

# 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

Stephen J. Neame and Clare M. Isacke

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

**Abstract.** A number of recent reports on the trafficking of receptor proteins in MDCK epithelial cells have provided evidence that delivery to the basolateral domain requires a specific targeting sequence and that deletion of this sequence results in constitutive expression on the apical surface. To date, these studies have concentrated on receptors which are competent for internalization via the clathrin coated pits. We have examined the localization of a resident plasma membrane protein by transfecting human CD44 into MDCK cells. Using human specific and cross-species reactive antibodies, we show that in MDCK cells both the endogenous and transfected wild-type CD44 are found on the basolateral surface where they are restricted to the lateral domain. Deletion of the CD44 cytoplasmic tail reduces the half life of this mutant protein and causes

it to be expressed both on the apical surface and to a significant extent within the cell. We have also used biochemical and morphological analysis to investigate the interaction of CD44 with the cytoskeleton in detergent extracted cells. Strikingly different extraction results were obtained between epithelial and fibroblast cells. However, there is no difference in the Triton X-100 solubility of the transfected wild-type and tail-less CD44 in fibroblasts and both forms of the protein remain associated with the cortical cytoskeleton after Triton X-100 extraction. These results demonstrate that the sequence present in the cytoplasmic domain of CD44 responsible for its distribution in epithelial cells is functionally and spatially separate from the ability of this protein to associate with the cytoskeleton.

CD44 is an abundant transmembrane glycoprotein which has been implicated to play a role in a number of important physiological processes including the homing of lymphocytes and prothymocytes, T cell activation, cell adhesion, cell migration and metastatic spread (reviewed in Haynes et al., 1989, 1991). The question arises as to how one molecule might fulfil these somewhat diverse functions.

One observed mechanism is the expression of multiple forms of the CD44 protein. The most abundant form of CD44 (CD44H) is an 80–100 kD glycoprotein present on fibroblasts and haematopoietic cells which functions as a receptor for the extracellular matrix glycosaminoglycan, hyaluronate (Aruffo et al., 1990; Culty et al., 1990; Lesley et al., 1990). Higher molecular weight CD44 forms have been detected on a variety of other cell types (Omary et al., 1988). cDNA cloning has demonstrated that these result from the insertion of a variable stretch of amino acids into the extracellular domain of CD44 (Brown et al., 1991; Dougherty

et al., 1991; Gunthert et al., 1991; Stamenkovic et al., 1991; Jackson et al., 1992) and in some cases these higher molecular weight forms have been demonstrated to have an altered behavior as compared to their CD44H counterpart (Gunthert et al., 1991; Stamenkovic et al., 1991; Sy et al., 1991; Arch et al., 1992).

A second potential mechanism for the regulation of CD44 function is via the interaction of its cytoplasmic tail with intracellular components as there is increasing evidence that the binding function of other adhesion molecules is regulated in this manner (Nagafuchi and Takeichi, 1988; Jaffe et al., 1990). Detergent extraction experiments (Tarone et al., 1984; Lacy and Underhill, 1987; Carter and Wayner, 1988; Camp et al., 1991), membrane mobility studies (Jacobson et al., 1984) and in vitro analysis (Kalomiris and Bourguignon, 1988; Lokeshwar and Bourguignon, 1991) have all suggested that CD44 is associated with the underlying cytoskeleton whereas the experiments of Lesley et al. (1992) have demonstrated a role for the CD44 cytoplasmic tail in the control of hyaluronate binding. The cytoplasmic tail of CD44 is highly conserved between species and subject to phosphorylation on serine residues (Carter and Wayner, 1988; Neame and Isacke, 1992).

Address correspondence to Clare M. Isacke, Department of Biology, Imperial College of Science, Technology and Medicine, Prince Consort Road, London SW7 2BB, United Kingdom.

Alho and Underhill (1989) have shown that CD44 is expressed on a variety of different epithelia *in vivo* but that it is particularly abundant on cells which are rapidly proliferating. They argue that this pattern of CD44 expression may be important in epithelial maturation and in this respect it is interesting that CD44 is found expressed at high levels in epithelial tumours (Stamenkovic et al., 1989; 1991). An understanding of the mechanisms regulating CD44 function may prove important in understanding normal epithelial biogenesis and the aberrant events associated with tumor growth. For this purpose we have taken the approach of studying the wild-type CD44H and a tailless CD44H mutant expressed in MDCK cells. MDCK cells provide an *in vitro* model of simple epithelia whereby an upper apical surface, which faces outwards in the body (e.g., towards the lumen of the kidney tubules), is delineated from the basolateral surface by the presence of tight junctions. The basolateral surface can be further divided into the lateral region (involved in cell-cell interactions) and the basal region (in contact with the underlying basal lamina). The formation of these functionally distinct domains is associated with the functional segregation of membrane proteins (reviewed in Nelson, 1989; Hopkins, 1991; Mostov et al., 1992). In the experiments described here, we demonstrate that the CD44 cytoplasmic tail plays an important role in CD44 distribution in epithelial cells but this function appears to be distinct from the ability of the CD44 protein to interact with the fibroblast cortical cytoskeleton.

## Materials and Methods

### Isolation of a Human CD44H cDNA Clone and Generation of CD44H Tailless Mutants

A cDNA clone encoding the human haematopoietic form of CD44 (CD44H) was isolated by PCR using oligonucleotides based upon the published sequence of Stamenkovic et al. (1989). The reaction conditions were as follows: 1  $\mu$ g each of oligonucleotides C3 (5'GGATCCTCCAGCTCC-TTTCG; bases 58 to 77) and C4 (5'GTCCAGCTCCCTGTAATGG; bases 1247 to 1266), 1  $\mu$ g  $\lambda$ ZAP human fibroblast cDNA library, 1 mM dNTPs (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), 2.5 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) in 2.5 mM MgSO<sub>4</sub>, 10 mM Tris pH 8.3, 50 mM KCl, 0.01% gelatin (final vol 50  $\mu$ l). PCR was performed using a Tempcycler (Coy Lab Products, Inc., Ann Arbor, MI) with 30 cycles of 1 min at 94°C, 2 min at 60°C and 2 min at 72°C followed by 30 min at 37°C with additional dNTPs and Klenow fragment of DNA polymerase I to fill in any single stranded ends. The reaction products were separated on a 1% agarose gel and a band with the expected size of 1199 bp was excised, purified and ligated into the Sma I site of pUC19. Restriction enzyme digests and partial sequencing were performed to check the orientation and identity of the CD44H insert.

To generate a CD44H tailless mutant, oligonucleotide C5 (5'CAGTCA-ACAGTCGAAGAAGGTGAGGGC; corresponding to bases 978 to 1004) as synthesized. A is a change from a T in the wild-type (WT)<sup>1</sup> CD44H sequence, changing a cys codon to a TGA stop codon. Under the same PCR conditions as described above using oligonucleotides C5 and C4 with a template of WT CD44H in pUC19, a 287-bp fragment was produced which encoded part of the putative transmembrane and COOH-terminal domain of CD44H including the desired mutation. This fragment was then digested with BstX I and Hinc II and the resulting 235-bp mutant fragment was isolated from a 1% agarose gel. WT CD44H in pUC19 was cleaved with BstX I and the 3474-bp and 411-bp fragments were purified, the 411-bp fragment was further digested with Hinc II giving a 176-bp fragment which was purified and a 235-bp fragment which was discarded. The 3474-bp and 176-

bp WT CD44H/pUC19 fragments and the mutated 235-bp fragment were then ligated. The WT CD44H and tailless (T-) CD44H were excised from the pUC19 vector with EcoR I and Xba I. A eukaryotic expression vector pSR $\alpha$ -neo (Takebe et al., 1988; DNAX, Palo Alto, California) was linearized with BamH I. 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. To generate a CD44H tailless variant, the WT CD44H cDNA in pUC19 was digested with Pst I and the 826- and 118-bp fragments were purified. The 118-bp fragment was treated with S I nuclease, end filled and digested with BstX I. The 826-bp fragment was digested with Taq I, the 641-bp fragment was purified, end filled, digested with BstX I and the 182-bp fragment was purified. CD44H cDNA in pSR $\alpha$ -neo was digested with BstX I, the large fragment consisting of the vector and much of the CD44H cDNA was purified and ligated with equimolar concentrations of the 118 bp BstX I digest and 182-bp fragment. Several of the ligation products were sequenced, one of these sequences encoded a tailless CD44H mutant in which the carboxy-terminal arg-arg-arg of the original T- CD44H had been substituted by arg-trp-thr. This second tailless mutant was designated PT16 CD44H.

### Cell Lines and Antibodies

All cell lines were maintained in DME supplemented with 10% FCS. F1084 human embryonic diploid fibroblasts and MDCK epithelial cells were obtained from C. R. Hopkins (University College, London). Swiss 3T3 fibroblasts were obtained from E. Rozengurt, (ICRF, London). Cell lines were transfected by electroporation using a modification of the method of Chu et al. (1987). 0.5-1.0  $\times 10^7$  MDCK or 2-4  $\times 10^6$  Swiss or NIH 3T3 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<sub>2</sub>HPO<sub>4</sub>, 6 mM D-glucose) and resuspended in 250  $\mu$ l of HeBS containing 20  $\mu$ g of the pSR $\alpha$ -neo construct. Electroporation was at 250  $\mu$ Farads, 270 V and infinite ohms in 0.4 cm cuvettes in a Gene Pulser (BioRad Laboratories, Richmond, CA). The cells were allowed to recover for 10 min and then plated into 10-cm tissue culture dishes in DME plus 10% FCS for 24-48 h. The cells were then trypsinized and replated into 96 well tissue culture plates at a range of densities (90, 9, and 1% of an electroporation per 96 well plate) in DME plus 10% FCS supplemented with 0.5 mg/ml (MDCK cells), 0.4 mg/ml (Swiss 3T3 cells), or 0.2 mg/ml (NIH 3T3 cells) of G418 (GIBCO-BRL, Gaithersburg, MD). G418 resistant clones were screened by immunofluorescence for expression of transfected human CD44H and positive clones were then maintained in 0.3 mg/ml (MDCK), 0.2 mg/ml (Swiss 3T3), or 0.1 mg/ml (NIH 3T3) of G418. For each of the transfected MDCK cell lines, at least five individual clones were characterized and for the transfected fibroblast cell lines, at least two individual clones were characterized. Where indicated, cultures of Swiss 3T3 cells in which 60-80% of the cells were expressing either WT, T- or PT16 forms of CD44H were produced by electroporating 5  $\times 10^6$  cells with 50  $\mu$ g of the pSR $\alpha$ -neo construct at 300 V and 250  $\mu$ Farads, culturing for 2-3 d in DME plus 10% FCS followed by 15-20 d in DME plus 10% FCS and 0.3 mg/ml G418.

E1/2.1 is a mouse mAb which is specific for human CD44 (Isacke et al., 1986). IM7.8.1 is a rat anti-CD44 mAb (Trowbridge et al., 1982). H68.4 is a mouse anti-transferrin receptor mAb (White et al., 1992) and anti-annexin II is a rabbit polyclonal antiserum (Cooper and Hunter, 1983). FITC and HRP-conjugated anti-mouse, anti-rat and anti-rabbit Ig and HRP-conjugated streptavidin were obtained from Jackson ImmunoResearch. Other antibodies were <sup>125</sup>I-labeled by the chloramine T method (Hunter and Greenwood, 1962).

### Immunofluorescence

For general studies cells were cultured on glass coverslips, fixed in 3% paraformaldehyde (Agar Scientific, Stansted, Essex, UK) for 10 min and blocked with 10 mM glycine pH 8.0. To look at polarized expression in MDCK cell clones, 1-2  $\times 10^6$  cells were cultured on 24 mm transwell 0.4- $\mu$ m filters (Costar Corp., Cambridge, MA) for 2-3 d and tested for tight junction formation as previously described (Neame and Isacke, 1992). Immunofluorescence was performed exactly as described by Isacke et al., (1990) with 0.2% saponin present throughout the experiment to permeabilize the cells. An ascites preparation of E1/2 was used at a dilution of 1:100, hybridoma supernatant preparations of IM7 were used at a dilution of 1:2 and FITC-conjugated anti-mouse or anti-rat Ig were used at 10  $\mu$ g/ml. Coverslips were mounted cells down on glass slides and filter sections were placed cells upward in mounting medium on glass slides and then sealed under a coverslip. Microscopy was performed using a Leitz Aristoplan

1. *Abbreviations used in this paper:* ECL, enhanced chemiluminescence; LDL, low density lipoprotein; WT, wild-type.

fluorescence microscope, Nikon Microphot SX or a Nikon Optiphot microscope in conjunction with a confocal laser scanning unit (model MRC600, BioRad Laboratories).

### Biotinylation of Plasma Membrane Proteins

1–2 × 10<sup>6</sup> MDCK cells were cultured for 3–4 d on transwell filters and the integrity of the tight junctions was checked. Duplicate filters were washed twice in PBS, incubated in PBS with 0.5 mg/ml NHS-SS-Biotin (Pierce Chemical Co., Rockford, IL) on either the apical or basal side for 30 min at 4°C, washed twice in 0.2 M glycine in PBS and lysed in 0.4 ml NDET (1% Nonidet P40, 0.4% sodium deoxycholate, 66 mM EDTA, 10 mM Tris pH 7.4). Lysates were incubated on ice for 15 min, clarified by centrifugation at 12,000 g for 10 min, the supernatants were removed to a fresh tube and SDS added to a concentration of 0.3%. All subsequent procedures were carried out on ice. Lysates were precleared by incubation with 100 µl of fixed *Staphylococcus aureus* bacteria (Pansorbin, Calbiochem Corp., La Jolla, CA) for 30 min on ice, human CD44H was immunoprecipitated by incubating the lysates with mAb E1/2 for 60 min and then with anti-mouse Ig bound to 50 µl protein-A agarose (BioRad Laboratories) for 60 min. The immunoprecipitates were washed four times in NDET plus 0.3% SDS, resuspended in nonreducing sample buffer, boiled for 2 min, resolved by electrophoresis on a 10% SDS polyacrylamide gel and then transferred to nitrocellulose membranes (Hybond C extra, Amersham Corp., Arlington Heights, IL). The membranes were blocked in PBS containing 5% low fat milk powder and 0.2% Tween 20 for 20 min and then incubated in fresh blocking solution with HRP-conjugated streptavidin at 1 µg/ml for 1 h at room temperature. Membranes were washed for 20 min in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20) and HRP-streptavidin was visualized using an enhanced chemiluminescence (ECL) kit (Amersham Corp.) and exposure to x-ray film (Fuji XR) at room temperature.

### Determination of the Transfected CD44H Half Life

3 × 10<sup>6</sup> MDCK cells were plated into 35-mm tissue culture dishes and incubated overnight in low calcium (5 µM CaCl<sub>2</sub>)-DME plus 10% dialyzed FCS. The cells were then washed twice with high calcium (1.8 mM CaCl<sub>2</sub>) DME without cysteine or methionine (DME-cys/met), and then incubated in 1 ml of (DME-cys/met) plus 5% dialyzed FCS, 0.3 mCi <sup>35</sup>S-trans label (ICN Biomedicals, Inc., Costa Mesa, CA) and 5% complete DME for 18 h. Cells were then washed and incubated in complete DME plus 10% FCS for a further 0 to 24 h. Cells were lysed in 0.4 ml NDET, CD44H was immunoprecipitated as described above, solubilized in reducing sample buffer and separated on a 10% SDS-polyacrylamide gel. The gel was fixed, enhanced in 1 M sodium salicylate for 40 min, dried and exposed to x-ray film at -76°C. Labeled protein was quantified by excising sections of the polyacrylamide gel, rehydrating in 50–100 µl H<sub>2</sub>O, incubating in 0.45 ml NCS tissue solubilizer (Amersham Corp.) for 24 h at room temperature followed by 1 h at 45°C, neutralizing with the addition of 25 µl glacial acetic acid and counting in 4.5 ml Aquasint H (National Diagnostics Inc., Manville, NJ) scintillation fluid.

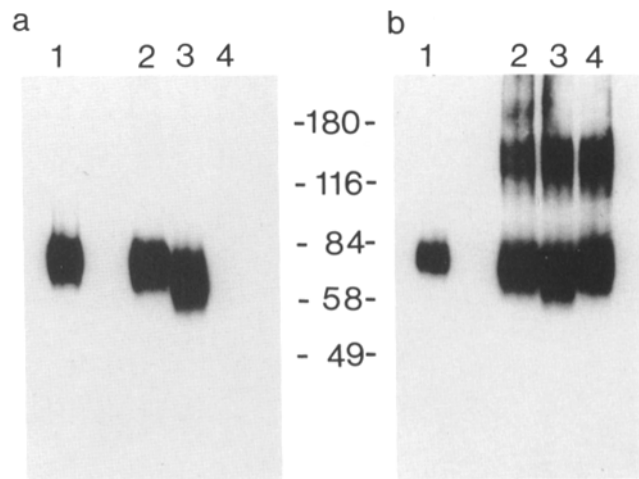
### Detergent Extraction of Cells

Cells were grown to confluence on 35-mm tissue culture dishes, extracted with varying concentrations of Triton X-100 and the distribution of protein between the detergent soluble and insoluble fractions was assayed by immunoblotting as previously described (Neame and Isacke, 1992). CD44 was detected using an ascites preparation of mAb E1/2 at a dilution of 1:2,500 or mAb IM7 hybridoma supernatant at a dilution of 1:4, transferrin receptor and annexin II were detected using mAb H68.4 at 0.2 µg/ml and anti-annexin II polyclonal antiserum at a dilution of 1:10,000, respectively followed by <sup>125</sup>I-anti-mouse or anti-rat Ig (50,000 cpm/ml) or HRP-conjugated anti-mouse or anti-rabbit Ig (0.2 µg/ml). <sup>125</sup>I was detected by exposure of the blot to x-ray film with an intensifying screen at -76°C, HRP-antibodies were visualized using the ECL kit.

## Results

### Expression of Wild-type CD44H and a Tailless (T-) Mutant in MDCK Cells

A cDNA clone encoding the haematopoietic form of human CD44 (CD44H) was isolated and a tailless (T-) mutant was

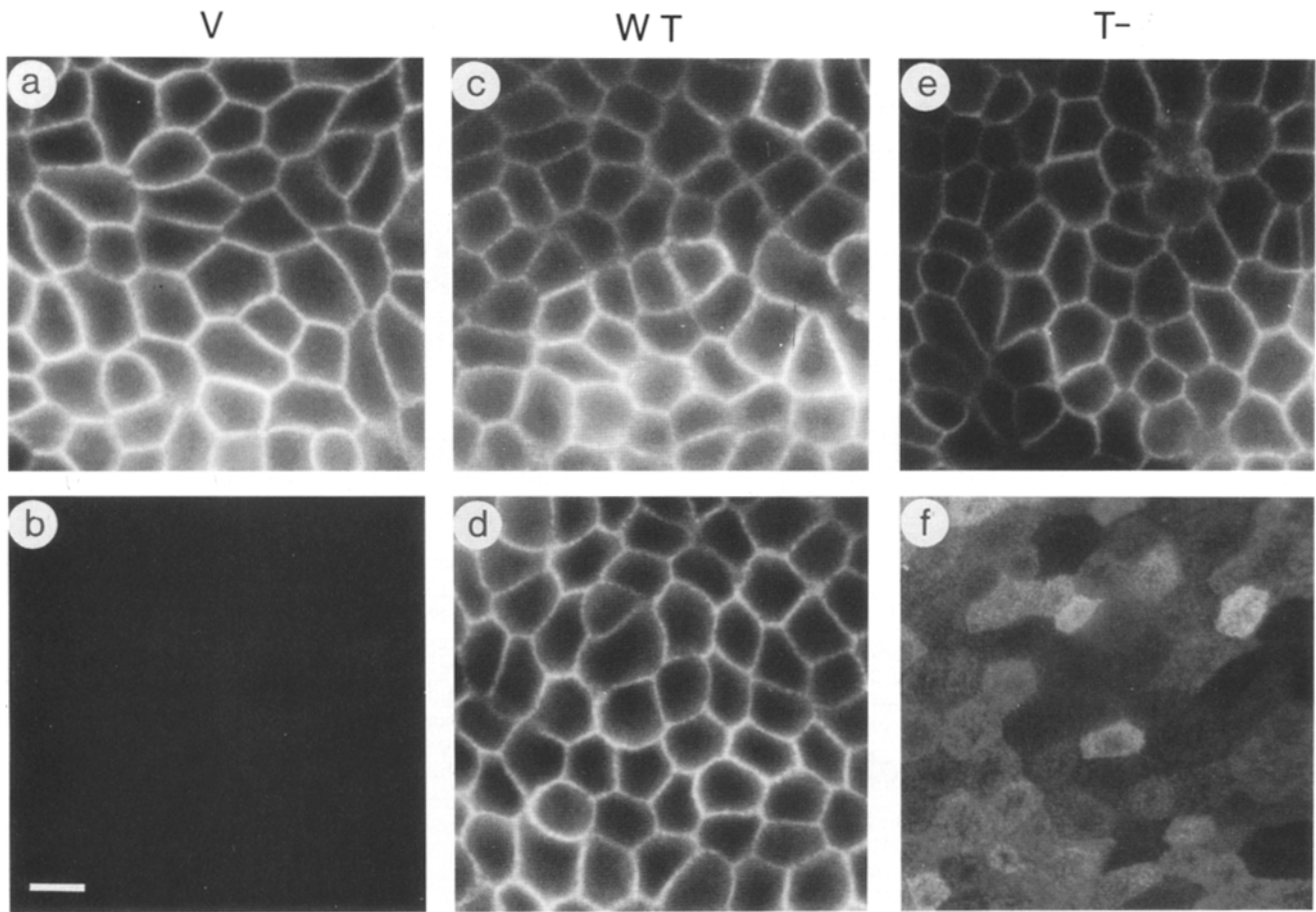


**Figure 1.** Expression of CD44 in transfected MDCK cell lines. Semi-confluent cultures of MDCK cells were lysed in nonreducing sample buffer and samples containing 20 µg of protein were run in duplicate lanes on a 10% polyacrylamide gel and blotted onto nitrocellulose. *a* was probed with the human specific anti-CD44 mAb, E1/2, and an HRP-conjugated anti-mouse Ig. *b* was probed with a species cross-reactive anti-CD44 mAb, IM7, and an HRP-conjugated anti-rat Ig. (Lane 1) F1084 human fibroblasts; (lane 2) clonal MDCK cell line expressing human WT CD44H; (lane 3) clonal MDCK cell line expressing human T-CD44H; (lane 4) clonal MDCK cell line transfected with pSRα-neo vector alone. Blots were developed with the ECL reagent and exposed to x-ray film for 30 s. Molecular size markers are in kilodaltons.

generated whereby a cysteine codon located three amino acids after the putative transmembrane domain was changed to a TGA stop codon. For permanent expression, both the WT and T-CD44H were inserted into the eukaryotic expression vector, pSRα-neo, and transfected into MDCK epithelial cells. G418 resistant clonal cells lines were tested for expression using the E1/2 mAb which is specific for human CD44 and does not react with the endogenous canine CD44. Immunoblots of MDCK cells transfected with the human WT CD44H demonstrates the presence of a protein species with mol wt 80–90 kD similar to that recognized in human fibroblasts (Fig. 1). Cells transfected with the T-CD44H mutant produce a protein with reduced molecular weight consistent with the loss of a 7-kD cytoplasmic domain. Probing parallel immunoblots with a species cross-reactive antibody, mAb IM7, demonstrates that MDCK cells express two major forms of CD44. Approximately 30–50% of the CD44 is the haematopoietic form and 50–70% resolves as higher molecular weight proteins consistent with the presence of the alternatively spliced forms of CD44 previously observed in epithelial cells (Omary et al., 1988; Stamenkovic et al., 1991; Jackson et al., 1992; Fig. 1).

### Localization of WT and T-CD44H in MDCK Monolayers

Transfected MDCK cells were examined by immunofluorescence to determine the cellular localization of the endogenous and human CD44 protein. Initially, the cells were cultured to confluence on glass coverslips and stained with either mAb IM7 or mAb E1/2 (Fig. 2). It can be seen that



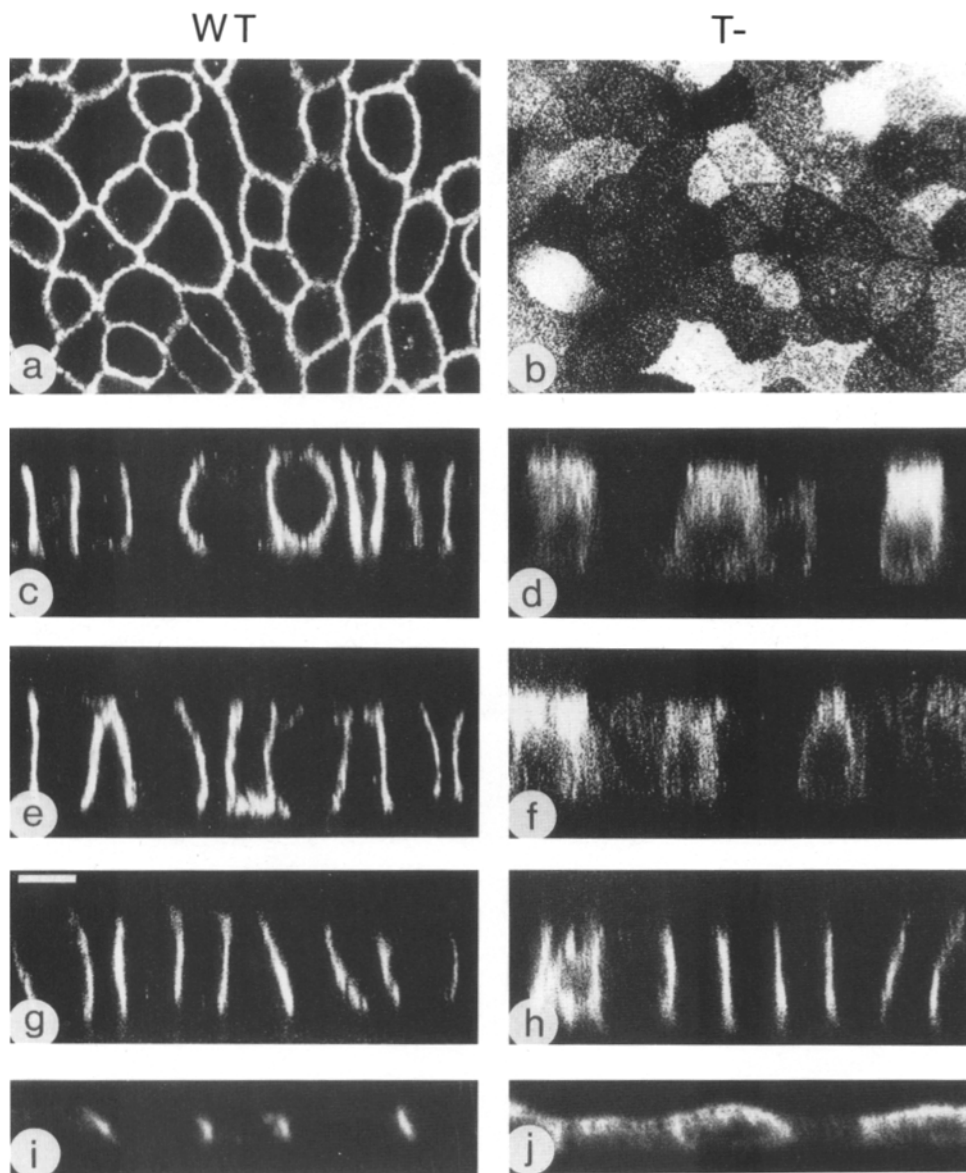
**Figure 2.** Localization of CD44 in polarized MDCK cells by fluorescence microscopy. Clonal MDCK cell lines transfected with pSR $\alpha$ -neo vector alone (*a* and *b*), WT CD44H (*c* and *d*) or T- CD44H (*e* and *f*) were cultured to confluence on glass coverslips and then stained with mAb IM7 and an FITC-anti-rat Ig (*a*, *c*, and *e*) or mAb E1/2 and an FITC-anti-mouse Ig (*b*, *d*, and *f*). Bar, 10  $\mu$ m.

the endogenous canine CD44, recognized by mAb IM7, is localized to the lateral surface and focusing through the monolayer showed little or no basal or apical staining. An identical pattern of staining is observed with mAb E1/2 in cells transfected with the human WT CD44H. From these data it appears that the presence of additional exons in the extracellular domain of the epithelial form of CD44 does not alter its pattern of cellular expression. By contrast, removal of the cytoplasmic tail dramatically alters the localization of the protein in MDCK cells. There is little evidence of basolateral staining, rather the T- protein appears to be localized to the apical plasma membrane. This was confirmed by repeating this experiment with non-permeabilized cells (data not shown). In addition, there is a distinct punctate cytoplasmic distribution of the T- CD44H protein with the nuclei being clearly defined. Finally, we consistently find that the expression of the T- CD44H in these confluent monolayers exhibits cell to cell heterogeneity.

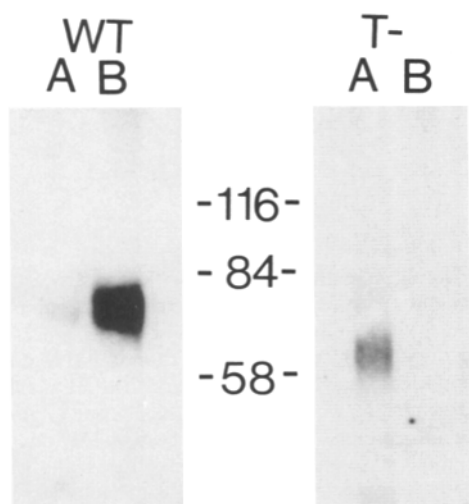
To study these distributions in more detail, transfected cells cultured on transwell filters were examined in the confocal microscope. The confocal microscope allows optical sections to be cut either in the horizontal plane (XY section; see Fig. 3, *a* and *b*) or in the vertical plane (XZ section). The XZ sections show that both the endogenous and transfected WT protein (Fig. 3, *c*, *e*, *g*, and *h*) are tightly confined to

the lateral membrane with no detectable CD44 on the apical or basal plasma membranes. To ensure that the lack of observable basal WT CD44H staining was not due to interference from the nitrocellulose filter, vertical XZ confocal sections were also examined on cells cultured to confluence on glass coverslips. Under these culture conditions, the MDCK cells do not achieve the height observed with cells cultured on filters. Again, only lateral staining of the WT CD44H is observed (Fig. 3 *i*) even though the mAb clearly penetrates the permeabilized cell monolayer to the level of the coverslip. The T- CD44H (Fig. 3, *d*, *f*, and *j*) is observed on the apical surface but it is not restricted to the plasma membrane as punctate intracellular staining is seen in the apical half of the cell with the nucleus clearly excluded. Again, these sections show the cell to cell heterogeneity of the T- CD44H expression. The distribution of WT CD44H and T- CD44H described here was identical in all individual clonal transfectants examined.

To quantify the expression of WT and T- CD44H to the different plasma membrane domains of epithelial cells, confluent monolayers of transfected cells were biotinylated on either the apical or basolateral surfaces as described in Materials and Methods. The cells were then lysed, the transfected CD44H was immunoprecipitated and the extent of biotinylation was assessed by probing nitrocellulose blots with



**Figure 3.** Localization of CD44 in polarized MDCK cells by confocal microscopy. Clonal transfected MDCK cell lines expressing WT CD44H (a, c, e, g, and i) or T-CD44H (b, d, f, h, and j) were cultured to confluence on transwell filters (a-h) or glass coverslips (i and j) and stained with mAb E1/2 and FITC-anti-mouse Ig (a-f, i and j) or mAb IM7 and FITC-anti-rat Ig (g and h). a and b show XY sections through the cells in the same plane as shown in Fig. 2. The lower panels (c-j) show XZ sections taken in 0.1  $\mu\text{m}$  steps through the cells at 90° to the XY sections; the apical region of the cells is orientated topmost. Bar, 10  $\mu\text{m}$ .



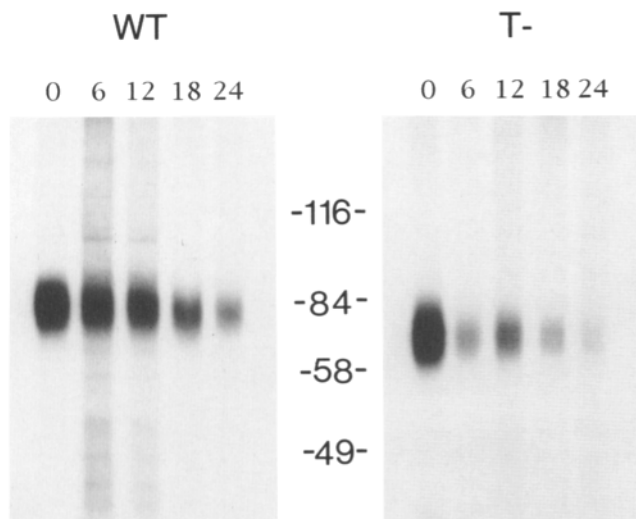
**Figure 4.** Cell surface biotinylation of CD44H in polarized MDCK cells. Clonal transfected MDCK cell lines expressing WT CD44H or T-CD44H were grown to confluence on duplicate transwell fil-

HRP-conjugated streptavidin (Fig. 4). In agreement with the microscopic observations, <7% of the WT CD44H is accessible to biotinylation from the apical side and conversely, <5% of the T-CD44H is accessible to biotinylation from the basolateral side.

#### ***The Cytoplasmic Tail of CD44H Is Important for Protein Stability***

Mistargeting of proteins in epithelial cells can lead to reduced stability (Wollner et al., 1992). To determine whether this is true for the T-CD44H, transfected MDCK cells were labeled to steady state with  $^{35}\text{S}$ -*trans* label and then chased

and biotinylated either from the apical (A) or from the basolateral (B) side. CD44H was immunoprecipitated with mAb E1/2, resolved on a 10% polyacrylamide gel and blotted onto nitrocellulose. Biotinylated protein was visualized using HRP-streptavidin and the ECL reagent. Blots were exposed to x-ray film for 1 h. Molecular size markers are in kilodaltons.

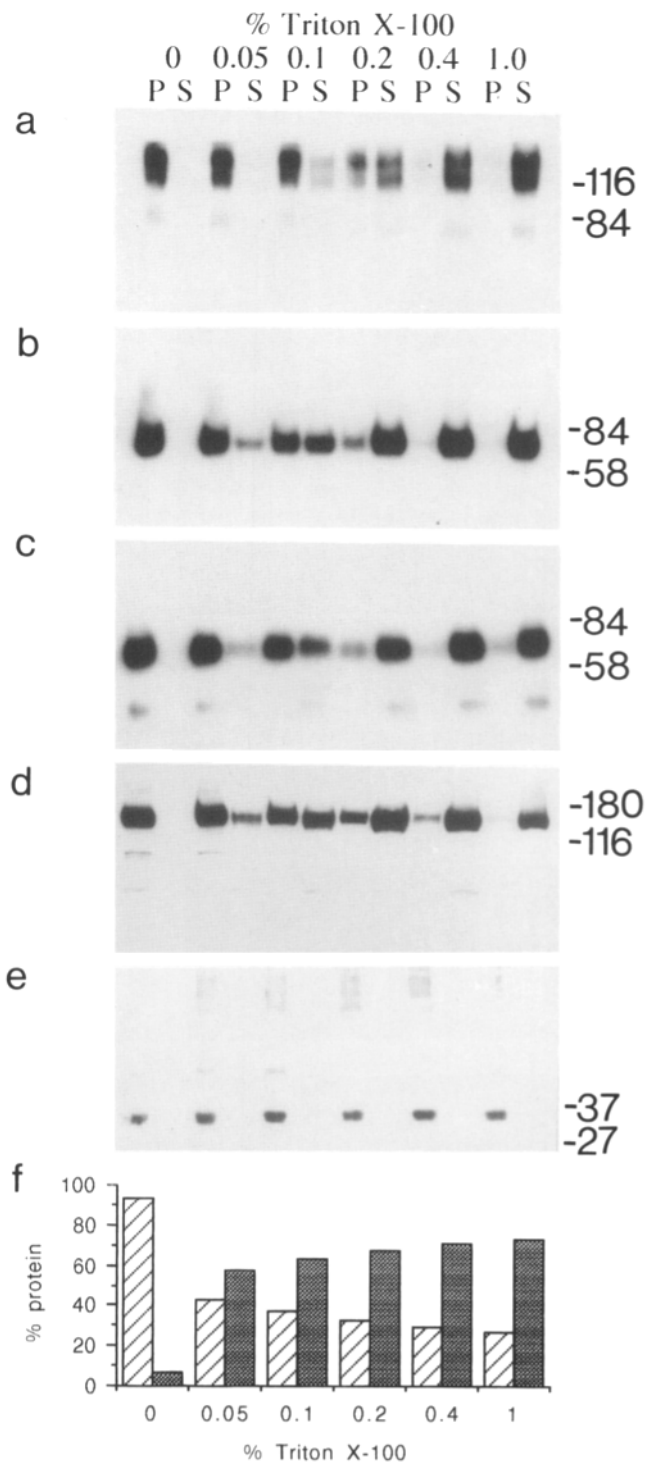


**Figure 5.** Half life determination of WT and T- CD44H. Clonal transfected MDCK cell lines expressing WT CD44H or T- CD44H were labeled with  $^{35}\text{S}$ -*trans* label to steady state and then chased in unlabeled medium for 0–24 h as described in Materials and Methods. CD44 was immunoprecipitated with mAb E1/2 and resolved on a 10% polyacrylamide gel. Enhanced gels were exposed to x-ray film for 5 d. Molecular size markers are in kilodaltons. CD44 protein was excised from the gel, solubilized and the amount of label incorporated measured by scintillation counting.

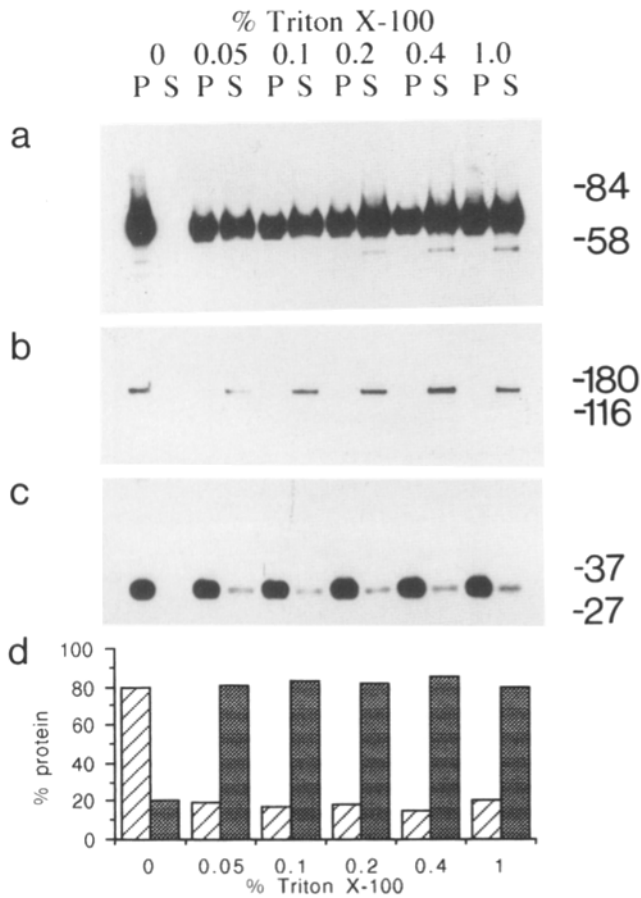
in unlabeled medium for 0–24 h (Fig. 5). Quantification of this data, and that from separate experiments, indicates that the WT CD44H has a half life of 15–17 h. The stability of the T- CD44H is two- to fourfold reduced compared to its WT CD44H counterpart.

#### **The Cytoplasmic Tail of CD44H Is Not Required for Interaction with the Cytoskeleton**

Several laboratories have previously demonstrated that a proportion of the CD44H population is resistant to 0.5–1% Triton X-100 detergent extraction. These and other experiments have led to the suggestion that CD44 is associated with the underlying cytoskeleton. To determine whether the removal of the cytoplasmic tail or the presence of additional exons in the higher molecular weight epithelial forms of CD44 alters this association with the cytoskeleton, MDCK cell transfectants were incubated in varying concentrations of Triton X-100 and the amount of CD44 in the detergent soluble and insoluble extracts was assayed by immunoblotting. All forms of endogenous canine CD44 are completely extracted from the MDCK cells with 0.4% Triton X-100 (Fig. 6 *a*). Under these experimental conditions, using three independently isolated clonal cell lines for each transfectant, we were unable to detect any reproducible differences between the detergent extraction profile of the endogenous canine CD44 (Fig. 6 *a*), the transfected WT CD44H (Fig. 6 *b*), the T- CD44H (Fig. 6 *c*) and endogenous canine transferrin receptor (Fig. 6 *d*). As a control for these experiments, the detergent solubility of annexin II (previously known as p36, calpactin I and lipocortin II) was assayed in parallel. Annexin II is known to associate with the cortical cytoskeleton (Greenberg and Edelman, 1983; Lehto et al., 1983;



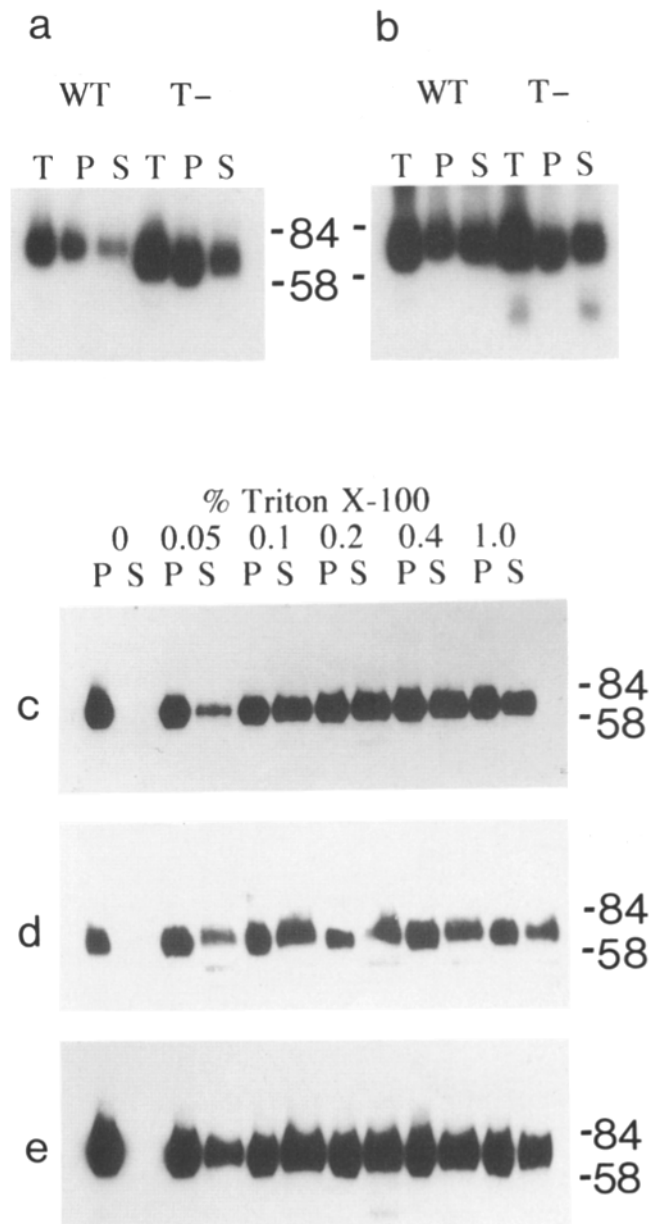
**Figure 6.** Detergent extraction of CD44H from MDCK cells. Confluent cultures of MDCK cell clones transfected with pSR $\alpha$ -neo vector alone (*a*), WT CD44H (*b*) or T- CD44H (*c-e*) were extracted with varying concentrations of Triton X-100 as detailed in Materials and Methods. The insoluble cell pellet (P) and the detergent soluble extract (S) were resolved on 10% polyacrylamide gels, blotted onto nitrocellulose and probed with mAb IM7 and  $^{125}\text{I}$ -anti-rat Ig (*a*), mAb E1/2 and  $^{125}\text{I}$ -anti-mouse Ig (*b* and *c*), H68.4 anti-transferrin receptor mAb and HRP-anti-mouse Ig (*d*), and anti-annexin II antiserum and HRP-anti-rabbit Ig (*e*). Blots *a-c* were exposed to x-ray film for 1–3 d; blots *d* and *e* were visualized using an ECL kit and exposed to x-ray film for 60 and 10 min, respectively. Molecular size markers are in kilodaltons. *f* shows the



**Figure 7.** Detergent extraction of CD44H from human fibroblasts. Parallel dishes of F1084 human fibroblasts were extracted with varying concentrations of Triton X-100 and the amount of CD44H (a), transferrin receptor (b) and annexin II (c) in the insoluble cell pellet (P) and detergent soluble extract (S) was assayed. d shows the average protein content of the insoluble cell pellet (P: hatched bars) and detergent soluble extract (S: shaded bars) as a percentage of total protein, i.e., S + P. Molecular size markers are in kilodaltons.

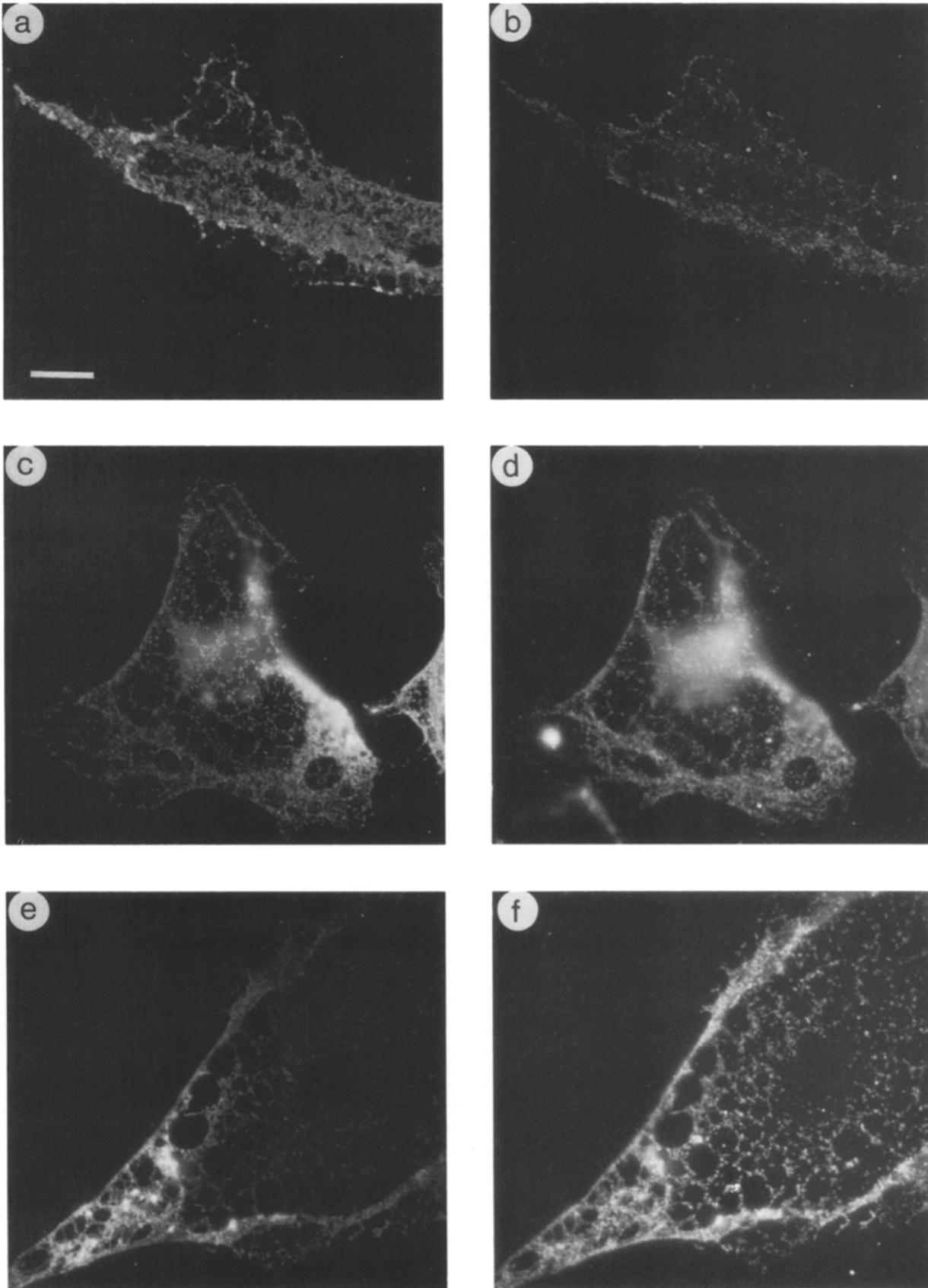
Nigg et al., 1983; Gerke and Weber, 1984) and in MDCK cells, >90% of the annexin II is insoluble in the presence of 0.4% Triton X-100 (Fig. 6 e). As these CD44 extraction results were in disagreement with data from other laboratories it was important to repeat these experiments in another cell type. Initially, F1084 human fibroblasts, which have endogenous expression of CD44H, were examined. Approximately 50% of the F1084 CD44H remains in the detergent insoluble cell pellet in the presence of 0.4% Triton X-100 while 75–85% of the cell protein is extracted (Fig. 7, a and d). In parallel immunoblots, in the presence of 0.4% Triton X-100 >90% of the transferrin receptor is detergent soluble (Fig. 7 b) whereas >90% of the annexin II remains in the detergent insoluble cell pellet (Fig. 7 c). The solubility of the

average protein content of the insoluble cell pellet (P: hatched bars) and detergent soluble extract (S: shaded bars) as a percentage of total protein, i.e., S + P. Each value varied <7% between the different cell lines.



**Figure 8.** Detergent extraction of CD44H from transfected murine fibroblasts. Parallel dishes of clonal Swiss 3T3 cell lines (a) and NIH 3T3 cell lines (b) expressing WT and T- CD44H were either lysed whole or extracted with 0.4% Triton X-100. The amount of CD44H in the total cell lysate (T), insoluble cell pellet (P), and detergent soluble extract (S) was assayed. Parallel dishes of Swiss 3T3 cells in which 60–80% of the cells were expressing WT (c), T- (d) or PT16 tailless (e) CD44H were extracted with varying concentrations of Triton X-100 and the amount of CD44H in the insoluble cell pellet (P) and detergent soluble extract (S) was assayed. Molecular size markers are in kilodaltons.

transferrin receptor suggests that under these experimental conditions the fibroblast plasma membrane is fully soluble and that the retention of CD44 does not result from lipid entrapment as has been recently reported for certain GPI anchored proteins (Brown and Rose, 1992). Swiss 3T3 and NIH 3T3 fibroblasts transfected with the human WT or T- forms of CD44H were then examined under the same con-



**Figure 9.** Localization of CD44H and annexin II in detergent extracted fibroblasts by fluorescence microscopy. F1084 human fibroblasts (*a* and *b*) and Swiss 3T3 cells expressing WT (*c* and *d*) or T- (*e* and *f*) CD44H were cultured at low density on glass coverslips, extracted with 0.4% Triton X-100, fixed with paraformaldehyde and stained with E1/2 anti-CD44 mAb and anti-annexin II antiserum followed by noncross-reactive FITC-anti-mouse and rhodamine-anti-rabbit Ig. The CD44 associated FITC stain is shown in *a*, *c*, and *e* while the anti-annexin II associated rhodamine stain is shown in *b*, *d*, and *f*. Bar, 10  $\mu$ m.



ditions. No difference could be detected between the detergent solubility of the WT and T- CD44H (Fig. 8) with >50% of both the WT and T- CD44H remaining in the detergent insoluble cell pellet in the presence of 0.4% Triton X-100. The T- CD44H mutant contains three carboxy terminal arginine residues after the predicted transmembrane domain. To determine whether these basic residues were responsible for the detergent insolubility of the T- CD44H, a second tailless mutant, PT16 CD44H, was generated whereby the three arginine residues were replaced by arg-trp-thr-COOH. No difference was detected in the detergent solubility of the PT16 CD44H and T- CD44H (Fig. 8, *d* and *e*).

To further investigate the association of endogenous and transfected CD44 with the cytoskeleton, fibroblast cells extracted with detergent were examined by immunofluorescence (Fig. 9). The staining pattern of both WT and T- CD44H in detergent extracted fibroblasts was in a three dimensional punctate reticulum which defined the edges of the cell. A significant degree of colocalization was observed between CD44 and cytoskeletally-associated annexin II.

## Discussion

### *Removal of the CD44 Cytoplasmic Tail Results in a Redistribution of the Protein in MDCK Cells*

In this paper we have examined the influence of the cytoplasmic domain on the behavior of CD44H. Biochemical and morphological data (Figs. 2, 3, and 4) demonstrate that both CD44H and the higher molecular weight epithelial forms of CD44 are localized to the basolateral surface of MDCK cells. However, a mutant of CD44H which lacks the cytoplasmic tail shows a different distribution in that it is found on the apical plasma membrane and a proportion within the cell.

The basolateral localization of the WT CD44H suggests that it is either directly targeted to this site or it is initially delivered elsewhere and rapidly redistributed basolaterally. By contrast, the T- form must be either excluded from the basolateral membranes or rapidly redistributed subsequent to basolateral delivery. This implies that the CD44 cytoplasmic tail contains sequences required for basolateral targeting and/or stabilization in the basolateral membrane. CD44H is normally a resident plasma membrane protein (Bretscher et al., 1980; Jacobson et al., 1984) and therefore, it cannot be involved in endocytotic events usually associated with the rapid redistribution of transmembrane proteins from one plasma membrane domain to another. This suggests that the steady state distribution of CD44 is likely to be determined by its primary targeting. Recent experiments have demonstrated the existence of specific basolateral targeting sequences (reviewed Hopkins, 1991; Mostov et al., 1992) which in turn suggest that the T- CD44H cannot reach its final destination via the basolateral domain. Mutational analysis of basolaterally targeted proteins has suggested that they fall into two classes; (*a*) those such as the IgG Fc receptor and lysosomal membrane glycoprotein, lgp 120, where the basolateral targeting information is intimately linked to the coated pit internalization signal (Hunziker et al., 1991), and (*b*) proteins such as the low density lipoprotein (LDL) receptor and the polymeric IgA/IgM (pIg) receptor where

the basolateral sorting signal is spatially distinct from the internalization signal (Casanova et al. 1991; Hunziker et al. 1991; Yokode et al., 1992). Sequence comparison of the region encompassing the basolateral sorting signal in the LDL and pIg receptors shows a 10 amino acid consensus (Yokode et al., 1992). An examination of the CD44 cytoplasmic tail shows no evidence for a clathrin coated pit internalization signal. There is a partial sequence homology at the extreme carboxyl terminus of CD44 with the potential basolateral sorting consensus region and a somewhat stronger homology when compared directly to the rabbit poly Ig receptor. Central to this partial homology is the amino acid stretch asn-val-asp (amino acids 354-356 in human CD44H).

### *CD44 is Restricted to the Lateral Surface of Polarized MDCK Cells*

In MDCK cells, the majority of the basolateral CD44, both CD44H and the alternatively spliced epithelial forms of CD44, is found on the lateral membranes with little being apparent on the basal membranes (Figs. 2 and 3). This suggests that CD44 might play a role in cell to cell interaction in epithelial cells. Other laboratories have reported that a significant proportion of CD44 is associated with the cytoskeleton (Jacobson et al., 1984; Tarone et al., 1984; Lacy and Underhill, 1987; Carter and Wayner, 1988; Kalomiris and Bourguignon, 1988; Camp et al., 1991; Lokeshwar and Bourguignon, 1991). Initially we investigated the possibility that the lateral distribution of CD44 was due to an interaction with the cytoskeleton by extracting the MDCK cells with detergent. These experiments gave an unexpected result. All the endogenous canine CD44 forms and the transfected WT and T- CD44H were completely extracted from the MDCK cells in 0.4% Triton X-100 (Fig. 6). This result is not due to an inability of MDCK proteins to form Triton X-100 insoluble interactions as Salas et al. (1988) and Nelson et al. (1990) have demonstrated that a number of cytoskeletally associated plasma membrane proteins in MDCK cells are resistant to detergent extraction. Further, in our MDCK cell experiments, we have demonstrated that a cytoskeletally associated protein, annexin II, is essentially detergent insoluble. Although the CD44 proteins are fully extracted in 0.4% Triton X-100, they are mostly insoluble in 0.05% Triton X-100. At this lower detergent concentration almost 60% of the total cellular protein is released into the soluble fraction. One interpretation of these results is that CD44 does interact with the MDCK cytoskeleton albeit in a manner that it sensitive to higher detergent concentrations. However, a transmembrane protein which does not associate with the cytoskeleton, the transferrin receptor, showed a detergent extraction profile similar to the CD44 proteins (Fig. 6). These data suggests that in MDCK cells, CD44 has no detergent insoluble association with the cytoskeleton and therefore such an association cannot regulate the tight lateral restriction of CD44.

An alternative possibility for the control of CD44 localization to the lateral membrane is an interaction between the extracellular domain and proteins expressed on neighboring cells or an element of the extracellular matrix found only in these lateral regions. CD44H is a receptor for hyaluronate (Aruffo et al., 1990; Lesley et al., 1990) but binding to hyaluronate is unlikely to be the main mechanism for deter-

mining lateral membrane localization in MDCK cells as (a) the higher molecular weight epithelial forms of CD44 have been shown to have a diminished ability to bind hyaluronate (Stamenkovic et al., 1991) and yet they are tightly localized to the lateral surface, and (b) when intestinal epithelial sections are examined, CD44 can be clearly observed on the lateral membranes whereas hyaluronate is excluded from this area but is abundant in the basement membranes (Alho and Underhill, 1989). CD44 has been reported to bind to the extracellular matrix molecules, type I and type IV collagen (Wayner and Carter, 1987; Faassen et al., 1992) and fibronectin (Jalkanen and Jalkanen, 1992) and to a heterotypic ligand on the plasma membrane of mouse fibroblasts cells (St. John et al., 1990). It remains to be determined whether any of these types of interaction might have some role in localizing CD44 to the lateral membrane of MDCK cells as opposed to the general basolateral domain.

### ***The Association of CD44 with the Cytoskeleton Does Not Require the Cytoplasmic Tail and Is Dependent upon Cell Type***

The detergent extraction experiments were extended to look at CD44 in fibroblasts. In agreement with data from other laboratories, a significant proportion of the endogenous CD44H in human fibroblasts is resistant to Triton X-100 extraction (Fig. 7) suggesting that in these cells, CD44 can associate with the cytoskeleton. To further investigate this phenomena we examined the distribution of CD44 in detergent extracted human fibroblasts by immunofluorescence. Carter and Wayner (1988) have previously described the distribution of CD44 in detergent extracted WI-38 human fibroblasts as having a "distinct fibrillar pattern defining the cell periphery and many closed loops over the cells body" and an essentially similar distribution was observed in our F1084 human fibroblast experiments (Fig. 9). This fibrillar pattern does not codistribute with fibronectin, collagen or actin containing stress fibres (Carter and Wayner, 1988) although disruption of the actin cytoskeleton does increase the amount of detergent soluble CD44 (Lacy and Underhill, 1987). Using CD44 reconstituted into liposomes, Kalomiris and Bourguignon (1988) have demonstrated an in vitro association of CD44 with ankyrin while Carter and Wayner (1988) have demonstrated a codistribution of CD44 with the intermediate filament protein, vimentin. There is evidence that ankyrin and vimentin can interact directly with each other (Georgatos and Marchesi, 1985) and also with spectrin, a major component of the cortical cytoskeleton (Mangeat and Burrige, 1984). As annexin II is known to codistribute with spectrin (Greenberg and Edelman, 1983; Lehto et al., 1983; Gerke and Weber, 1984), we performed double-labeled immunofluorescence experiments with an anti-annexin II antisera. A considerable degree of overlap was observed between the staining patterns for CD44 and annexin II (Fig. 9) implying that the detergent insoluble pool of CD44 is associated with the submembraneous skeleton of fibroblasts.

Deletion of the CD44 cytoplasmic tail does not alter its detergent extraction profile (Fig. 8) nor the distribution of the protein in Triton X-100 treated cells (Fig. 9). In a recent report, Carpen et al. (1992) have demonstrated that  $\alpha$ -actinin can bind to the cytoplasmic domain of ICAM-1 and that this

binding can be mimicked using a 5 amino acid positively charged peptide identical to a short ICAM-1 sequence close to the transmembrane spanning domain. To exclude the possibility that the interaction of T-CD44H with the cytoskeleton was mediated by a similar mechanism, a second tailless mutant, PT16 CD44H, was examined and shown to have properties indistinguishable from the T-CD44H. (Fig. 8, *d* and *e*). This leads to the conclusion that the cytoplasmic domain is not responsible for the detergent insoluble interaction and that this function must therefore be mediated by some other portion of the CD44 molecule. This interaction could be either direct, as has been suggested for the CD8 molecule (André et al., 1991), or indirect, for example through co-association with the extracellular matrix. This latter possibility is not supported by experiments from other laboratories where extracellular matrix components including hyaluronate were removed by enzyme digestion with little effect upon the detergent extraction profile of CD44 (Tarone et al., 1984; Lacy and Underhill, 1987).

A comparison between the detergent extraction of CD44 in the epithelial and fibroblast cell lines (Figs. 6, 7, and 8) clearly demonstrates that in the presence of 0.4% Triton X-100, a significant proportion of fibroblast CD44 remains in the detergent insoluble cell pellet whereas in epithelial cells essentially all the CD44 is detergent soluble. These results imply that whatever the mechanism is by which CD44 is associated with the cytoskeleton in fibroblasts, this mechanism does not operate in MDCK cells. If a second protein interfaces between CD44 and the fibroblast cytoskeleton then the absence of such a protein, or its modification, could explain the results obtained with the MDCK cells. In this context it is interesting that Camp et al. (1991) have shown that in resident peritoneal macrophages there is a population of detergent insoluble CD44 while in elicited macrophages all of the CD44 is detergent soluble.

### ***A Population of T-CD44H is Localized Intracellularly***

In immunofluorescence experiments we observe a proportion of T-CD44H localized with a distinct punctate distribution within MDCK cells. This distribution suggests that intracellular T-CD44H probably resides in membranes of small vesicles. Immunoblotting of transfected cells shows that there is a smaller molecular weight form of T-CD44H (see Figs. 6 *c* and 7 *f*; mol wt 55–65 kD) which is found in all the T-CD44H cell lines, however, the presence of this form is variable and may be dependent on culture condition. What is known is that this smaller form is not expressed on the cell surface as it is never detected when cells are surface labeled with biotin or  $^{125}\text{I}$ , it is consistently more susceptible to detergent extraction than the higher molecular weight form (see Fig. 8 *b*) and it retains at least a portion of the extracellular domain containing the mAb epitope. It remains to be determined whether this smaller T-CD44H form, which contributes to the intracellularly localized T-CD44H, results from protein which has not reached the cell surface or protein which has been expressed on the cell surface, internalized and then partially degraded.

To enable us to examine the stability of the T-CD44H protein, MDCK cells were plated into culture dishes in low calcium medium for 24 h prior to the  $^{35}\text{S}$ -labeling in complete medium. The low calcium medium prevents the formation

of tight junctions and hence, cell polarization and targeting of proteins to specific membrane domains (Nelson et al., 1990), and under these culture conditions we only detect the higher molecular weight form of T-CD44H. In these experiments, it was demonstrated that removal of the cytoplasmic tail reduces the stability of the CD44H protein (Fig. 5). Electron microscopy studies have shown that CD44H is excluded from the coated pits on the cell surface (Bretscher et al., 1980; Hopkins 1986). One explanation for the reduction in the T-CD44H stability is that removal of the cytoplasmic tail allows entry into the coated pit and hence, the protein is more rapidly internalized and degraded than the WT counterpart. A similar conclusion has been drawn for the CD4 protein which in lymphocytes is excluded from the coated pits due to its association with p56<sup>lck</sup>. Deletion of the cytoplasmic tail abolishes this interaction and results in an increased rate of CD4 endocytosis (Pelchen-Matthews et al., 1991). An increased rate of internalization might account, at least in part, for the intracellular localization of the T-CD44H and the presence of the 55–65 kD form. Morphological examination of MDCK cells has shown that in confluent monolayers there is consistent heterogeneity of T-CD44H expression (Figs. 2 and 3). The reason for this heterogeneity of expression is not known but it does not result from heterogeneity within the clonal cell line illustrated here, as all the clonal T-CD44H transfected MDCK cell lines tested gave the same result, and when plated sparsely and examined by immunofluorescence each cell expresses T-CD44H to a uniform level.

Goldstein et al. (1989) have isolated a cDNA clone encoding a tailless CD44H molecule with almost identical structure to the T-CD44H described here. In the cell lines examined, a truncated CD44 was only detected at very low levels (Goldstein and Butcher, 1990) and it is therefore unlikely to play an important physiological role unless its expression is elevated in cells which are as yet untested. A tailless mutant was used by Lesley et al. (1992) to examine the hyaluronate binding capacity of CD44H molecules transfected into the CD44-ve AKR1 T lymphoma cell line. This mutant had a much reduced capacity to bind hyaluronate in solution but partially retained the ability to adhere to immobilized hyaluronate. Anti-CD44 mAb could enhance the hyaluronate binding capacity of the truncated CD44 protein suggesting that hyaluronate binding requires a conformational change in CD44 or a redistribution of the protein on the cell surface. These results are compatible with the data presented here showing that the CD44 cytoplasmic tail does not directly mediate interaction with the cytoskeleton but is important for membrane localization.

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