer without Ca^{2+} or Mg^{2+} (k). The distribution of CD44 between the detergent insoluble (P) and soluble (S) fractions was assessed by immunoblotting. In A, human CD44 was detected using mAb E1/2 followed by HRP-anti-mouse Ig. In B parallel blots were incubated with mAb IM7 followed by HRP-anti-rat Ig and an anti-annexin II antiserum followed by HRP-anti-rabbit Ig. Blots were developed using the ECL reagent and exposed to X-ray film for 15 seconds (A) and 1 minute (B). Molecular size markers are in kDa.

that reported for glycosylphosphatidylinositol (GPI) anchored proteins which are found associated with membrane subdomains enriched in glycolipids (Brown and Rose, 1992). Furthermore, a similar extraction pattern is observed with annexin II (Fig. 5b) which again is found associated with Triton X-100 insoluble, lipid rich domains (Lisanti et al., 1994).

A cell type dependence of CD44 Triton X-100 solubility

In fibroblasts, 30-70% of CD44 is recovered in a non-ionic detergent insoluble fraction (Tarone et al., 1984; Lacy and Underhill, 1987; Carter and Wayner, 1988; Figs 2,3,4). As reported previously, we have been unable to demonstrate any association of CD44 with a detergent insoluble fraction in polarized MDCK epithelial cells (Neame and Isacke, 1992, 1993). We describe here experiments to investigate this cell type dependence. First, these previous results are not an artefact of the cell lines originally employed as this difference between fibroblastic and epithelioid cells extends to a wide range of cell lines originating from a number of different species (Table 1). The only exceptions are the cervical epithelioid HeLa cell line which has a profile characteristic of fibroblasts with approximately 25% of the CD44 retained in the detergent insoluble fraction, and the SV40 T antigen expressing COS-1 monkey fibroblast line which has no detectable CD44 retained in the Triton X-100 insoluble cell fraction. Two lymphoid cell lines were also examined: AKR1 cells, which are CD44-negative; and BW5147 cells, which express large amounts of endogenous CD44 (Trowbridge et al., 1982). Like epithelial cells, greater than 95% of the AKR transfected CD44 and BW5147 endogenous CD44 is detergent soluble.

In all of the fibroblast cell lines examined, the predominant CD44 species expressed is the 80-100 kDa CD44H isoform (Figs 2 to 5) which contains none of the alternatively spliced exons. By contrast the majority of the epithelioid cell lines express one or more CD44 splice variants (for example HC11 cells (Wheatley and Isacke, 1995); MDCK cells, Fig. 6; HT-29 cells, Fig. 8). However, there is no evidence that the detergent solubility of CD44 in epithelial cells is related to the presence of the alternatively spliced exons as no difference is observed in the detergent solubility of the different isoforms over a range of detergent concentrations, and transfected CD44H in epithelial cells exhibits an identical extraction profile to the endogenous epithelial CD44 (Fig. 6; Neame and Isacke, 1993).

The distinctly different detergent solubility of CD44 expressed in fibroblastic cells compared to that expressed in epithelial/lymphoid cells suggests the possibility of different functions for CD44 in these functionally distinct cell types. The most obvious differences between these cell types is that fibroblasts have a motile solitary nature involving cell:extracellular matrix interactions as opposed to the more stationary cell:cell oriented interactions of the epithelial cell types. To address these issues, the migration of cells was manipulated. By time lapse video microscopy it can be seen that exponentially growing MDCK cells exhibit limited motility which becomes completely restricted as the cells assume confluence (data not shown). A comparison of sparse and stationary cultures reveals no difference in the detergent solubility of either transfected or endogenous CD44 (Fig. 6A). To exert a more dramatic change, MDCK cells were cultured in the presence of scatter factor (reviewed by Furlong, 1992). Scatter factor/hepatocyte growth factor obtained either in purified form or as a component of MRC-5 fibroblast conditioned medium induces the breakdown of epithelial junctions and the migration of cells across the tissue culture plates (Fig. 6B). However, there is no increase in the detergent insolubility of endogenous CD44 (Fig. 6C) or transfected human CD44H (Fig. 6D) after 24 hours. Similarly, culturing cells for between 1 and 9 hours (data not shown) or for periods of time longer than 24 hours (Fig. 6C) in the presence of scatter factor has no effect. As an alternative approach, fibroblasts were cultured in semi-solid medium to prevent their migration on a tissue culture substratum. A comparison of cells extracted from methylcellulose after 24 or 48 hours with cells growing on plates revealed no differences in the detergent solubility of CD44 (Fig. 7).

To address the issue as to whether an association with extracellular matrix induces the association of CD44 with the Triton X-100 insoluble fraction, the interaction of CD44 with hyaluronan was examined. We have already demonstrated that removal of fibroblast associated hyaluronan does not increase the amount of detergent soluble CD44 (Fig. 2). An alternative approach was to determine whether there was an increase in the amount of Triton X-100 insoluble CD44 in BW5147 cells

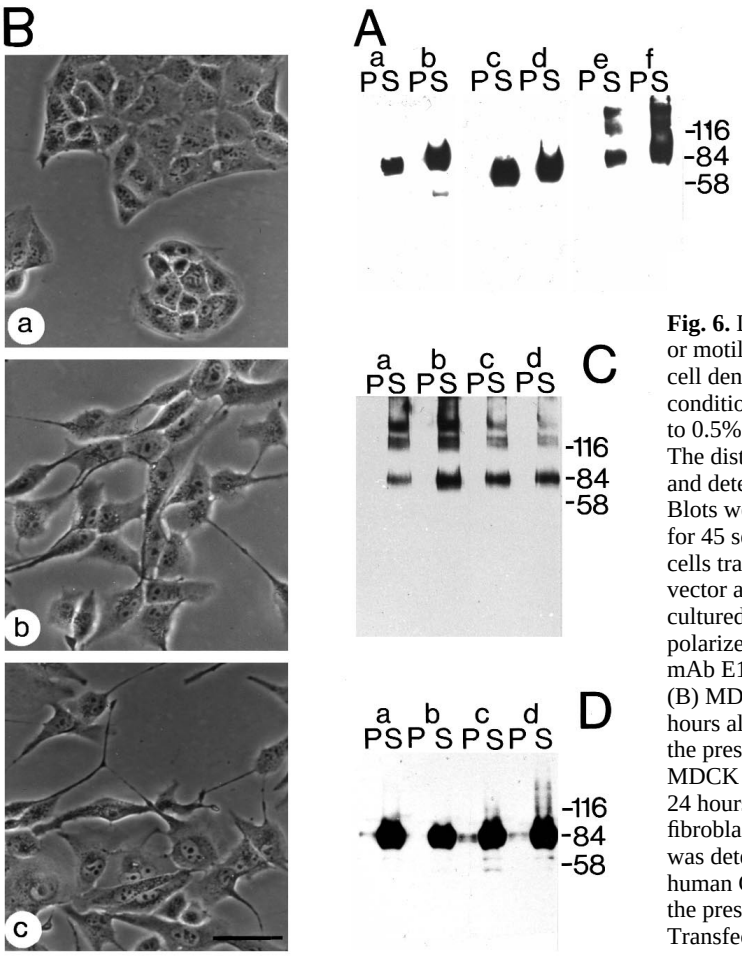

Fig. 6. Detergent solubility of CD44 is not modulated by the growth state or motility of epithelial cells. Epithelial cells were cultured at different cell densities (A) or treated with scatter factor or scatter factor conditioned medium (B-D). Cells were inspected visually (B) or subjected to 0.5% Triton X-100 detergent extraction on ice for 15 minutes (A,C,D). The distribution of CD44 between the detergent insoluble cell pellet (P) and detergent soluble supernatant (S) was assessed by immunoblotting. Blots were developed using the ECL reagent and exposed to X-ray film for 45 seconds. Molecular size markers are in kDa. (A) 1×10^6 MDCK cells transfected with WT human CD44 (a,b), T– CD44 (c,d) or pSRα vector alone (e,f) were plated into 6 cm tissue culture dishes and either cultured for 24 hours (a,c,e) or for 7 days (b,d,f) to obtain sparse and fully polarized cultures, respectively. Transfected CD44 was detected using mAb E1/2 (a-d); endogenous CD44 was detected using mAb IM7 (e,f). (B) MDCK cells were cultured for 24 hours and then for a further 24 hours alone (a), in the presence of MRC-5 conditioned medium (b) or in the presence of 5 ng/ml purified scatter factor (c). Bar, 100 μm. (C) 1×10^6 MDCK cells were plated onto 6 cm tissue culture plates and cultured for 24 hours. The medium was then removed and replaced either with MRC-5 fibroblast conditioned medium for 0 (a), 1 (b), 2 (c) or 3 (d) days. CD44 was detected using mAb IM7. (D) 1×10^6 MDCK cells expressing WT human CD44 were cultured for 24 hours and then for a further 24 hours in the presence of 0 (a), 2 (b), 10 (c) or 50 (d) ng/ml scatter factor. Transfected CD44 was detected using mAb E1/2.

cultured on a hyaluronan-coated substratum or in the presence of soluble hyaluronan. These cells are competent to bind hyaluronan in solution and adhere to hyaluronan-coated plates (Lesley et al., 1990) but the presence of hyaluronan does not increase the detergent insolubility of BW5147 CD44 (Fig. 8A). Similarly, if MDCK cells are plated onto a hyaluronan-coated substratum, there is no change in the detergent extraction of the WT transfected human CD44 (Fig. 8B) or endogenous canine CD44 (data not shown). One problem with this latter approach is that if hyaluronan is responsible for retaining fibroblast CD44 in the detergent insoluble fraction, this effect may require further modification of the ligand by the secreting cell. To address this, HT-29 epithelial cells were co-cultured with Swiss 3T3 fibroblasts and then the detergent solubility of HT-29 CD44 was determined using a human CD44 specific mAb, E1/2. Co-culture did not alter the detergent solubility of HT-29 CD44 (Fig. 8C) or Swiss 3T3 CD44 (data not shown).

Together these data indicate that the mechanism which retains the CD44 molecules in the detergent insoluble fraction of fibroblasts is not down regulated when fibroblasts lose their attachment to the substratum, nor is it upregulated when epithelial cells are induced to migrate and lose their intimate association with neighbouring epithelial cells.

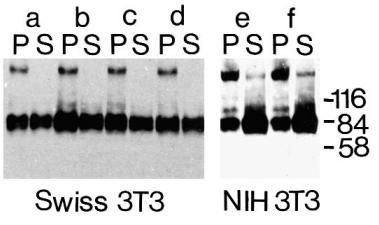

Fig. 7. Expression and distribution of CD44 in fibroblasts is not modulated by loss of matrix attachment. 1×10^6 Swiss 3T3 fibroblasts (a-d) or NIH 3T3 fibroblasts (e,f) were cultured for 24 (a,b,e,f) or 48 (c,d) hours on tissue culture plates (a,c,e) or suspended in methylcellulose (b,d,f). Cells were extracted on ice with 0.5% Triton X-100 for 15 minutes and the distribution of CD44 between the detergent insoluble cell pellet (P) and the soluble supernatant (S) was assessed by immunoblotting using mAb IM7 followed by an HRP-anti-rat Ig. Blots were developed using the ECL reagent and exposed to X-ray film for 40 seconds. Molecular size markers are in kDa.

DISCUSSION

It is often considered that adhesion protein function is influenced by interactions with intracellular components as well as interaction with extracellular ligand. One frequently cited method to examine transmembrane protein associations with the underlying cytoskeleton is extraction of cells at 4°C with non-ionic detergents. Proteins which are not solubilized are considered to be cytoskeletally associated. In the case of fibroblasts, 30-70% of CD44 is found to be insoluble in Triton X-100. This together with membrane motility and in vitro studies was taken as supporting evidence that CD44 is associ-

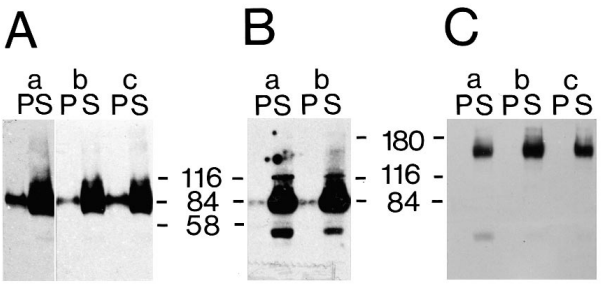

Fig. 8. Addition of hyaluronan does not induce an association of epithelial or lymphoid CD44 with the detergent insoluble fraction. Cells were cultured on hyaluronan coated plates, with soluble hyaluronan or in the presence of hyaluronan producing cells. Subsequently cells were extracted with 0.5% Triton X-100 for 15 minutes on ice and the distribution of CD44 between the detergent insoluble pellet (P) and the detergent soluble supernatant (S) was assessed by immunoblotting. Blots were developed using the ECL reagent and exposed to film for 45 seconds. Molecular size markers are in kDa. (A) 1×10^5 BW5147 cells were cultured in 24-well plates previously coated with 5 mg/ml hyaluronan (a), in the presence of 0.5 mg/ml soluble hyaluronan (b) or in the absence of hyaluronan (c) for 2 hours. CD44 was detected using mAb IM7. (B) 1×10^5 MDCK cells expressing WT human CD44 were cultured on 35 mm plates previously coated with 5 mg/ml hyaluronan (a) or uncoated dishes (b) for 24 hours. Transfected human CD44 was detected using mAb E1/2. (C) 1×10^6 HT-29 cells were cultured alone (a) or co-cultured with 1×10^6 Swiss 3T3 cells for 48 (b) or 72 (c) hours. Human CD44 was detected using mAb E1/2.

ated with the underlying cytoskeleton (reviewed by Isacke, 1994; Tsukita et al., 1994; Lokeshwar et al., 1994). However, we have previously observed that a cytoplasmically truncated T– CD44 is also retained in the detergent insoluble cytoskeletal fraction and that the distribution of detergent insoluble T– CD44 is identical to that of detergent insoluble WT CD44 (Neame and Isacke, 1993). This behaviour of T– CD44 is not due to the Arg-Arg-Arg sequence remaining as a cytoplasmic stub as substitution of these three amino acids with Arg-Trp-Thr (Neame and Isacke, 1993), with Arg-Ser or with Pro (data not shown) generates tailless proteins with properties identical to T– CD44. Therefore, the most likely explanations for the Triton X-100 insolubility of T– CD44 are: (a) that transfected T– CD44 interacts with endogenous WT CD44 and that the latter is directly associated with the cytoskeleton. If this were the case it would be expected that the proportion of detergent insoluble CD44 would decrease with increased expression levels. Such a dilution effect is not observed. Moreover, antibody patching experiments provide no evidence for an homotypic association of CD44 (Fig. 1). Similarly, Herrlich et al. (1993) have reported that in cross-linking experiments there is no evidence for the formation of dimers or oligomers of the CD44H isoform. (b) That CD44 binding via its extracellular domain to hyaluronan causes the formation of detergent insoluble multimeric structures or links CD44 indirectly to other hyaluronan-binding cytoskeletally-attached proteins. Hyaluronan is a large (up to 5×10^6 Da), unbranched glycosaminoglycan composed of repeating *D*-glucuronic acid and *N*-acetyl glucosamine which when hydrated can form random coils of up to 500 nm in diameter (reviewed by Laurent, 1989). It has been well documented that fibroblasts synthesise and secrete hyaluronan, and hyaluronan can be detected associated with the fibroblasts employed here using a bovine proteoglycan probe (Wheatley and Isacke, 1995). Moreover, the observation that addition of hyaluronan to some CD44-positive lymphoid cells results in cell aggregation (Lesley et al., 1990) demonstrates that this ligand has multiple CD44 binding sites. However, experiments shown here suggest that such a mechanism is not responsible for the detergent insoluble pool of CD44 in fibroblasts. Fibroblast associated hyaluronan can be removed with hyaluronidase but this removal does not increase the amount of Triton X-100 soluble CD44 (Fig. 2). BW5147 cells which have the capacity to bind hyaluronan (Lesley et al., 1990) can be cultured either on hyaluronan coated plates or in the presence of soluble hyaluronan. Neither treatment increases the proportion of detergent insoluble CD44 (Fig. 8). Similar experiments employing MDCK and HT-29 epithelial cell lines including the co-culture with hyaluronan producing Swiss 3T3 cells similarly has no effect (Fig. 8). Finally, the observation that the presence of hyaluronan, heparan sulphate or chondroitin sulphate in the extraction buffer does not increase the solubility of fibroblast CD44 (Fig. 4) argues against a role for glycosaminoglycan side chains in mediating CD44 insolubility. (c) That the transmembrane domain of CD44 is associated with a transmembrane cytoskeletally-associated linker protein. Chimera experiments in which the transmembrane domain of CD44 has been replaced with that from the CD45 molecule or the CD3 ζ chain of the T cell receptor have demonstrated that it is the transmembrane domain of CD44 which is responsible for retaining a proportion of the protein in the fibroblast Triton X-100 insoluble fraction (Perschl et al., 1995a). The evidence indicating that the Triton X-100 insolubility of fibroblast CD44 does not result from the CD44 transmembrane domain interacting with a cytoskeletally-associated linker protein is that Triton X-100 insoluble CD44 migrates in sucrose density gradients to the upper part of the gradient indicating an association with more buoyant material whereas Triton X-100 soluble CD44 is found predominantly in the 40% sucrose cushion (Perschl et al., 1995a). Plasma membrane domains which have identical buoyancy characteristics to these CD44 containing domains are stable under the same detergent conditions in which CD44 is insoluble, and are destabilized by detergent conditions that solubilize CD44 (Fig. 5; Brown and Rose, 1992). In similar experiments sucrose gradient buoyancy has been used to isolate a caveoli rich fraction which contains GPI linked proteins, transmembrane proteins and plasma membrane associated cytosolic proteins (Lisanti et al., 1994). One of these latter proteins, annexin II, also shows increased solubility in *n*-octylglucoside and has a similar detergent solubility profile to that of CD44 (Fig. 5b).

Taken together these data indicate that the transmembrane domain of CD44 ensures its inclusion in Triton X-100 insoluble, lipid rich, plasma membrane complexes. However, although there are striking similarities between the detergent extraction of GPI linked proteins such as PLAP from the apical surface of MDCK cells (Brown and Rose, 1992) and CD44 from fibroblasts, several issues remain unresolved: (a) CD44, unlike PLAP, is a transmembrane protein and therefore the association with plasma membrane lipids must be functionally distinct. It remains to be determined whether as is the case for PLAP, CD44 associated membrane structures are

enriched in glycosphingolipids. Certainly CD44 cannot be associated with the same glycosphingolipid enriched complexes as PLAP because both PLAP and T− CD44 are expressed on the apical plasma membrane of MDCK cells and yet there is no evidence of T− CD44 Triton X-100 insolubility in these cells (Brown and Rose, 1992; Neame and Isacke, 1993). This indicates that T− CD44 and PLAP are in distinct apical plasma membrane subdomains. Furthermore in these experiments we cannot determine whether the retention of CD44 in Triton X-100 insoluble complexes results from an association with lipids and/or with other transmembrane proteins concentrated in such domains. (b) It is not known why there is always a significant proportion of Triton X-100 soluble CD44 in fibroblasts in addition to the 30-70% detergent insoluble fraction. This is not due to saturation by CD44 of a detergent-insoluble complex as transfection of CD44 into fibroblasts does not change the proportion of detergent insoluble endogenous material and moreover, the behaviour of transfected CD44 mirrors that of endogenous CD44. Jacobson et al. (1984a,b) found that CD44 was more rapidly redistributed in the leading edge of fibroblasts than in the trailing edge and proposed that CD44 associated with cellular components in a dynamic manner with the association being weaker at the leading edge. Together, these data would be consistent with a model whereby the association of CD44 with the detergent-insoluble complexes in fibroblasts is transient. (c) The ability of CD44 to associate with Triton X-100 insoluble, lipid rich, complexes is cell type dependent (Table 1). No CD44 is found associated with a Triton X-100 insoluble fraction in epithelial cells and yet such cells contain Triton X-100 insoluble lipid complexes (Brown and Rose, 1992). This suggests that there is a significant difference between the localization of CD44 in membrane subdomains between fibroblasts and epithelial/lymphoid cells and raises the question as to how this difference in distribution might be regulated and whether it reflects different functions for CD44 in different cell types. The most obvious differences between fibroblasts and epithelial/lymphoid cells is that the former have a migratory phenotype and are primarily involved in cell:matrix interactions whereas the latter are non-motile cells involved in cell:cell interactions. However, although cell motility and interaction with the extracellular matrix can be manipulated, such manipulations do not alter the Triton X-100 solubility of CD44 (Figs 2,6,7,8). In conclusion, the data presented here argues for a mechanism whereby CD44 associates with Triton X-100 insoluble, lipid rich, complexes in a transient, cell type and temperature dependent manner.

We would like to thank Peter Clark (St Mary's Hospital Medical School, London, UK) for advice with the scatter factor experiments, Fiona Lucas who initiated this work during her undergraduate project and Jayne Lesley and Robert Hyman (The Salk Institute, La Jolla, USA) for reagents and many helpful discussions. This work was supported by project grants from the Cancer Research Campaign and the Medical Research Council.

REFERENCES

Arch, R., Wirth, K., Hofmann, M., Ponta, H., Matzku, S., Herrlich, P. and Zoller, M. (1992). Participation in normal immune responses of a metastasis-inducing splice variant of CD44. *Science* **257**, 682-685.

Bennett, K. L., Jackson, D. G., Simon, J. C., Tanczos, E., Peach, R., Modrell, B., Stamenkovic, I., Plowman, G. and Aruffo, A. (1995). CD44 isoforms containing exon V3 are responsible for the presentation of heparin-binding growth factor. *J. Cell Biol.* **128**, 687-698.

Brown, D. A. and Rose, J. K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**, 533-544.

Camp, R. L., Kraus, T. A. and Puré, E. (1991). Variations in the cytoskeletal interaction and posttranslational modification of the CD44 homing receptor in macrophages. *J. Cell Biol.* **115**, 1283-1292.

Carter, W. G. and Wayner, E. A. (1988). Characterization of the class III collagen receptor, a phosphorylated transmembrane glycoprotein expressed in nucleated human cells. *J. Cell Biol.* **263**, 4193-4201.

Chen, W. Y. J., Grant, M. E., Schor, A. M. and Schor, S. L. (1989). Differences between adult and foetal fibroblasts in the regulation of hyaluronate synthesis: correlation with migratory activity. *J. Cell Sci.* **94**, 577-584.

Cooper, J. A. and Hunter, T. (1983). Identification and characterization of cellular targets for tyrosine protein kinases. *J. Biol. Chem.* **258**, 1108-1115.

Faassen, A. E., Schrager, J. A., Klein, D. J., Oegema, T. R., Couchman, J. R. and McCarthy, J. B. (1992). A cell surface chondroitin sulfate proteoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. *J. Cell Biol.* **116**, 521-531.

Furlong, R. A. (1992). The biology of hepatocyte growth factor/scatter factor. *BioEssays* **14**, 613-617.

Green, S. J., Tarone, G. and Underhill, C. B. (1988). Distribution of hyaluronate and hyaluronate receptors in the adult lung. *J. Cell Sci.* **90**, 145-156.

Günthert, U., Hofmann, M., Rudy, W., Reber, S., Zöller, M., Haussmann, I., Matzku, S., Wenzel, A., Ponta, H. and Herrlich, P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* **65**, 13-24.

Haggerty, J. G., Bretton R. H. and Milstone, L. M. (1992). Identification and characterization of a cell surface proteoglycan on keratinocytes. *J. Invest. Dermatol.* **99**, 374-380.

Heldin, P., Laurent, T. C. and Heldin, C.-H. (1989). Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts. *Biochem. J.* **258**, 919-922.

Herrlich, P., Zoller, M., Pals, S. T. and Ponta, H. (1993). CD44 splice variants: metastases meet lymphocytes. *Immunol. Today* **14**, 395-399.

Isacke, C. M., Sauvage, C. A., Hyman, R., Lesley, J., Schulte, R. and Trowbridge, I. S. (1986). Identification and characterization of the human Pgp-1 glycoprotein. *Immunogenetics* **23**, 326-332.

Isacke, C. M. (1994). The role of the cytoplasmic domain in regulating CD44 function. *J. Cell Sci.* **107**, 2353-2359.

Jackson, D. G., Bell, J. I., Dickinson, R., Timans, J., Sheilds, J. and Whittle, N. (1995). Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. *J. Cell Biol.* **128**, 673-685.

Jacobson, K., O'Dell, D. and August, J. T. (1984a). Lateral diffusion of an 80,000-dalton glycoprotein in the plasma membrane of murine fibroblasts.: Relationships to cell structure and function. *J. Cell Biol.* **99**, 1624-1633.

Jacobson, K., O'Dell, D., Holifield, B., Murphy, T. L. and August, J. T. (1984b). Redistribution of a major cell surface glycoprotein during cell movement. *J. Cell Biol.* **99**, 1613-1623.

Jalkanen, S., Jalkanen, M., Bargatze, R., Tammi, M. and Butcher, E. C. (1988). Biochemical properties of glycoproteins involved in lymphocyte recognition of high endothelial venules in man. *J. Immunol.* **141**, 1615-1623.

Jalkanen, S. and Jalkanen, M. (1992). Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. *J. Cell Biol.* **116**, 817-825.

Kalomiris, E. L. and Bourguignon, L. Y. W. (1988). Mouse T lymphoma cells contain a transmembrane glycoprotein (GP85) that binds ankyrin. *J. Cell Biol.* **106**, 319-327.

Knudson, C. B. and Knudson, W. (1993). Hyaluronan-binding protein in development, tissue homeostasis, and disease. *FASEB J.* **7**, 1233-1241.

Koopman, G., Heider, K.-H., Horst, E., Adolf, G. R., van der Berg, F., Ponta, H., Herrlich, P. and Pals, S. (1993). Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. *J. Exp. Med.* **177**, 897-904.

Lacy, B. E. and Underhill, C. B. (1987). The hyaluronate receptor is associated with actin filaments. *J. Cell Biol.* **105**, 1395-1404.

Laurent, T. (1989). *The Biology of Hyaluronan*, Ciba Foundation Symposium vol. 143 (ed. D. Evered and J. Whelan). Wiley, UK.

Lesley, J., Schulte, R. and Hyman, R. (1990). Binding of hyaluronic acid to lymphoid cell lines is inhibited by monoclonal antibodies against Pgp-1. *Exp. Cell Res.* **187**, 224-233.

Lesley, J., He, Q., Miyake, K., Hamann, A., Hyman, R. and Kincade, P. W. (1992). Requirements for hyaluronic acid binding by CD44: a role for the cytoplasmic domain and activation by antibody. *J. Exp. Med.* **175**, 257-266.

Lesley, J., Hyman, R. and Kincade, P. W. (1993). CD44 and its interaction with extracellular matrix. *Advan. Immunol.* **54**, 271-335.

Liao, H.-X., Levesque, M. C., Patton, K., Bergamo, B., Jones, D., Moody, M. A., Telen, M. J. and Haynes, B. F. (1993). Regulation of human CD44H and CD44E isoform binding to hyaluronan by phorbol myristate acetate and anti-CD44 monoclonal and polyclonal antibodies. *J. Immunol.* **151**, 6490-6499.

Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y.-H., Cook, R. F. and Sargiacomo, M. (1994). Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: Implication for human disease. *J. Cell Biol.* **126**, 111-126.

Lokeshwar, V. B. and Bourguignon, L. Y. W. (1991). Post-translational protein modification and expression of ankyrin-binding site(s) in GP85 (Pgp-1/CD44) and its biosynthetic precursors during T-lymphoma membrane biogenesis. *J. Biol. Chem.* **266**, 17983-17989.

Lokeshwar, V. B., Fregien, N. and Bourguignon, L. Y. W. (1994). Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. *J Cell Biol.* **126**, 1099-1109.

Mackay, C. R., Terpe, H.-J., Stauder, R., Marston, W. L., Stark, H. and Gunthert, U. (1994). Expression and modulation of CD44 variant isoforms in humans. *J. Cell Biol.* **124**, 71-82.

Miettinen, H. M. and Jalkanen, M. (1994). The cytoplasmic domain of syndecan-1 is not required for association with Triton X-100-insoluble material. *J. Cell Sci.* **107**, 1571-1581.

Milde, K. F., Alejandro, R., Mintz, D. H. and Pastori, R. L. (1994a). Molecular cloning of the canine CD44 antigen cDNA. *Biochem. Biophys. Acta* **1218**, 112-114.

Milde, K. F., Alejandro, R. and Pastori, R. L. (1994b). Expression of *CD44* variant transcripts in dog lymphatic tissue. *Immunogenetics* **40**, 437-444.

Morris, A. and Tannenbaum, J. (1980). Cytochalasin D does not produce net depolymerization of actin filaments in HEp-2 cells. *Nature* **287**, 637-639.

Murakami, S., Shimabukuro, Y., Miki, Y., Saho, T., Hino, E., Kasai, D., Nozaki, T., Kusumoto, Y. and Okada, H. (1994). Inducible binding of human lymphocytes to hyaluronate via CD44 does not require cytoskeleton association but does require new protein synthesis. *J. Immunol.* **152**, 467-477.

Neame, S. J. and Isacke, C. M. (1992). Phosphorylation of CD44 *in vivo* requires both Ser323 and Ser325, but does not regulate membrane localization or cytoskeletal interaction in epithelial cells. *EMBO J.* **11**, 4733-4738.

Neame, S. J. and Isacke, C. M. (1993). The cytoplasmic tail of CD44 is required for basolateral localization in epithelial MDCK cells but does not mediate association with the detergent-insoluble cytoskeleton of fibroblasts. *J. Cell Biol.* **121**, 1299-1310.

Perschl, A., Lesley, J., English, N., Hyman, R. and Trowbridge, I. (1995a). Transmembrane domain of CD44 is required for its detergent-insolubility in fibroblasts. *J. Cell Sci.* **108**, 1033-1041.

Perschl, A., Lesley, J., English, N., Trowbridge, I. and Hyman, R. (1995b). Role of CD44 cytoplasmic domain in hyaluronan binding. *Eur. J. Immunol.* **25**, 495-501.

Rudy, W., Hofmann, M., Schwartz-Albiez, R., Zoller, M., Heider, K.-H., Ponta, H. and Herrlich, P. (1993). The two major CD44 proteins expressed on a metastatic rat tumor cell line are derived from different splice variants: each one individually suffices to confer metastatic behavior. *Cancer Res.* **53**, 1262-1268.

Tarone, G., Ferracini, R., Galetto, G. and Comoglio, P. (1984). A cell surface integral membrane glycoprotein of 85,000 mol wt (gp85) associated with the Triton X-100-insoluble cell skeleton. *J. Cell Biol.* **99**, 512-519.

Thomas, L., Byers, R. H., Vink, J. and Stamenkovic, I. (1992). CD44H regulates tumor migration on hyaluronate-coated surfaces. *J. Cell Biol.* **118**, 971-977.

Trowbridge, I. S., Lesley, J., Schulte, R., Hyman, R. and Trotter, J. (1982). Biochemical characterization and cellular distribution of a polymorphic, murine cell-surface glycoprotein expressed on lymphoid tissues. *Immunogenetics* **15**, 299-312.

Tsukita, S., Oishi, K., Satao, N., Kawai, A. and Tsukita, S. (1994). ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeleton. *J. Cell Biol.* **126**, 391-401.

Wheatley, S. C. and Isacke, C. M. (1995). Induction of a hyaluronan receptor, CD44, during embryonal carcinoma and embryonic stem cell differentiation. *Cell Adhes. Commun.* (in press).

(Received 24 February 1995 - Accepted 28 June 1995)