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Phosphorylation of CD44 *in vivo* requires both Ser323 and Ser325, but does not regulate membrane localization or cytoskeletal interaction in epithelial cells

Stephen J.Neame and Clare M.Isacke[1]

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

[1]Corresponding author

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CD44 has been implicated to play an important role in a diverse range of physiological processes, which involve cell-matrix recognition, cell-cell adhesion and cell motility. There is increasing evidence that the highly conserved intracellular domain of CD44 may be involved in influencing these activities. CD44 is phosphorylated *in vivo* on serine residue(s). In view of the importance that phosphorylation has been accorded in a multitude of cellular regulatory processes, we have investigated the role of phosphorylation in the control of CD44. In this report we identify the sites of human CD44 phosphorylation by mutating the three conserved cytoplasmic serine residues. We show that both Ser323 and Ser325, but not Ser316, are required for phosphorylation *in vivo* and demonstrate that this event is not stimulated by phorbol esters. Clonal MDCK cell lines expressing both the single and double CD44 phosphorylation mutants have been generated. These cell lines have been used to directly assess the role of phosphorylation on CD44 localization in polarized epithelial cells and its association with the cytoskeleton.

Key words: CD44/cytoskeleton/epithelial cells/phosphorylation/protein kinase C

Introduction

The hyaluronate receptor, CD44, presents a conundrum as to how an abundant transmembrane glycoprotein, expressed on a wide variety of cell types (Flanagan *et al.*, 1989; Picker *et al.*, 1989), can play a role in a diverse range of important physiological processes such as lymphocyte and prothymocyte homing, T cell activation, cell adhesion, cell migration and metastatic spread (reviewed in Haynes *et al.*, 1989, 1991). Recently, it has become clear that one mechanism by which this is achieved is that CD44 exists in a number of different forms due to alternative splicing of additional amino acid stretches into the extracellular domain (Brown *et al.*, 1991; Dougherty *et al.*, 1991; Gunthert *et al.*, 1991; Stamenkovic *et al.*, 1991; Jackson *et al.*, 1992), and it has been shown that these different forms of CD44 can exhibit altered functions (Gunthert *et al.*, 1991; Stamenkovic *et al.*, 1991; Sy *et al.*, 1991). A second potential mechanism for regulating CD44 function and expression is via intracellular interactions, as all of the forms of CD44 isolated so far have a common 70 amino acid cytoplasmic domain, which is also highly conserved between species. It is known that this cytoplasmic domain is subject to phosphorylation by a serine/threonine protein kinase (Isacke *et al.*, 1986; Carter and Wayner, 1988) and that CD44 can associate with the underlying cytoskeleton (Jacobson *et al.*, 1984; Tarone *et al.*, 1984; Lacy and Underhill, 1987; Carter and Wayner, 1988). There have been some reports showing a potential relationship between these two events (Kalomiris and Bourguignon, 1989; Camp *et al.*, 1991; Geppert and Lipsky, 1991), which raises the question as to whether the CD44-cytoskeleton association is regulated by phosphorylation. In order to address this question directly we have identified the sites of CD44 phosphorylation by mutational analysis and examined the behaviour of these mutants in cultured epithelial cells by morphological and biochemical techniques.

Results

Generation of CD44H phosphorylation mutants

A cDNA clone encoding the haematopoietic form of CD44 (CD44H) was isolated from a human fibroblast library and subjected to site-directed mutagenesis using two mutagenic oligonucleotides. Oligonucleotide C7 encoded a two base change converting Ser316 to Ala316. Oligonucleotide C8 was designed to give the following mixture of alterations: Ser323 to Gly323 or Ala323, Ser325 to Ala325 and the double mutant combination of the 323 and 325 changes. In 23 single-stranded mutant products sequenced the change Ser323 to Ala323 was not found and hence the Gly323 mutation was used throughout (Figure 1). In addition, a tailless CD44H mutant (T-CD44H) was generated, whereby a TGA stop codon was incorporated three amino acids after the proposed transmembrane domain replacing a cysteine residue at amino acid 295 (Figure 1; S.Neame and C.Isacke, manuscript submitted).

Identification of the CD44H phosphorylation sites

Transfected Cos-1 cells were labelled with [^{32}P]orthophosphate overnight and then immunoprecipitated with mAb E1/2, which is specific for human CD44. From Figure 2a it can clearly be seen that in a control labelling of F1084 human fibroblasts, the endogenous CD44H is heavily phosphorylated and phosphoamino acid analysis demonstrated that this occurred solely on serine residues (see Figure 3). The human wild type (WT) CD44H transfected into Cos-1 cells was phosphorylated, again on serine residues (data not shown), and as expected complete removal of the CD44H cytoplasmic tail abolished this phosphorylation. Mutation of the three phosphorylation sites demonstrated that Ser316 was not a major site of phosphorylation as this mutant was labelled to the same extent as the WT CD44H. By contrast, mutation of Ser323 or Ser325, either alone or together, abolished phosphorylation (Figure 2a). To demonstrate that these results were not due to a reduction

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         280         290       .  300         310
WLIILASLLALA LILAVCIAVN SRRRCGQKKK LVINSGNGAV

         320         330         340         350
EDRKPSGLNG EASKSQEMVH LVNKESSETP DQFMTADETR
      A        G A

         360
NLQNVDMKIG V
```

Fig. 1. Amino acid sequence of the transmembrane and cytoplasmic domains of human CD44H. Arrowheads indicate the mutated serine residues and the amino acids that were substituted. Asterisk indicates the cysteine residue that was changed to a TGA stop codon to generate a tailless (T-) CD44H. The predicted transmembrane domain is underlined. Amino acid and nucleotide numbers correspond to the published sequence of Stamenkovic *et al.* (1989).

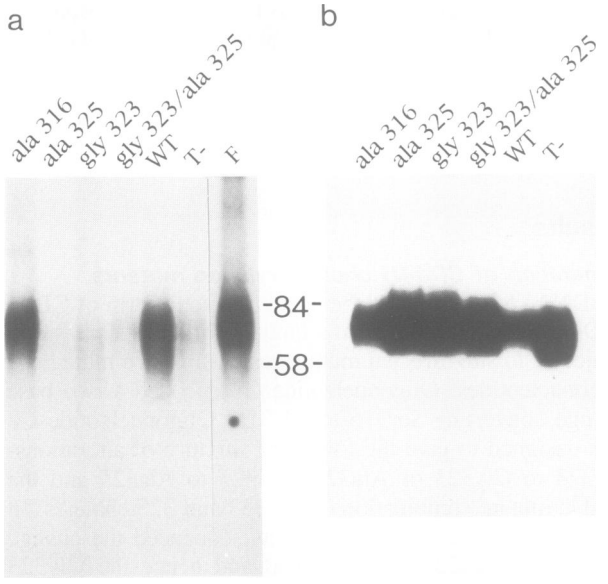

Fig. 2. Phosphorylation of CD44H mutants expressed in Cos-1 cells. **a**. Transfected Cos-1 cells were labelled overnight with [^{32}P]orthophosphate and the transfected CD44H was immunoprecipitated with the anti-human CD44 mAb, E1/2, as described in the Materials and methods. Ala316, change from serine to alanine at residue 316; Ala325, change from serine to alanine at residue 325; Gly323, change from serine to glycine at residue 323; Gly323/Ala325, double mutant where serines at residues 323 and 325 were changed to glycine and alanine respectively; WT, wild type; T-, tailless; F, F1084 human fibroblasts labelled in parallel with the transfected Cos-1 cells. The gel was exposed to film for 2 days. Molecular size markers are in kDa. **b**. Parallel dishes of transfected Cos-1 cells were lysed and the level of human CD44 expression was determined by quantitative immunoblotting using mAb E1/2 and [^{125}I]anti-mouse Ig antisera. Blots were exposed to X-ray film for 24 h.

of CD44H expression in the transfected Cos-1 cells, parallel immunoblots were run (Figure 2b).

As it has been previously reported that CD44H is a target for protein kinase C (PKC; Kalomiris and Bourguignon, 1989), we examined the phosphorylation of CD44H in human diploid fibroblasts treated for 10 min with TPA, which binds to and activates PKC. Although the phosphorylation of a known PKC substrate, p180 (Isacke *et al.*, 1990), was greatly enhanced under these conditions, there was no observable effect on the level of CD44H

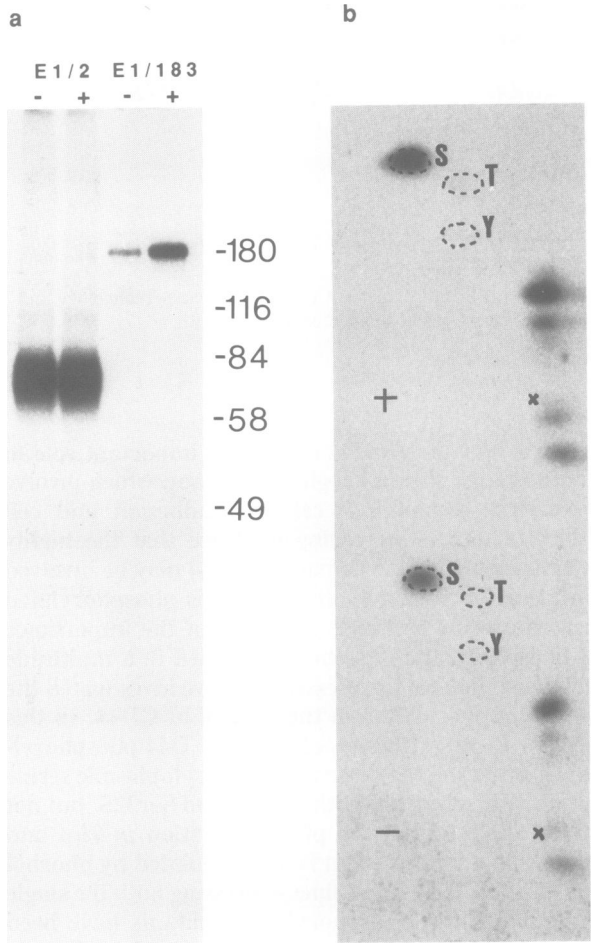

Fig. 3. Phosphorylation of CD44H in human fibroblasts treated with TPA. **a**. ^{32}P-labelled human fibroblasts were treated for 10 min without (−) or with (+) TPA. Lysates were immunoprecipitated with the anti-CD44 mAb, E1/2 or an anti-p180 mAb, E1/183 (Isacke *et al.*, 1990), resolved on a 10% polyacrylamide gel and exposed to film for 12 h. Molecular size markers are in kDa. **b**. Labelled CD44H protein was eluted from the gel shown in panel a and subjected to two dimensional phosphoamino acid analysis. Thin layer plates were exposed to X-ray film for 6 days with intensifying screens. x marks the point of sample application.

phosphorylation and again only phosphoserine residues were detected (Figure 3). Similarly, no difference was observed in two-dimensional phosphotryptic peptide maps of CD44H from fibroblasts treated with or without TPA (data not shown).

Localization of the CD44H phosphorylation mutants in polarized MDCK cells

To determine whether phosphorylation of CD44H on Ser323 or Ser325 is important for its localization in epithelial cells, MDCK cells were transfected with the CD44H mutants. MDCK cells can be cultured as a polarized monolayer, whereby the upper apical membrane is delineated from the basolateral membrane by tight junctions. The basolateral membrane consists of a lateral domain in contact with neighbouring cells and a basal domain in contact with the underlying substratum (Figure 4g; reviewed in Mostov *et al.*, 1992). Polarized MDCK cell lines were examined by confocal microscopy as the distribution of CD44H in these cells is most clearly seen in vertical (*xz*) optical sections.

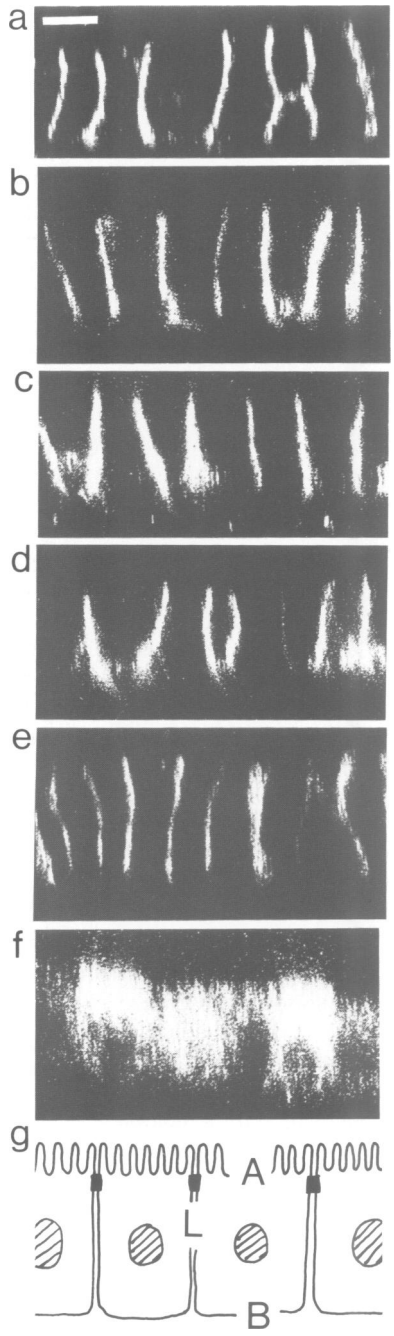

Fig. 4. Distribution of CD44H phosphorylation mutants in polarized MDCK cells. Confocal immunofluorescent micrographs through the vertical (*xz*) axis of MDCK cells expressing WT (**a**), Ala316 (**b**), Gly323 (**c**), Ala325 (**d**), Gly323/Ala325 (**e**) and T- (**f**) forms of human CD44H. CD44H was visualized with the anti-human CD44 mAb, E1/2 and FITC-conjugated anti-mouse Ig. **g** is a diagrammatic representation of the above cells showing the three plasma membrane domains: A, apical; L, lateral; B, basal. Scale bar represents 10 μm.

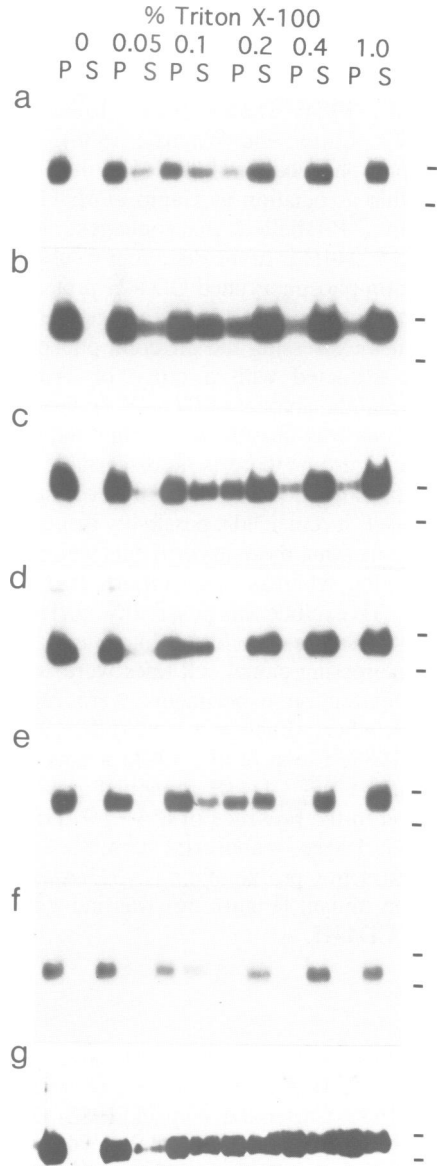

Fig. 5. Detergent solubility of CD44H phosphorylation mutants. The CD44 forms: WT (**a**), Ala316 (**b**), Gly323 (**c**), Ala325 (**d**), Gly323/Ala325 (**e**) and T- (**f**) expressed in MDCK cells and Gly323/Ala325 (**g**) expressed in Swiss 3T3 fibroblasts were extracted with different concentrations of Triton X-100 as detailed in the Materials and methods. The detergent soluble fraction (S) and insoluble cell pellet (P) was subjected to immunoblotting with mAb E1/2 and [^{125}I]anti-mouse Ig antisera. Blots were exposed to X-ray film for 3 days. Molecular size markers (right hand side of figure) represent 84 and 58 kDa.

The WT CD44H is localized to the basolateral membrane of MDCK cells, within which it is tightly restricted to the lateral domain (Figure 4a). Removal of the cytoplasmic tail of CD44H abolishes the basolateral localization and T-CD44H is seen on the apical membrane of the cells and a significant proportion is also found intracellularly (Figure 4f). All of the phosphorylation mutants had a distribution indistinguishable from the WT CD44H, that is the protein was localized exclusively to the lateral domain in a profile which was clearly distinct from the T-CD44H (Figure 4b–e). Conventional immunofluorescence microscopy and confocal microscopy in the horizontal (*xy*) axis gave equivalent results (data not shown). We have previously shown that removal of the CD44H cytoplasmic tail results in a reduction of protein stability (S.Neame and C.Isacke; manuscript submitted) and suggested that this may be as a consequence of incorrect targeting. Consistent with this suggestion was the finding that the Gly323/Ala325 double phosphorylation mutant and the WT CD44H both had a half-life of between 16 and 18 h in MDCK cells whereas the half-life of the T-CD44H was only between 5 and 8 h.

The effect of phosphorylation on the association of CD44 with the cytoskeleton

There have been numerous reports by other laboratories that CD44H is associated with the underlying cytoskeleton (Jacobson *et al.*, 1984; Tarone *et al.*, 1984; Lacy and Underhill, 1987; Carter and Wayner, 1988). It may be expected that phosphorylation of CD44H would play a role in regulating this association as Camp *et al.* (1991) have shown that in ^{32}P-labelled macrophages, all of the phosphorylated CD44H is in the detergent soluble fraction, whereas only non-phosphorylated CD44H protein is found in the detergent insoluble cytoskeletal fraction. Consequently, MDCK cell lines expressing the different phosphorylation mutants were extracted with a range of Triton X-100 concentrations and the amount of CD44H in the soluble and insoluble fractions was quantified by immunoblotting. As can be seen in Figure 5, there is no discernible difference in the detergent extraction profile of any of the forms of CD44H examined. It remained a possibility that these results were due to the fact that these experiments were performed with MDCK cells, whereas other reports had used non-epithelial cells. To exclude this possibility, different forms of human CD44H were transfected into Swiss 3T3 cells. Permanently expressing clonal cell lines were isolated and the detergent extraction experiments were repeated. In agreement with others (Lacy and Underhill, 1987; Carter and Wayner, 1988; Camp *et al.*, 1991) it was found that the majority of the WT CD44H remained associated with the cytoskeleton in the presence of 0.4–1% Triton X-100 (S.Neame and C.Isacke, manuscript submitted) and again, the detergent extraction profile of the Gly323/Ala325 double phosphorylation mutant (Figure 5g) was indistinguishable from the WT CD44H.

Discussion

It had been previously shown that the extensive phosphorylation of human CD44H *in vivo* occurs only on serine residues (Isacke *et al.*, 1986; Carter and Wayner, 1988). The human CD44 contains six serine residues in the cytoplasmic tail, but only three of these, Ser316, 323 and 325 (Figure 1), are conserved between the human (Stamenkovic *et al.*, 1989), baboon (Idzera *et al.*, 1989), bovine (Bosworth *et al.*, 1991), hamster (Aruffo *et al.*, 1990), rat (Gunthert *et al.*, 1991) and mouse (Nottenburg *et al.*, 1989; Zhou *et al.*, 1989) protein, and hence these three were chosen for mutagenesis. Ser316 is not a major site of phosphorylation, whereas mutation of either Ser323 or Ser325 abolishes the ability of CD44H to be phosphorylated (Figure 2). The amino acid sequence surrounding Ser323 and Ser325 (Figure 1) suggests they are not targets for any of the basophilic protein kinases, such as PKC or cAMP-dependent protein kinase. These kinases tend to phosphorylate serine or threonine residues that are two or three amino acids away from basic amino acids (Pearson and Kemp, 1991), whereas Ser323 and Ser325 are associated with upstream and downstream acidic residues. Due to previous reports that CD44H is a PKC substrate (Kalomiris and Bourguignon, 1989), we examined CD44H in human fibroblasts treated with phorbol ester, but were unable to detect any change in its phosphorylation (Figure 3). Camp *et al.* (1991) have reported the same result with ^{32}P-labelled macrophages. One explanation for the discrepancy between these data and those reported by Kalomiris and Bourguignon (1989) is that the experiments presented here were performed with intact cells as opposed to membrane preparations or immunoprecipitated CD44H protein. Under the latter conditions, it is possible that Ser291, which is in a potential PKC target sequence but very close to the predicted transmembrane domain (Figure 1), may become accessible to PKC *in vitro*. The presence of acidic amino acids in close proximity to Ser323 and Ser325, combined with the observation that both these serines are involved in CD44 phosphorylation, is more compatible with protein kinases such as casein kinase-2 (Pinna, 1990; Pearson and Kemp, 1991). It is not known whether phosphorylation occurs on both Ser323 and Ser325, nor is it known why mutation of either serine abolishes the phosphorylation of CD44H. Possibly, substitution of an alanine or glycine residue at one of these positions results in a local conformational change that disrupts phosphorylation at the second site. It is also important to elucidate the limits of the kinase recognition element. *In vitro* studies with purified protein kinases and synthetic CD44 peptides, and further mutagenic analysis will be required to resolve these issues.

CD44H is localized to the basolateral membrane of MDCK epithelial cells and this restriction appears to be regulated by the cytoplasmic tail of the protein as T-CD44H mutants are found localized on the apical plasma membrane. From the data presented in Figure 4, we conclude that any mechanism of basolateral sorting does not involve the phosphorylation of CD44H and that a sorting signal is probably not located in the vicinity of Ser316 or Ser323/Ser325, as mutation of these residues does not alter the distribution of the protein. Loss of the cytoplasmic tail also results in a significant proportion of the protein being localized intracellularly and a reduction in it stability. These events could be due to the absence of a lateral membrane specific mechanism, which retains CD44 on the plasma membrane, the lack of a cytoplasmic tail, which mediates exclusion from coated pits, or the delivery of T-CD44H from the Golgi apparatus directly to degradatory organelles with no plasma membrane placement for a portion of the newly synthesized material. None of the phosphorylation mutants were found to localize, to any noticeable extent, to the cytoplasm, indicating that they are resident in the plasma membrane and presumably not subject to any of the above mechanisms. In keeping with these observations we found that their half-lives are very similar to the WT protein, as compared with the reduced half-life of the T-form. It is also noteworthy that within the basolateral domain, both the WT and phosphorylation mutants of CD44H are localized to the lateral domain and are absent from the basal domain. This suggests that Ser316, 323 and 325 are not involved in the segregation of CD44 within the basolateral membrane.

Removal of the CD44H cytoplasmic tail does not alter the association of this protein with the cytoskeleton as determined by the detergent extraction (Figure 5) and we have previously proposed that this cytoskeletal association may be indirect and involve a putative linker molecule interacting with the transmembrane or extracellular domain of CD44H (S.Neame and C.Isacke; manuscript submitted). In contrast to this hypothesis, it has been reported by others that phosphorylation of CD44 can alter its ability to associate with the cytoskeleton, and therefore it remained a possibility that in polarized epithelial cells, the results obtained with the

T-CD44H mutant were due to its aberrant cellular localization and therefore they could not be accurately compared with the results obtained with the WT CD44H counterpart. Camp *et al.* (1991) have shown that in macrophages all of the phosphorylated CD44H was found in the detergent soluble fraction, while only non-phosphorylated CD44H was detected in the detergent insoluble cell pellet. The availability of mutated CD44H protein that cannot be phosphorylated allowed us to assess the role of phosphorylation directly. When we examined the detergent extraction profile of transfected CD44H in MDCK cells, we were unable to detect any difference between the WT and CD44H phosphorylation mutants (Figure 5). These experiments were repeated with transfected Swiss 3T3 fibroblasts, where in agreement with others (Tarone *et al.*, 1984; Lacy and Underhill, 1987; Carter and Wayner, 1988) the majority of the WT CD44H was detergent insoluble in the presence of 0.4% or 1% Triton X-100, but again there was no difference in the detergent extraction profile between the WT CD44H and the CD44H double phosphorylation mutant. Our data suggest that phosphorylation of CD44H does not directly regulate its association with the cytoskeleton, however, phosphorylation of the putative CD44 linker protein would explain how phorbol esters can rapidly decrease the level of CD44 association with the cytoskeleton (Geppert and Lipsky, 1991), without altering the phosphorylation of CD44 itself. Although the data presented by Camp *et al.* (1991) clearly show that all of the phosphorylated CD44H is found in the detergent soluble fraction, it does not show that all of the non-phosphorylated protein is detergent insoluble, and as they have suggested, an alternative possibility is that phosphorylation does not regulate the association with the cytoskeleton, but rather this association physically prevents access of the kinase to CD44H. If this is the case it is unsurprising that mutation of the CD44H phosphorylation sites does not alter the detergent extraction profile of the protein. In conclusion, in this experimental system there is no evidence that phosphorylation of CD44H regulates either the targeting of the protein to the basolateral membrane of epithelial cells or its association with the cytoskeleton.

Materials and methods

Mutation of CD44 phosphorylation sites

A cDNA clone encoding the haematopoietic form of human CD44 (CD44H) was isolated from an human fibroblast λZAP cDNA library by polymerase chain reaction (PCR) and ligated into the *Sma*I site of pUC19 (S.Neame and C.Isacke, manuscript submitted). CD44H was excised from pUC19 with *Eco*RI and *Xba*I and inserted into the Promega pSELECT vector at the same sites. Mutants of CD44H in which specific serine residues had been changed to glycine or alanine (Figure 1), were made with the Altered Sites mutagenesis kit (Promega) using oligonucleotides C7 (AGAAAGCCA-GCTGGACTCAA; bases 1052–1071) and C8 (CGGAGAGGCC(A/G)-(G/C)CAAG(T/G)CTCAGGAAATG; bases 1072–1099) where underlined bases are those that differ from the wild type nucleotide sequence. The brackets in C8 indicate single positions where more than one nucleotide was incorporated to create a mixture of oligonucleotides. Single-stranded preparations of the mutant clones were sequenced and clones verified as having the required mutations were excised from pSELECT with *Eco*RI and *Xba*I. A eukaryotic expression vector, pSRα-neo (Takebe *et al.*, 1988; DNAX, Palo Alto, CA), was linearized with *Bam*HI. This vector and the excised CD44 clones were treated with the Klenow fragment of DNA polymerase I and dNTPs, ligated together and the orientation of the insert was checked by restriction endonuclease digestion.

Cell lines and antibodies

All cell lines were maintained in DME supplemented with 10% FCS. Cells were transfected by electroporation using a modification of the method of Chu *et al.* (1987). Essentially, $2-4 \times 10^6$ Cos-1 cells or $0.5-1.0 \times 10^7$ MDCK cells were trypsinized, washed in DME plus 10% FCS, washed twice in HeBS (20 mM HEPES pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 6 mM D-glucose) and resuspended in 250 μl HeBS containing 20 μg of the pSRα-neo constructs. Electroporation was at 250 μF, 270 V and infinite ohms in 0.4 cm cuvettes in a Bio-Rad Gene Pulser; cells were cultured for a further 24–48 h. MDCK cells were then replated into 96-well tissue culture plates in medium containing 0.5 mg/ml G418 (Gibco-BRL). G418-resistant colonies were screened by immunofluorescence for expression of CD44H and positive clones were then maintained in 0.3 mg/ml of G418. E1/2.1 is a mouse monoclonal antibody (mAb) that is specific for human CD44 (Isacke *et al.*, 1986). IM7.8.1 is a cross-species reactive rat anti-CD44 mAb (Trowbridge *et al.*, 1982). FITC-conjugated anti-mouse Ig was obtained from Jackson ImmunoResearch. Unconjugated anti-mouse Ig was ^{125}I-labelled by the chloramine T method (Hunter and Greenwood, 1962).

CD44H phosphorylation

5 cm dishes of F1084 human fibroblasts or Cos-1 cells transfected with the pSRα-neo constructs were washed in phosphate-free DME and then incubated for 16 h in 2 ml of phosphate-free DME containing 2% FCS, 20 mM HEPES pH 7.5 and 2 mCi [^{32}P]orthophosphate (Amersham). Cells were then lysed in 400 μl NDET (1% Nonidet P40, 0.4% sodium deoxycholate, 66 mM EDTA and 10 mM Tris pH 7.4), the lysate was clarified by centrifugation and SDS added to a final concentration of 0.3%. All further procedures were carried out on ice. Lysates were precleared with 100 μl of fixed *Staphylococcus aureus* bacteria (Pansorbin, Calbiochem) for 30 min and then human CD44H was immunoprecipitated by incubating with 5 μg of mAb E1/2 for 60 min followed by rabbit anti-mouse Ig prebound to 50 μl of protein A–agarose (Bio-Rad) for 60 min. The immunoprecipitates were washed four times in NDET plus 0.3% SDS, resuspended in reducing sample buffer, boiled for 2 min and then separated by electrophoresis on a 10% SDS–polyacrylamide gel. Dried gels were exposed to X-ray film (Fuji XR) with an intensifying screen at −76°C. To assay the level of CD44H expression, parallel dishes of transfected Cos-1 cells were lysed in 0.2 ml of non-reducing sample buffer and subjected to immunoblotting. To examine the effects of treating cells with phorbol esters, parallel dishes of human fibroblasts were labelled overnight with [^{32}P]orthophosphate as described above and 100 ng of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; Sigma) was added to one dish for 10 min at the end of the labelling period. Lysates were immunoprecipitated with the anti-CD44 mAb, E1/2, or mAb E1/183, which recognizes the human p180 protein (Isacke *et al.*, 1990). Two-dimensional phosphoamino acid analysis was carried out as described by Cooper *et al.* (1983).

Immunofluorescence

$1-2 \times 10^6$ MDCK cells were cultured on 24 mm transwell 0.4 μm filters (Costar) for 2–3 days. Before use, each filter was tested for tight junction formation, taken as an indication of polarization of the cells, by rinsing twice with PBS and then incubating the filters with 2 ml of PBS on the basal side and with 1 ml of PBS containing 1 μCi [^{3}H]inulin (Amersham) on the apical side at 4°C. After 30 min, samples from both sides were counted and filters were only used when the basal counts were <0.5% of the total applied. The filters were then rinsed in PBS, fixed in 3% paraformaldehyde for 10 min, blocked with 10 mM glycine pH 8.0, excised and cut into 4–6 sections. Immunofluorescence was performed as described by Isacke *et al.* (1990) with 0.2% saponin present throughout to maintain cell permeability. Microscopy was performed using a Nikon Optiphot microscope in conjunction with a Bio-Rad MRC600 confocal laser scanning unit and vertical (*xz*) sections were taken in 0.1 μm steps.

Detergent extraction of cell lines expressing CD44H mutants

Cells were grown to confluence on 35 mm tissue culture dishes, washed twice in ice cold Tris–saline (15 mM Tris pH 7.5 and 120 mM NaCl) and incubated in 0.4 ml extraction buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM Tris pH 7.5 and 150 mM NaCl with Triton X-100 at 0, 0.05, 0.1, 0.4 or 1.0%) on ice for 10 min with gentle rocking. The extraction buffer was removed and added to 80 μl of 6× non-reducing sample buffer (without bromophenol blue), the remaining cellular material was rinsed briefly with ice cold Tris–saline and scraped into 480 μl of 1× non-reducing sample buffer (without bromophenol blue). The protein content of each sample was measured using a Pierce BCA kit, bromophenol blue was added and the relative levels of CD44 expression assayed by immunoblotting.

Immunoblotting

Samples were sonicated for 10 s at 18 amplitude microns, 10 μl was resolved by electrophoresis on a mini 10% SDS–polyacrylamide gel and then blotted onto nitrocellulose membrane (Hybond C extra; Amersham). The membranes

were blocked for 30 min at room temperature in PBS containing 5% low fat milk powder and 0.2% Tween 20, incubated for 1 h in fresh blocking buffer containing an ascites preparation of E1/2 at a dilution of 1:1000, washed for 20 min in TBST (10 mM Tris pH 8.0, 150 mM NaCl and 0.05% Tween 20), incubated for 1 h at room temperature in blocking buffer containing 50 000 c.p.m./ml ^{125}I-labelled anti-mouse Ig and then washed for a further 20 min in TBST. ^{125}I was detected by exposure of the blot of X-ray film with an intensifying screen at −76°C.

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