Variations in the Cytoskeletal Interaction and Posttranslational Modification of the CD44 Homing Receptor in Macrophages

Robert L. Camp, Thomas A. Kraus, and Ellen Puré

Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021

Abstract. Murine CD44 is a cell surface glycoprotein that is thought to play a role in leukocyte migration. We studied the structure and expression of CD44 on two populations of macrophages: those that reside in the peritoneum of unprimed mice, and those that have been elicited to migrate into the peritoneum by the intraperitoneal injection of agents that cause localized inflammatory responses. Our studies reveal structural variations in both the extracellular and intracellular domains of CD44 expressed by these two macrophage populations. The form of CD44 in elicited macrophages has an apparent molecular mass that is ~5 kD greater and more heterogenous than that in resident macrophages. This structural change is posttranslational, extracellular, and apparently reflects increases in N-linked glycosylation. It is also specific for CD44 and does not occur with several other glycoproteins examined. This novel regulation of glycosylation may play an important role in the ability of CD44 to bind to different substrates, particularly lectin-like ligands. In addition, we demonstrate that CD44 in resident macrophages is divided into two pools, one containing nonphosphorylated, cytoskeletally associated CD44, and one containing phosphorylated, unassociated CD44. In contrast, CD44 on the surface of elicited macrophages does not associate with the cytoskeleton. The attachment of CD44 to the cytoskeleton involves either direct or indirect association with actin. The regulated association of CD44 with the cytoskeleton suggests that it may influence or be influenced by macrophage mobility.

CD44 (H-CAM) plays multiple roles in the adhesion of leukocytes to a variety of substrates and cell types (Carter and Wayner, 1988; Miyake et al. 1990*b*). CD44 is principally known for its ability to mediate the attachment of lymphocytes to high endothelial venules (HEV),[1] presumably by binding to an endothelial cell "addressin," as well as through its affinity for hyaluronic acid (Nakache et al., 1989; Aruffo et al., 1990; Culty et al., 1990). The form of CD44 expressed by most leukocytes is 80–100 kD and is highly glycosylated with both N- and O-linked sugars (Hughes et al., 1981; Sutton et al., 1987). Recent studies have described the "restricted" expression of higher molecular mass (>150 kD) isoforms of CD44, known collectively as CD44R, that are the products of alternative exon splicing (Brown et al., 1991; Gunthert et al., 1991; Stamenkovic et al., 1991). The expression of these isoforms has been correlated with the metastatic potential of various carcinoma cell lines. Certain cell lines also express >150-kD forms that contain the CD44 core modified with chondroitin sulfate (Jalkanen et al., 1988; Brown et al., 1991).

We have analyzed the structure of CD44 expressed on resident and thioglycollate-elicited macrophages. Our studies demonstrate that resident macrophages express solely the ~85-kD isoform of CD44, whereas elicited, inflammatory macrophages express a form that is ~5 kD larger and more heterogenous when analyzed by SDS-PAGE. Unlike the recently described CD44/CD44R isoforms, the two variant forms described here differ in their posttranslational modifications.

Several studies have documented the cytoskeletal association of CD44 in cell lines (Lacy and Underhill, 1987; Carter and Wayner, 1988). We extend these observations to primary macrophages, and demonstrate that this association inversely correlates with the degree of CD44 phosphorylation. After elicited migration, macrophages contain little or no cytoskeletally associated CD44. Together with variations in the extracellular domain, which may influence ligand interaction, changes in the cytoskeletal association of CD44 may influence the migration and function of macrophages in sites of inflammation.

Materials and Methods

Animals

8–20-wk-old female CD_2F_1 ((BALB/c × DBA/2)F_1) and C57BL/6 mice were purchased from the Trudeau Institute (Saranac Lake, NY). CD_2F_1 mice are homozygous for the CD44-1.1 allele, whereas C57BL/6 mice are homozygous for the CD44-1.2 allele (Lesley and Trowbridge, 1982).

1. *Abbreviation used in this paper*: HEV, high endothelial venules.

Monoclonal Antibodies

All mAbs used in this study were generated in rats against murine antigens. KM201 (IgG_1) anti-CD44 was a generous gift from P. Kincade and K. Miyake (Oklahoma Medical Research Foundation, Oklahoma City, OK; Miyake et al., 1990*a*). Bet-2 (IgG_1) anti-IgM (Kung et al., 1981), M1/70.15.11.5.HL (IgG_{2b}) anti-Mac-1α (CD11b) (Springer et al., 1978), and M1.9.3.4.HL.2 (IgG_{2a}) anti-CD45 (Springer et al., 1978) were obtained from the American Type Culture Collection (Rockville, MD).

Preparation of Macrophages

Mice were injected intraperitoneally with brewer's yeast thioglycollate (Difco Laboratories, Detroit, MI), as described previously (Mishell and Shiigi, 1980). 3–4 d later, elicited peritoneal exudate cells (~90% macrophages) were harvested by peritoneal lavage with PBS (without Ca^{2+} or Mg^{2+}). Resident peritoneal exudate cells were similarly isolated from the peritoneal lavage of untreated mice and consisted of ~50% macrophages. Peritoneal cells were then washed and plated on 35-mm polystyrene tissue culture dishes (Falcon Labware, Oxnard, CA) at 5×10^6/plate (elicited) or 1×10^7/plate (resident) in RPMI (Gibco Laboratories, Grand Island, NY), supplemented with 10% heat-inactivated FCS (Hazelton Biologics, Lenexa, KS), β-mercaptoethanol, antibiotics, and antimycotics. After 2 h, the adherent macrophages were washed free of nonadherent cells with warm medium, incubated overnight, and then washed twice with warm medium. The final yield of both cell preparations was ~5×10^6/plate, each consisting of >95% macrophages as analyzed by fluorescence flow cytometry.

Radioisotope Labeling

Adherent macrophages were surface radiolabeled with Na^{125} Iodine (~17 Ci/mg; New England Nuclear, Boston, MA) using soluble lactoperoxidase/glucoseoxidase. For metabolic phosphorylation, adherent macrophages were incubated for 1 h in phosphate-free RPMI (Gibco Laboratories) supplemented with 5% FCS (dialyzed against Tris-buffered saline, pH 7.4), followed by the addition of 2 mCi ortho-^{32}phosphate (~9,000 Ci/mM; New England Nuclear), and an additional 1 hour incubation. Cells were washed free of unincorporated label and incubated for 1 hour in RPMI containing phosphate and 10% FCS. For biosynthetic labeling with ^{35}S-Met/Cys (~1,000 Ci/mM; Tran^{35}S-label™, ICN Biomedicals, Inc., Costa Mesa, CA), cells were washed and cultured in Met/Cys-free RPMI [Gibco Laboratories] supplemented with 5% dialyzed FCS for 1 h. Adherent cells were then pulsed with 1 mCi/ml of ^{35}S-Met/Cys for 5 min to 12 h (as indicated). After labeling, cells were washed three times and cultured for 0–60 min in complete RPMI with 10% FCS, to chase the radiolabel.

Tunicamycin (Boehringer Mannheim Biochemicals, Indianapolis, IN) was dissolved at 5 mg/ml in DMSO and added to culture medium as indicated at a final concentration of 10 μg/ml during a 1-h Met/Cys depletion and a 1-h [^{35}S]Met/Cys labeling. Cultures were then supplemented with unlabeled methionine and cysteine (50 μg/ml each) for 10 min at 37°C, before lysis.

Preparation of Detergent Lysates

Plate-bound macrophages were lysed on ice in 500 μl PBS containing 1% NP-40 (Sigma Chemical Co., St. Louis, MO), protease inhibitors (0.2 U/ml aprotinin, 1 mM PMSF, and 1 mM diisopropylfluorophosphate; Sigma Chemical Co.). During phosphate labeling, phosphatase inhibitors (50 mM NaH_2PO_4, 50 mM KF, and 10 mM sodium pyrophosphate; Sigma Chemical Co.) were added. After 1 min, the NP-40 lysates were removed from the plates after fully dislodging any remaining cellular debris from the plate surface with a micropipette. Lysates were incubated in 1.5-ml microcentrifuge tubes on ice for an additional 10 min. Lysates were then fractionated by centrifugation at 10,000 *g*, at 4°C for 10 min in a swinging bucket rotor (unless otherwise indicated). The NP-40–soluble supernatants were removed and the pellets were washed three times by gently resuspending them in 250 μl fresh 1% NP-40 lysis buffer and centrifuging as above, for 5 min. Washes were routinely assayed for labeled CD44, which was always undetectable by the third wash. The NP-40–insoluble pellets were then resuspended in PBS containing 1% Empigen-BB (Calbiochem, La Jolla, CA; Ranscht et al., 1984; Carter and Wayner, 1988) in the presence of the aforementioned protease and phosphatase inhibitors. After 10 min on ice, the lysates were centrifuged and the Empigen-BB–soluble supernatants were removed.

Immunoprecipitations

Lysates were precleared with normal rat Ig (50 μg/ml) and Sepharose-bound mouse anti-rat Ig (coupled at 1 mg/ml; Jackson Immuno Research Laboratories, West Grove, PA, and Pharmacia Fine Chemicals, Piscataway, NJ). Lysates were immunoprecipitated with rat anti-mouse CD44 mAb, KM201, or an isotype matched control anti-IgM, Bet-2. Immune complexes were recovered with mouse anti-rat Ig–coupled Sepharose. Sepharose pellets were washed with high salt (0.6 M NaCl, 0.0125 M KPO_4, pH 7.4, 0.02% NaN_3), mixed detergent buffer (0.05% NP-40, 0.1% SDS, 0.3 M NaCl, 10 mM Tris, pH 8.6) and PBS. Immune complexes were then eluted by boiling in 2× Laemmli running buffer (with 0.4% DTT) and electrophoresed on 7% or 8.5% polyacrylamide gels. High and low molecular weight standards were obtained from Bio-Rad Laboratories (Rockville Center, NY). Gels were dried and exposed to film for 24–48 h or 2 wk for ^{35}S-pulse-chase experiments.

In Vitro Cytoskeletal Release Assays

Plate-bound resident and elicited macrophages (5×10^6 cells per plate) were treated with iodinated anti-CD44 (KM201) or anti-CD11b (M1/70) at 1×10^6 cpm per plate for 30 min at 4°C. The specific activity of KM201 was ~2×10^6 cpm/μg, and M1/70 was ~7×10^5 cpm/μg. Unbound antibody was washed away, and NP-40 and Empigen-BB lysates were prepared as described above. The resulting NP-40 and Empigen-BB lysates, and Empigen-BB–insoluble pellets, were analyzed by use of an autogamma scintillation spectrometer (model 5220, Packard Instrument Co., Downers Grove, IL). The amount (cpm) of iodinated mAb was determined and used as a measure of the amount of CD44 present in each fraction. In other experiments, NP-40–insoluble material was resuspended in 50 mM Tris/4 mM $MgCl_2$ (pH 7.2, no detergent) with or without 1 mg/ml DNAase-I (Sigma Chemical Co.), and incubated at 25°C for the indicated times. After incubation, samples were centrifuged at 10,000 *g* for 10 min. Soluble and insoluble materials were separated and assayed for ^{125}I-KM201/CD44 immune complexes, as detailed above.

Tryptic Digests of Immunoprecipitated CD44

CD44 immunoprecipitated from lysates of iodinated resident and elicited macrophages was electrophoresed as described above. Bands corresponding to CD44 were cut out from the dried gels, reswollen in 2.5% acetic acid/10% methanol, and dehydrated in 50% methanol. The gel slices were dried by vacuum desiccation and rehydrated in 50 mM ammonium bicarbonate with 0.1 mg/ml TPCK-treated trypsin (Sigma Chemical Co.). Gel slices were incubated at 37°C for 12 h and the eluted peptide fragments were dried by vacuum desiccation. Peptides were resuspended in Laemmli running buffer containing 0.4% DTT, normalized according to cpm, and electrophoresed on a 15% polyacrylamide gel.

Glycosidase and Glycosaminoglycanase Treatments

Iodinated CD44 immune complexes coupled to Sepharose beads were treated as follows: (*a*)Alkaline Phosphatase; beads were washed twice in 50 mM Tris, pH 7.5/120 mM NaCl, incubated with 10 mg/ml alkaline phosphatase (#405-612, Boehringer Mannheim Biochemicals) for 6 h at 37°C, and then washed three times in PBS; (*b*) Neuraminidase; beads were resuspended in 100 μl (1 U/ml) neuraminidase from *Vibrio cholerae* for 6 h at 37°C, followed by three PBS washes; (*c*) *O*-glycosidase; immune complexes treated with neuraminidase were resuspended in 50 μl PBS containing 2.5 mU *O*-glycanase for 16 h at 37°C, followed by three washes in PBS; (*d*) *N*-glycosidase; CD44 was removed from Sepharose-coupled immune complexes by boiling the beads in 0.5% SDS/1% β-mercaptoethanol/20 mM sodium phosphate buffer (pH 7.6). The resulting mixture was diluted 1:10 with 0.5% NP-40/20 mM $NaPO_4$ buffer (pH 7.6) and incubated for 16 h with 0.5 U *N*-glycosidase. All enzymes were purchased from Boehringer Mannheim Biochemicals; (*e*) Chondroitinase ABC; immune complexes were resuspended in 30 μl PBS with 0.1 U/ml chondroitinase ABC (ICN Biomedicals, Inc., and Sigma Chemical Co.) for 2 h at 37°C, followed by three PBS washes. As a control, proteo-dermatin-sulfate (a generous gift of J. Gregory and S. Damle, Rockefeller University) was iodinated and incubated under identical conditions. Heparin lyase (Sigma Chemical Co.) and keratinase (ICN Biomedicals, Inc.) were similarly tested. In certain assays, complexes were treated as described for *N*-glycosidase (e.g., boiled in the presence of SDS and diluted in NP-40) and subsequently treated with chondroitinase ABC; and (*f*) Endoglycosidase H; CD44 immunoprecipitates

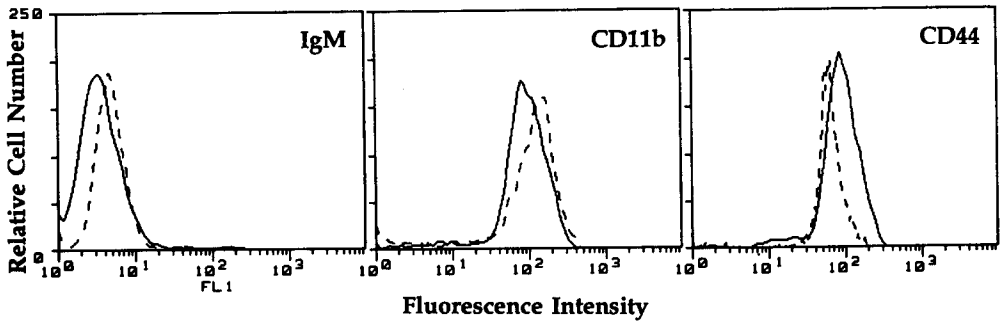

Figure 1. Resident and elicited macrophages express similar levels of CD44. Resident (- - -) and elicited (——) macrophages were stained with mAbs to CD44 (KM201), CD11b/CD18 (Mac-1) (M1/70), or IgM (Bet-2). Stained cells were visualized using a FITC-conjugated secondary antibody, and analyzed on a FACScan (a registered trademark of Becton Dickinson).

from ^{35}S-Met/Cys-labeled cells (10-min pulse) were boiled in 50 μl 0.02% SDS/0.1 M β-mercaptoethanol/100 mM citrate buffer (pH 5.5), cooled to room temperature, then treated with 5 mU endo-H for 2 h at 37°C.

Fluorescence Flow Cytometry

Adherent macrophages were washed twice and treated with 0.04% EDTA in PBS (without Ca^{2+} or Mg^{2+}). After a 30-min incubation at room temperature, adherent cells were dislodged by vigorous pipetting. Cells were washed, incubated on ice for 30 min with 5–10 μg/ml purified antibody (diluted in PBS containing 1 mg/ml BSA and 0.02% NaN_3), then incubated with FITC-conjugated F(ab')2 mouse anti-rat Ig antibody (Jackson Immuno Research Laboratories) for 30 min. Cells were then washed and analyzed on a FACScan (a registered trademark of Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Results

Elicited Macrophages Express a Form of CD44 with a Higher Apparent Molecular Weight and Greater Microheterogeneity

Macrophages residing in the peritoneum of untreated mice expressed high levels of an ~85-kD form of CD44, as demonstrated by fluorescence flow cytometry (Fig. 1) and SDS-PAGE (Fig. 2). Macrophages elicited to migrate into the peritoneum after the administration of the inflammatory agent thioglycollate, expressed comparable levels of CD44 protein (Fig. 1) and RNA (data not shown). However, elicited macrophages expressed a higher molecular mass form of CD44, averaging ~90 kD, which exhibited extensive microheterogeneity on polyacrylamide gels (Fig. 2). The heterodispersity of the ~90-kD form was a result of molecular mass variations, rather than an artifact of SDS-PAGE, since the reelectrophoresis of material from the top and the

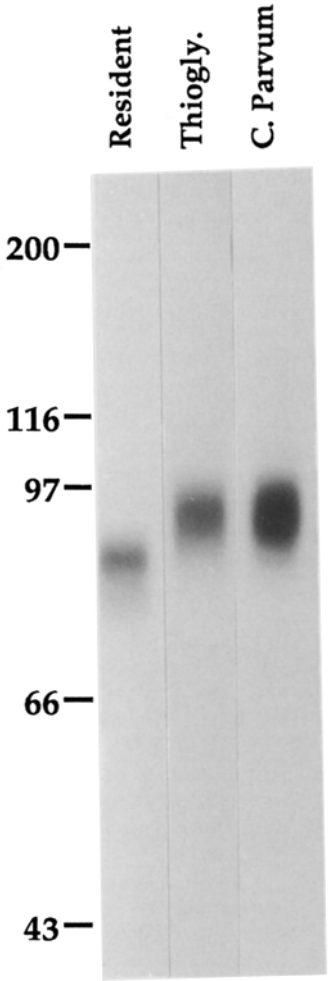

Figure 2. Elicited macrophages express a higher molecular weight form of CD44. Resident and thioglycollate and *C. parvum* elicited macrophages were surface labeled with sodium^{125} iodine and lysed in 1% NP-40. Lysates were immunoprecipitated with a mAb to CD44 (KM201) and analyzed by 7% SDS-PAGE. Standards are expressed in kilodaltons.

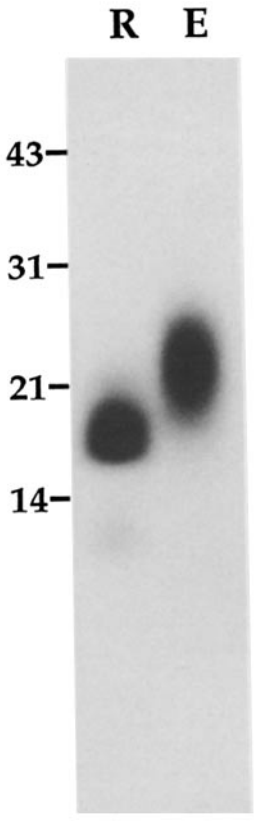

Figure 3. Tryptic peptide mapping of the extracellular domain of CD44 reveals differences between resident (*R*) and elicited (*E*) macrophage CD44. Purified CD44 isolated from surface iodinated macrophages was treated with trypsin, and the resulting peptide fragments were analyzed by 15% SDS-PAGE.

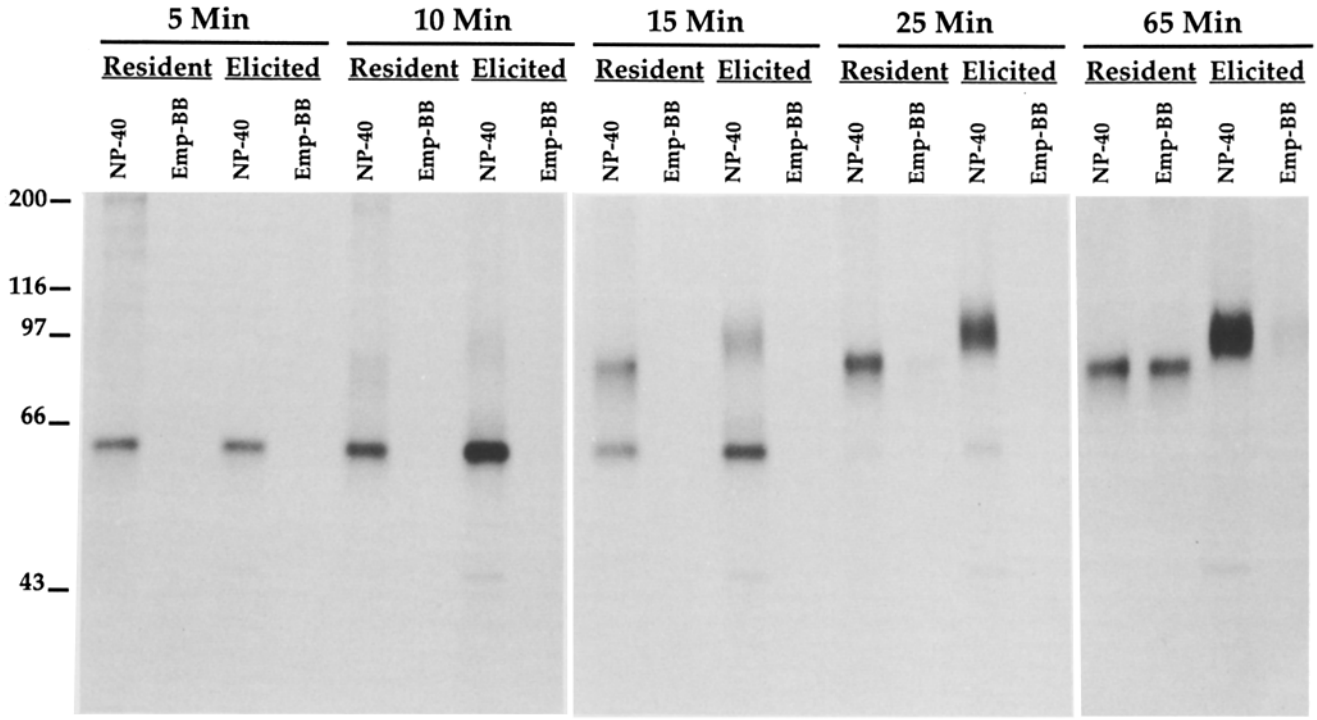

Figure 4. Resident and elicited macrophages express a common 58-kD immature form of CD44. Macrophages were depleted of Met/Cys, pulsed for 5 min with ^{35}S-Met/Cys, and chased for 0–60 min. Stated times include the 5-min pulse. NP-40 and Empigen-BB (*Emp-BB*) lysates were prepared sequentially, as described in Materials and Methods. CD44 was immunoprecipitated using the mAb KM201, and analyzed by 8.5% SDS-PAGE. The ~45-kD protein detected in trace amounts in some NP-40 lanes was also present in isotype-matched control samples, indicating that it is a background band.

bottom of this band ran as two distinct bands, both of which were larger than the ~85-kD form (data not shown). Administration of *C. parvum*, which activates as well as elicits corynebacterium, induced macrophages that express the 90-kD form (Fig. 2). Two other macrophage surface glycoproteins, MAC-1 (CD11b/CD18) and CD45, immunoprecipitated from resident and elicited macrophages, did not exhibit different apparent molecular masses (Fig. 10, *NP-40* lanes). Interestingly, the in vitro stimulation of murine B cells with anti-immunoglobulin induced the expression of a higher molecular mass form of CD44 as well (Camp, R. L., and E. Pure, manuscript in preparation).

Tryptic mapping of peptide fragments of surface iodinated CD44, demonstrated that extracellular modifications were largely responsible for the apparent molecular mass difference. All of the potential extracellular iodination sites are separated from the internal domain by multiple, potential trypsin cleavage sites (Nottenburg et al., 1989; Zhou et al., 1989). The major iodinated tryptic fragment of CD44 isolated from elicited macrophages was ~5 kD larger than that from resident macrophages (Fig. 3). The amino acid sequence of CD44 predicts that no tryptic fragment is >4 kD. Therefore, the heterogenous ~17- and ~22-kD fragments detected by 15% SDS-PAGE presumably underwent post-translational modification, most likely with glycosidic residues, and/or were the result of incomplete tryptic proteolysis.

To determine at what stage of biosynthesis the differential modifications of CD44 are introduced, we analyzed the metabolic processing of CD44 in resident and elicited macrophages. Macrophages were pulsed with ^{35}S-Met/Cys for 5 min, then chased for 0–60 min (Fig. 4, *NP-40* lanes). Within 5 min, resident and elicited macrophages expressed CD44 as a comigrating 58-kD species. This immature form of CD44 was presumably composed of protein modified with N-linked core sugars. Treatment with endoglycosidase-H (endo-H) reduced the molecular mass of the 58-kD band by 11 kD, bringing it close to the predicted molecular weight for the core protein (38 kD; Fig. 5). 10–15 min after the addition of radiolabel, the 58-kD band was rapidly converted into

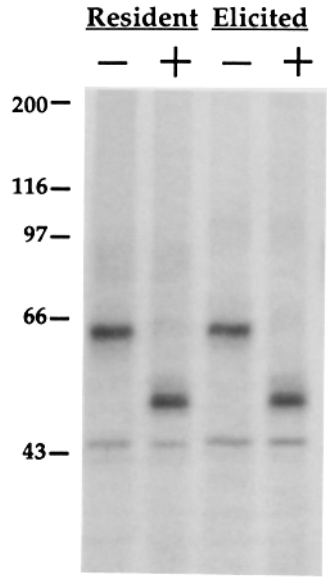

Figure 5. Identification of a common endo-H–sensitive precursor of CD44 in resident and elicited macrophages. Macrophages were depleted of methionine and cysteine, labeled for 10 min with ^{35}S-Met/Cys, and lysed in 1% NP-40. CD44 was immunoprecipitated with the mAb, KM201, and the resulting immune complex was subjected to cleavage with endo-H (+ lanes) and analyzed by 8.5% SDS-PAGE. The ~45-kD protein detected in trace amounts in all lanes is a background band, similarly expressed in control samples.

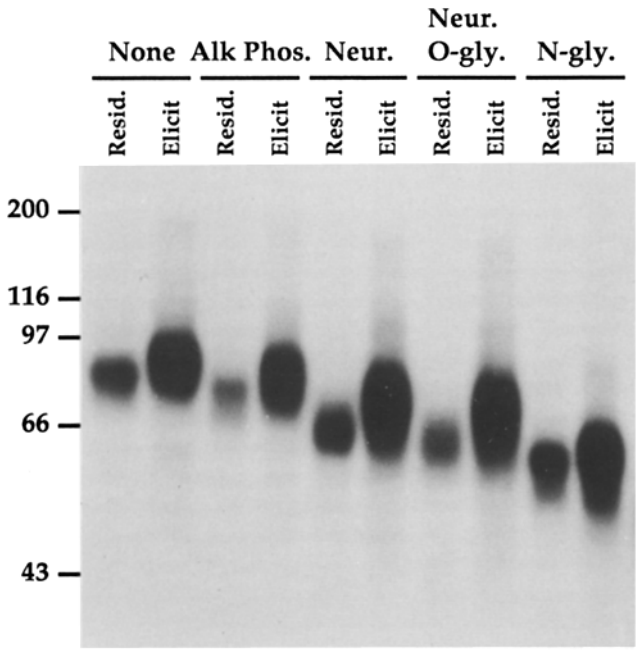

Figure 6. Contribution of phosphorylation, sialylation, and N- and O-linked glycosylation to the apparent molecular weight of resident and elicited macrophage CD44. Immunoprecipitated CD44 was treated with alkaline phosphatase, neuraminidase, *O*-glycosidase, or *N*-glycosidase, either alone or in combination, and analyzed by 7% SDS-PAGE.

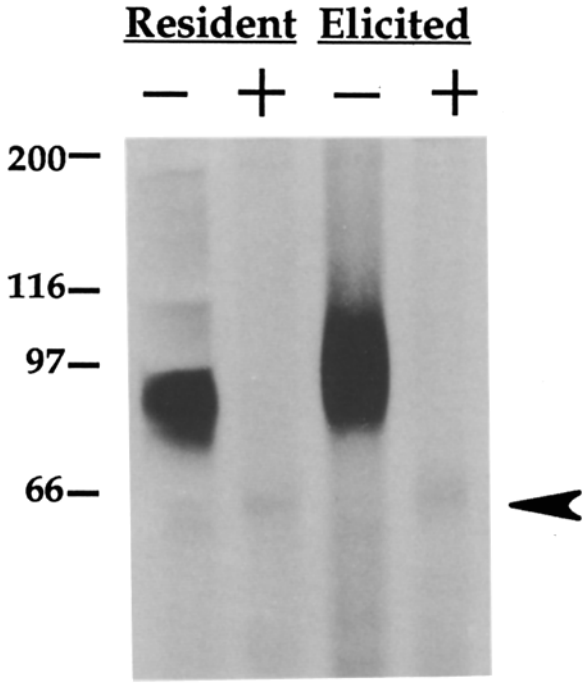

Figure 7. Treatment of resident and elicited macrophages with tunicamycin results in the production of a common 67-kD CD44 precursor. Macrophages were depleted of Met/Cys for 1 h, labeled for 1 h with ^{35}S-Met/Cys, and chased for 10 min, all in the presence of 10 μg/ml tunicamycin. Immunoprecipitated CD44 was analyzed by 7% SDS-PAGE.

the distinct mature forms of CD44 (resident, ~85 kD; elicited, ~90 kD). These mature forms of CD44 were resistant to cleavage with endo-H (data not shown). After 25 min, most of the 58-kD species was chased into the mature forms (Fig. 4, *NP-40* lanes).

To compare the posttranslational modifications of CD44, we determined the molecule's sensitivity to various combinations of neuraminidase, alkaline phosphatase, *N*-glycosidase, and *O*-glycosidase (Fig. 6). In primary macrophages, a substantial portion of the apparent mass of CD44 was attributable to a number of posttranslational modifications, including phosphorylation (~4 kD), N-linked glycosylation (~18 kD), sialylation (~14 kD), and O-linked glycosylation (~2 kD; Fig. 6). Because of the narrow substrate specificity of *O*-glycanase, this represents a minimal estimate of O-linked glycosylation.

Whereas treatment with enzymes that catalyze the removal of phosphate, sialic acid, or O-linked sugars failed to reduce the 5-kD mass difference between resident and elicited macrophage CD44, *N*-glycanase eliminated much of this difference, although it did not reduce the heterodispersity of elicited macrophage CD44 (Fig. 6). This heterodispersity may be due to differences in the amount of non-N-linked sugars on individual CD44 molecules, particularly O-linked sugars.

To further assess whether N-linked sugars are a key factor in the molecular weight difference, we treated macrophages with 10 μg/ml tunicamycin during a 60-min Met/Cys depletion, 60-min label, and 10-min chase (Fig. 7). Treatment with tunicamycin resulted in the production of relatively homogeneous, comigrating ~66-kD species of CD44 in resident and elicited macrophages. Because of the reduction in CD44 synthesis in treated cells, we cannot rule out the possibility that the drug also caused retention of CD44 in the endoplasmic reticulum, thus preventing later modifications (e.g., O-linked glycosylation). However, the fact that tunicamycin treated cells produced a ~66-kD species of CD44 which was ~20 kD larger than the endo-H–treated CD44 core (Fig. 5) suggests that the ~66-kD molecule was extensively modified with O-linked sugars.

Treatment of CD44 from either macrophage population with chondroitinase ABC, even under denaturing conditions, failed to alter the migration of CD44 in SDS-PAGE, demonstrating that the CD44 on macrophages was not modified with chondroitin or dermatin sulfate (Fig. 8). Treatment with heparin lyase and keratinase also had no effect on CD44 except for the effects of contaminating neuraminidase (as specified by the manufacturer), indicating that CD44 was not modified with heparin sulfate or keratin sulfate (data not shown). The labeling of macrophages with ^{35}sulfate demonstrated that both forms of CD44 contained small and equivalent amounts of sulfate, either in the form of sulfated sugars or tyrosine sulfate (data not shown).

Non-Phosphorylated CD44 on the Surface of Resident, but not Elicited Macrophages, Is Associated with the Cytoskeleton

Previous studies have demonstrated that CD44 associates with the cytoskeleton in T cells, cell lines and tumors of several cell types (Lacy and Underhill, 1987; Carter and Wayner, 1988; Geppert and Lipsky, 1991). Consequently, we analyzed the factors influencing the cytoskeletal association of CD44 in primary macrophages. Several reports have demon-

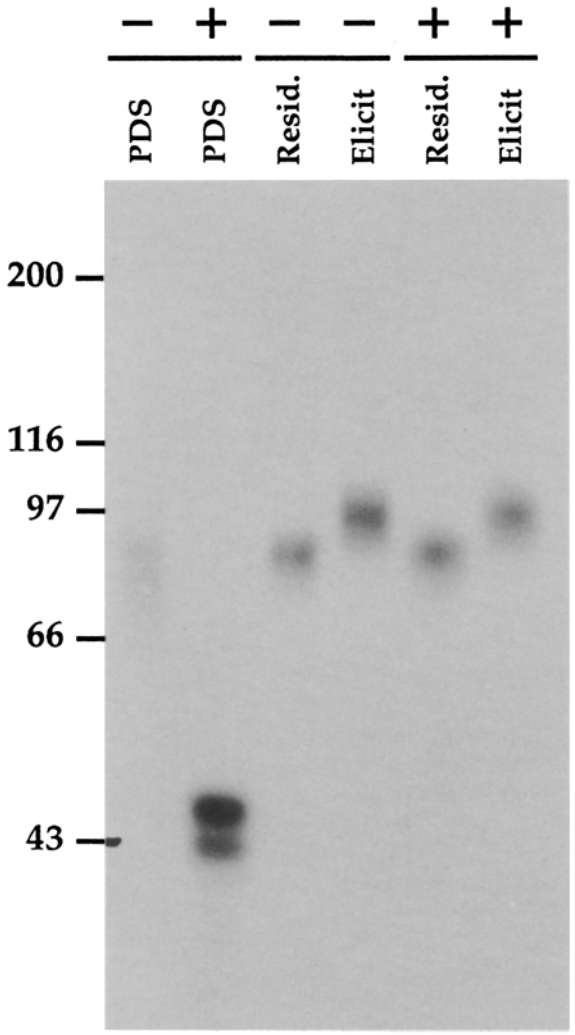

Figure 8. CD44 on resident and elicited macrophages is not modified with chondroitin sulfate. Immunoprecipitated CD44 was treated with chondroitinase ABC (+ lanes). As a positive control, iodinated, proteo-dermatin-sulfate (*PDS*) was similarly treated and reduced to its protein cores (43 and 45 kD).

strated the efficacy of a zwitterionic detergent, Empigen-BB, in liberating cytoskeletally associated proteins from NP-40–insoluble material (Ranscht et al., 1984; Carter and Wayner, 1988). We employed a procedure whereby cells are first lysed in NP-40 to solubilize proteins that are not complexed with the cytoskeleton. The resulting NP-40–insoluble residue is then washed and solubilized in Empigen-BB which liberates many proteins associated with the cytoskeleton. Empigen-BB–insoluble material includes cytoskeletal elements, nondissociated proteins, and nuclei.

We analyzed the detergent solubility of CD44 isolated from ^{35}S-Met/Cys–labeled resident and elicited macrophage lysates. In resident macrophages, ~50% of biosynthetically labeled CD44 was NP-40 insoluble, Empigen-BB soluble (Fig. 9). In contrast, elicited macrophages contained little or no NP-40–insoluble CD44 (Fig. 9 and Table I). Other surface antigens, including CD11b/CD18 and CD45, exclusively partitioned into the NP-40–soluble fraction in both resident and elicited macrophages (Fig. 10 and Table I).

Since NP-40–insoluble material contains cytoskeletal elements as well as nuclei, we studied the detergent solubility of CD44 on the surface of resident macrophages to rule out a nuclear association of CD44. Whole cell iodination and mAb binding assays demonstrated that >50% of surface-labeled CD44 was NP-40 insoluble (Fig. 9 and Table I). Thus, the NP-40–insoluble pool of CD44 was not associated with nuclear membranes.

The attachment of CD44 to the cytoskeleton occurred late (after 60 min) in the molecule's biosynthesis, subsequent to the formation of its mature form, and probably only after CD44 reached the cell surface (Fig. 4). To determine if this association involves actin, we treated preparations of cytoskeletally complexed CD44 with DNAse-I, which destabilizes actin filaments and can lead to the solubilization of proteins associated with the cytoskeleton (Hitchcock et al., 1976; Woda and Woodin, 1984; Lacy and Underhill, 1987). DNAse-I mediated a rapid and almost complete dissociation of CD44 from the cytoskeleton, releasing >40% of the insoluble CD44 after 5 min and >70% after 30 min (Fig. 11).

Previous studies have demonstrated that CD44 isolated from cell lines contains phosphoserine (Isacke et al., 1986; Carter and Wayner, 1988). Phosphopeptide and phospho–amino-acid analysis of CD44 immunoprecipitated from the NP-40 lysates of primary macrophages demonstrated that CD44 was constitutively phosphorylated on one or more serine residues. The site(s) of phosphorylation appeared similar in resident and elicited macrophages (data not shown). Virtually all of the CD44 on macrophages that was phosphorylated was expressed at the cell surface, as demonstrated by treating ortho^{32}phosphate–loaded macrophages with anti-CD44 mAb prior to lysis, washing away the excess antibody, then lysing the cells and immunoprecipitating without additional anti-CD44 mAb (data not shown). Subsequent immunoprecipitation of internal CD44 by the addition of more anti-CD44 mAb revealed little additional phosphorylated CD44, either in the precursor or mature form (data not shown).

To determine if CD44 phosphorylation correlates with cytoskeletal association, we examined the detergent solubility of phosphorylated CD44 in macrophages loaded with ortho^{32}phosphate. In resident macrophages, CD44 recovered in the NP-40 soluble fraction was readily phosphorylated, whereas NP-40–insoluble/Empigen-BB–soluble CD44 was not (Fig. 9). As expected, elicited macrophages contained only NP-40–soluble phosphorylated CD44 (Fig. 9). Thus, the association of CD44 with the cytoskeleton may be related to, and perhaps regulated by, its state of phosphorylation.

Discussion

Our studies distinguish between two pools of CD44 in resident macrophages, each containing roughly 50% of surface-associated CD44. One pool of CD44 is associated with the cytoskeleton, either directly or indirectly with actin, and is not phosphorylated. The other is not cytoskeletally associated, but is phosphorylated. In elicited macrophages, the distribution of CD44 in these two pools is dramatically different; >93% of the surface-associated CD44 is contained

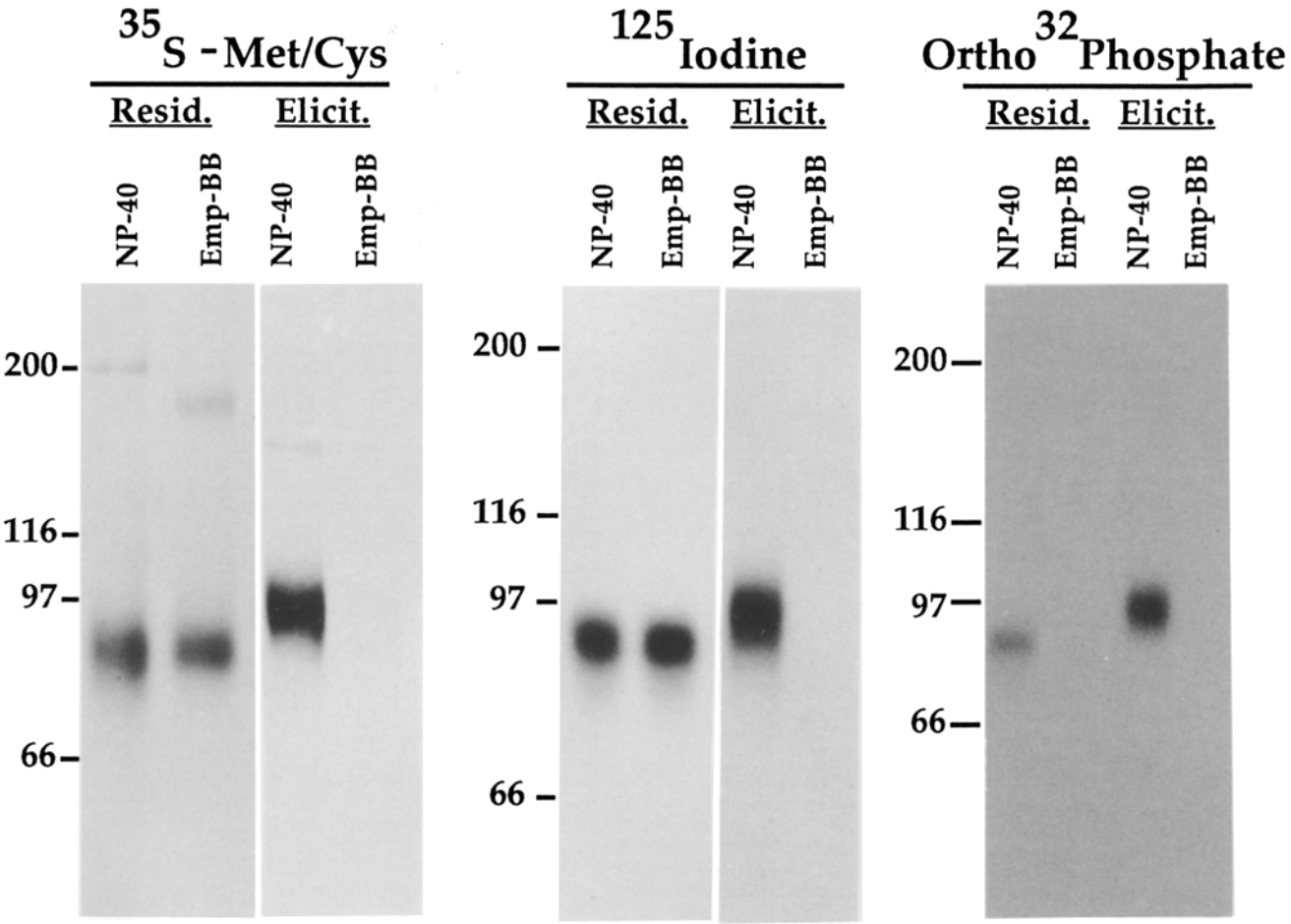

Figure 9. Resident, but not elicited, macrophages express a non-phosphorylated, cytoskeletally associated, form of CD44 on their surface. Macrophages were metabolically labeled with ^{35}S-Met/Cys [12-hr pulse, no chase], ortho^{32}phosphate, or surface labeled with sodium^{125}iodine. NP-40 and Empigen-BB (*Emp-BB*) lysates were prepared sequentially as described in Materials and Methods. CD44 was immunoprecipitated with the mAb KM201, and analyzed by 7% SDS-PAGE.

in the phosphorylated, non-cytoskeletally associated pool. In addition, elicited macrophages express a form of CD44 that is modified with more N-linked sugar, which contributes to a ~5-kD increase in the apparent molecular mass of CD44.

The regulated expression of CD44 influences leukocyte binding to hyaluronate and HEV in vitro (Jalkanen et al., 1987; Aruffo et al., 1990; Miyake et al., 1990*b*), and possibly cellular migration and differentiation in vivo (Lesley et al., 1985; Miyake et al., 1990*a*). Such regulation involves changes in the amount of CD44 expressed, as well as alterations in the structure of the molecule. Various reports have described the upregulation of CD44 following activation of B and T lymphocytes (Budd et al., 1987; Camp et al., 1991; Murakami et al., 1991). In addition, several studies have distinguished between two isoforms of CD44: the ~85-kD CD44 molecule, primarily expressed in hematopoietic cells, and a ~150-kD CD44R, expressed in epithelial cells. These isoforms are generated by alternative splicing of a single gene, and appear to play separate and distinct roles in cellular adhesion; CD44R potentiates the metastasis of carcinomas, whereas CD44 promotes the binding of cells to hyaluronate and HEV in vitro (Brown et al., 1991; Gunthert et al., 1991; Stamenkovic et al., 1991).

In addition to variations in the core protein of CD44/CD44R, other studies have demonstrated that CD44 ranges in size from 50 to >200 kD, depending on the cell type analyzed (Sutton et al., 1987; Omary et al., 1988; Stamenkovic et al., 1989). This wide range in apparent molecular mass is presumably due to alternative glycosylation as well as to the addition of chondroitin sulfate or heparin sulfate to one of several potential attachment sites in the external domain (Jalkanen et al., 1988; Zhou et al., 1989; Brown et al., 1991). However, we did not detect significant amounts of

Table I. Determination of the Amount of NP-40–Soluble, Empigen-BB–Soluble, and Empigen-BB–Insoluble CD44 in Resident and Elicited Macrophages

Cell type	Antigen	Total cpm	NP-40	Empigen-BB	Pellet
			%	%	%
Resident	CD44	1.2×10^5	46	42	12
Elicited	CD44	1.4×10^5	93	7	<1
Resident	CD11b	1.8×10^5	99	<1	<1

Plate-bound macrophages were labeled with iodinated anti-CD44 (KM201) or anti-CD11b/CD18 (M1/70). NP-40 and Empigen-BB-lysates were prepared sequentially as described in Materials and Methods and counted. The data shown is from a typical experiment.

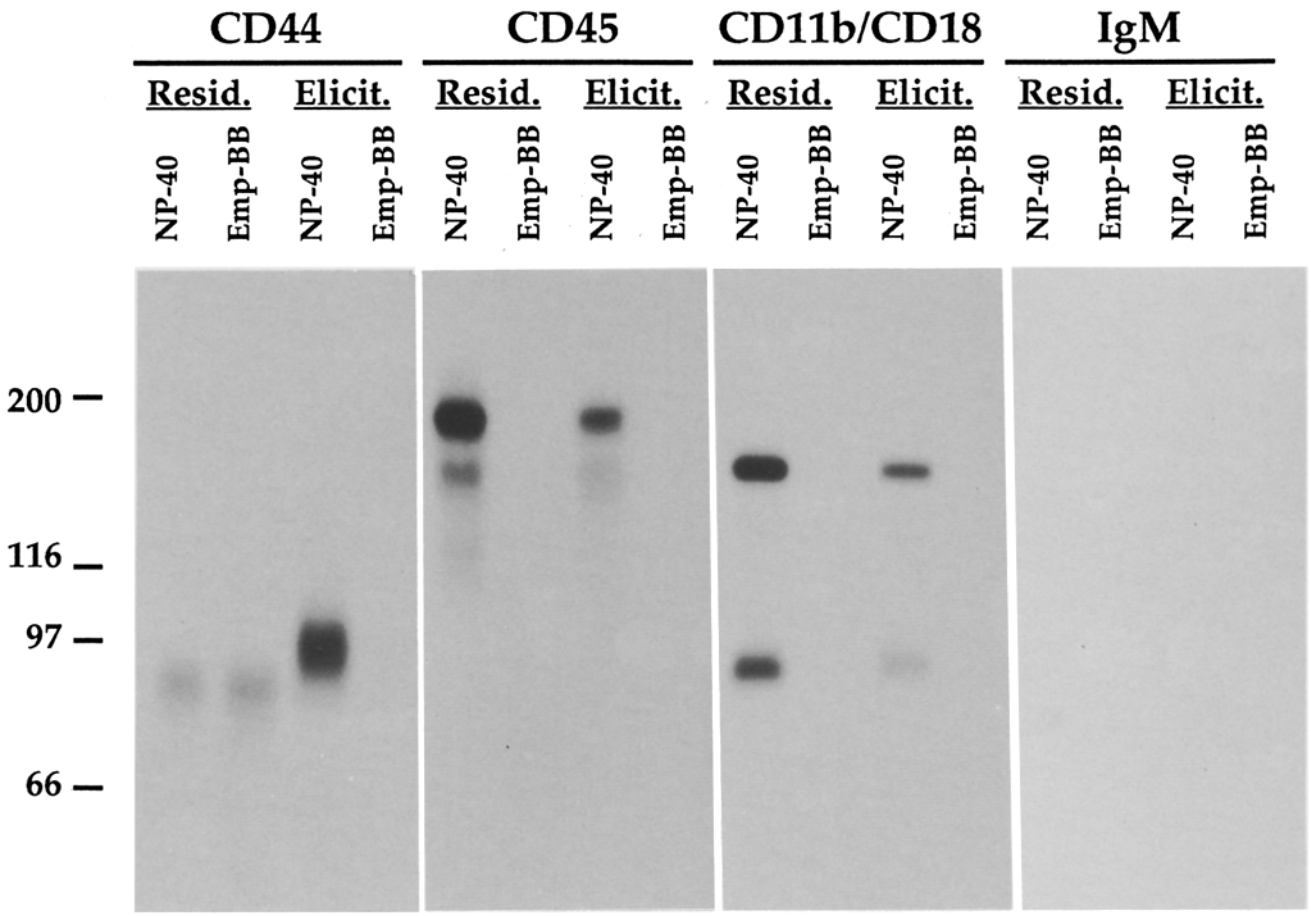

Figure 10. Resident and elicited macrophages do not differentially glycosylate CD11b/CD18 and CD45 and these antigens are not associated with the cytoskeleton. Macrophages were surface labeled with sodium^{125}iodine, NP-40, and Empigen-BB (*Emp-BB*) lysates were prepared as described in Materials and Methods. Lysates were treated with mAbs to CD44 (KM201), CD11b/CD18 (M1/70), CD45 (M1.9.3.4), or IgM (Bet-2), and immunoprecipitated proteins were analyzed by 7% SDS-PAGE.

chondroitin or heparin sulfate-modified CD44 on the surface of primary leukocytes, including macrophages. Despite the documentation of molecular weight heterogeneity, the factors influencing CD44 glycosylation in various cell types are unknown.

The results of this study demonstrate that the altered molecular mass of CD44 expressed by elicited macrophages is primarily, if not exclusively, due to posttranslational modifications of N-linked glycosidic residues. The terminal addition of sugar resulting in this structural change appears to occur in one cotemporal, biosynthetic step, with no detectable intermediates. Thus the ~90-kD elicited macrophage form does not go through an ~85-kD intermediate form, suggesting that the ~5-kD increase is not due to later glycosidic alterations (e.g., at the cell surface). Given that we are unable to detect intermediates in the biosynthetic glycosylation of CD44, it is impossible to conclusively determine from our present data, whether the ~90-kD form contains additional sites of glycosylation, more complex N-linked sugars, or both. However, the fact that the non-endo-H–treated core of resident and elicited macrophage CD44 is the same molecular weight suggests that the glycosidic difference is not due to an increased number of utilized glycosylation sites. It is important to note that this increase in N-linked glycosylation is specific for CD44 and does not occur with other macrophage proteins containing N-linked sugars, such as CD45 or CD11b/CD18.

The functional consequences of alterations in the N-linked sugars of CD44 on leukocyte binding and migration are not yet clear. All of the five consensus sequences for N-linked glycosylation are located in the membrane distal domain of CD44 that bears homology to proteoglycan core and link proteins (Nottenburg et al., 1989; Zhou et al., 1989). Since this portion of the molecule is presumably critical to the substrate binding capacity of CD44, even subtle changes in glycosylation may dramatically alter adhesion to extracellular matrix proteins such as hyaluronic acid. Despite the significant levels of CD44-mediated myeloma and hybridoma cell binding to hyaluronic acid coated plates, resting and activated primary leukocytes, including macrophages, exhibit little affinity for hyaluronic acid presented in this manner (Murakami et al., 1991). However, this does not exclude the possibility that CD44 on primary leukocytes might bind to different substrates under more physiologic conditions, such as HEV cell glycoproteins or hyaluronic acid as a component of an extracellular matrix complex. Changes in N-linked glycosylation would also be important if CD44 binds to lectin-like molecules that recognize specific glyco-

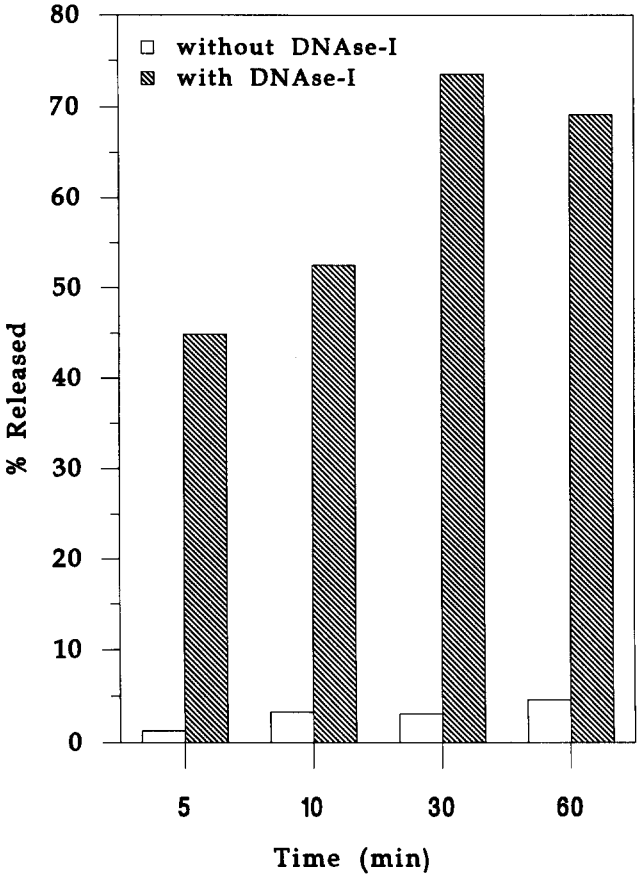

Figure 11. Treatment with DNAse-I liberates CD44 from the cytoskeleton of resident macrophages. Plate-bound macrophages were labeled with iodinated anti-CD44 (KM201). NP-40 soluble and insoluble fractions were prepared as described in Materials and Methods. NP-40 insoluble material was resuspended in the presence or absence of 1 mg/ml DNAse-I at 25°C. At the indicated times, samples were centrifuged, and soluble and insoluble materials were separated and counted. The data shown is from a typical experiment. □, without DNAse-I; ▨, with DNAse-I.

sidic residues. We have evidence that the glycosidic difference is accessible to lectins since the two forms exhibit differential binding to wheat germ agglutinin (data not shown).

The results of our experiments suggest that the structural differences in CD44 are related to cellular differentiation. Thioglycollate induces an inflammatory response in murine peritoneums characterized by an initial influx of polymorphonuclear leukocytes, that peaks 14 h after injection, followed by an influx of macrophages that peaks between 4–5 d after injection (Wiltrout et al., 1983). In addition to eliciting macrophage migration, thioglycollate induces a number of morphologic and functional changes (e.g., increased ingestion of complement coated RBCs) (Bianco et al., 1975). Another eliciting agent, *C. parvum*, activates macrophages and stimulates phagocytosis (Sljivic and Watson, 1977). The present study demonstrates that macrophages recruited to the peritoneum by either of these agents express a form of CD44 that manifests different posttranslational modifications and does not associate with the cytoskeleton. These alterations could reflect differences between two distinct macrophage populations, those residing in the peritoneum and those that are elicited to migrate there from the blood and lymphatics. Alternatively, the alterations may be due to changes during migration, or to changes induced by inflammatory stimulation. We have not observed a change in CD44 following the in vitro stimulation of resident macrophages with lipopolysaccharide or γ-interferon (data not shown).

Several groups have previously demonstrated the cytoskeletal association of CD44, possibly through an ankyrin-like protein (Lacy and Underhill, 1987; Carter and Wayner, 1988; Kalomiris and Bourguignon, 1988). The association of the hyaluronate receptor, CD44, with the actin cytoskeleton, has been well documented using a variety of actin stabilizing and destabilizing buffers, phalloidin, DNAse-I, and detergent extraction (Lacy and Underhill, 1987). Our DNAse-I extraction experiments suggest that CD44 also associates, either directly or indirectly, with the actin meshwork found in resident, but not elicited, macrophages. As predicted by the known mechanism of cytochalasins, which prevent the polymerization of new filaments without dissociating already polymerized F-actin, we were unable to demonstrate an effect of cytochalasin B on the cytoskeletal association of CD44 (data not shown).

The cytoskeletal attachment of CD44 may be similar to that of other glycoproteins, particularly those in the integrin and selectin families. Certain members of these families associate with a cytoskeletal complex that can include vinculin or vinculin-like proteins, talin, and actin (Horwitz et al., 1986; Nagafuchi et al., 1991). In one case, the PMA-induced binding of macrophages to laminin enhances the association of $\alpha_6\beta_1$ integrin with the cytoskeleton. This is associated with α_6 phosphorylation (Shaw et al., 1990).

Phosphorylation may play a role in the cytoskeletal association of CD44 as well. The correlation between the phosphorylation state of CD44 and its cytoskeletal association suggests that there may be a dynamic equilibrium of CD44 phosphorylation maintained by kinases and/or phosphatases. Biochemical events that change the balance of this equilibrium may, in turn, alter the association of CD44 with the cytoskeleton. Alternatively, the cytoskeletal association of CD44 may physically interfere with phosphorylation.

The cytoplasmic domain of CD44 contains one potential protein kinase C (PKC) and one protein kinase A (PKA) phosphorylation site (serines 293 and 318, respectively). However, we have not yet been able to detect changes in the phosphorylation of CD44 upon treatment with PKC or PKA stimulators including PMA, dibutyryl-cAMP, and forskolin (data not shown).

The cytoskeletal attachment of CD44 may influence or be influenced by leukocyte migration. Little is known about the migratory capacity of elicited macrophages except that, unlike resident macrophages, which migrate directly to the spleen after intravenous transfer, elicited macrophages tend to accumulate in the lungs (Wiltrout et al., 1983). Although studies with human leukocytes have demonstrated that CD44 is important to leukocyte binding to HEV in vitro (Jalkanen et al., 1987; Nakache et al., 1989), the importance of CD44 in in vivo migration into lymph nodes or inflammatory sites is as yet unknown, due to the unavailability of antibodies to murine CD44 that can effectively block leukocyte attachment to HEVs (Culty et al., 1990). It is of interest to note

that another homing receptor, the MEL-14 antigen, which promotes leukocyte binding to lymph node HEV, mediates the migration of neutrophils into the peritoneum after thioglycollate-induced inflammation (Watson et al., 1991).

We hypothesize that the association of CD44 with one or more endothelial ligands or extracellular matrix moieties, perhaps in the context of hyaluronic acid, may orient macrophages and prepare them for transendothelial migration into sites of inflammation (e.g., peritoneums exposed to thioglycollate). In macrophages that have been elicited to migrate into the peritoneum, CD44 is not associated with the cytoskeleton. This may be regulated through a phosphorylation-dependent mechanism. In addition, the N-linked glycosylation of newly synthesized CD44 is altered, resulting in the accumulation of the ~90-kD form. These two events may allow CD44 to alter the migratory capacity of elicited macrophages or to perform other functions within inflammatory sites, such as binding to lectin-like ligands.

The authors thank Drs. Z. Cohn, B. Cunningham, and D. Wojciechowicz, and M. Forman, R. Haspel, and L. Zeltser for critical review of this manuscript; Drs. M. Thelen, A. Aderem, R. Steinman, J. Gregory, and S. Damle for helpful discussions; J. Adams for graphics; and L. Tardelli for expert technical assistance.

This work was supported by U.S. Public Health Service grant AI25185 to E. Puré. R. L. Camp is a Merinoff Family Cancer Research Fund Graduate Fellow. E. Puré is a New York Heart Association Crawford-Maynard Established Fellow.

Received for publication 14 June 1991 and in revised form 26 August 1991.

References

Aruffo, A., I. Stamenkovic, M. Meinick, C. B. Underhill, and B. Seed. 1990. CD44 is the principal cell surface receptor for hyaluronate. *Cell.* 61:1303–1313.

Bianco, C., F. M. Griffin, and S. C. Silverstein. 1975. Studies of the macrophage complement receptor. *J. Exp. Med.* 141:1278–1290.

Brown, T. A., T. Bouchard, T. St. John, E. A. Wayner, and W. G. Carter. 1991. Human keratinocytes express a new CD44 core protein (CD44E) as a heparan-sulfate intrinsic membrane proteoglycan with additional exons. *J. Cell Biol.* 113:207–221.

Budd, R. C., J.-C. Cerottini, C. Horvath, C. Bron, T. Pedrazzini, R. C. Howe, and H. R. MacDonald. 1987. Distinction of virgin and memory T lymphocytes: Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *J. Immunol.* 138:3120–3129.

Camp, R. L., T. S. Kraus, M. L. Birkeland, and E. Puré. 1991. High levels of CD44 expression distinguish virgin from antigen primed B cells. *J. Exp. Med.* 173:763–766.

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

Culty, M., K. Miyake, P. W. Kincade, E. Silorski, E. C. Butcher, and C. B. Underhill. 1990. The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins. *J. Cell Biol.* 111:2765–2774.

Geppert, T. D., and P. E. Lipsky. 1991. Association of various T cell-surface molecules with the cytoskeleton: effect of cross-linking and activation. *J. Immunol.* 146:3298–3305.

Gunthert, U., M. Hofmann, W. Rudy, S. Reber, M. Zoller, I. Haußmann, S. Matzku, A. Wenzel, H. Ponta, and P. Herrlich. 1991. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell.* 65:13–24.

Hitchcock, S. E., L. Carlsson, and U. Lindberg. 1976. Depolymerization of F-actin by deoxyribonuclease I. *Cell.* 7:531–542.

Horwitz, A., K. Duggin, C. Buck, M. C. Berckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. *Nature (Lond.).* 320:531–533.

Hughes, E. N., G. Mengod, and J. T. August. 1981. Murine cell surface glycoproteins. Characterization of a major component of 80,000 daltons as a polymorphic differentiation antigen of mesenchymal cells. *J. Biol. Chem.* 256: 7023–7027.

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

Jalkanen, S., R. F. Bargatze, J. De Los Toyos, and E. C. Butcher. 1987. Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85–95-kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal, or synovial endothelial cells. *J. Cell Biol.* 105:983–990.

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

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

Kung, J. T., S. O. Sharrow, D. G. Sieckmann, R. Lieberman, and W. E. Paul. 1981. A mouse IgM allotypic determinant (Igh-6.5) recognized by a monoclonal rat antibody. *J. Immunol.* 127:873–876.

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

Lesley, J., and I. S. Trowbridge. 1982. Genetic characterization of a polymorphic murine cell surface glycoprotein. *Immunogenetics.* 15:313–320.

Lesley, J., R. Hyman, and R. Schulte. 1985. Evidence that Pgp-1 glycoprotein is expressed on thymus-homing progenitor cells of the thymus. *Cell. Immunol.* 91:397–403.

Mishell, B. B., and S. M. Shiigi. 1980. Selected Methods in Cellular Immunology. W. H. Freeman, San Francisco. 6–8.

Miyake, K., K. L. Medina, S.-I. Hayashi, S. Ono, T. Hamaoka, and P. W. Kincade. 1990*a*. Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. *J. Exp. Med.* 171:477–488.

Miyake, K., C. B. Underhill, J. Lesley, and P. W. Kincade. 1990*b*. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J. Exp. Med.* 172:69–76.

Murakami, S., K. Miyake, R. Abe, P. W. Kincade, and R. J. Hodes. 1991. Characterization of autoantibody-secreting B cells in mice undergoing stimulatory (chronic) graft-versus-host reactions. *J. Immunol.* 146:1422–1427.

Nagafuchi, A., M. Takeichi, and S. Tsukita. 1991. The 102-kD cadherin-associated protein: similarity to vinculin and posttranscriptional regulation of expression. *Cell.* 65:849–857.

Nakache, M., E. L. Berg, P. R. Streeter, and E. C. Butcher. 1989. The mucosal vascular addressin is a tissue-specific endothelial cell adhesion molecule for circulating lymphocytes. *Nature (Lond.).* 337:179–181.

Nottenburg, C., G. Rees, and T. St. John. 1989. Isolation of mouse CD44 cDNA: Structural features are distinct from the primate cDNA. *Proc. Natl. Acad. Sci. USA.* 86:8521–8525.

Omary, M. B., I. S. Trowbridge, M. Letarte, M. F. Kagnoff, and C. M. Isacke. 1988. Structural heterogeneity of human Pgp-1 and its relationship with p85. *Immunogenetics.* 27:460–464.

Ranscht, B., D. J. Moss, and C. Thomas. 1984. A neuronal surface glycoprotein associated with the cytoskeleton. *J. Cell Biol.* 99:1803–1813.

Shaw, L. M., J. M. Messier, and A. M. Mercurio. 1990. The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the alpha6/beta1 integrin. *J. Cell Biol.* 110:2167–2174.

Sljivic, V. S., and S. R. Watson. 1977. The adjuvant effect of Corynebacterium Parvum: T-cell dependence of macrophage activation. *J. Exp. Med.* 145: 45–57.

Springer, T., G. Galfre, D. S. Secher, and C. Milstein. 1978. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. *Eur. J. Immunol.* 8:539–551.

Stamenkovic, I., M. Amiot, J. M. Pesando, and B. Seed. 1989. A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. *Cell.* 56:1057–1062.

Stamenkovic, I., A. Aruffo, M. Amiot, and B. Seed. 1991. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:343–348.

Sutton, V. R., G. L. Wijffels, I. D. Walker, P. M. Hogarth, and I. F. C. McKenzie. 1987. Genetic and biochemical characterization of antigens encoded by the LT-24 (pgp-1) locus. *J. Immunogen.* 14:43–57.

Watson, S. R., C. Fennie, and L. A. Lasky. 1991. Neutrophil influx into an inflammatory site inhibited by a soluble homing receptor-IgG chimaera. *Nature (Lond.).* 349:164–167.

Wiltrout, R. H., M. J. Brunda, E. Gorelik, E. S. Peterson, J. J. Dunn, J. Leonhardt, L. Varesio, C. W. Reynolds, and H. T. Holden. 1983. Distribution of peritoneal macrophage populations after intravenous injection in mice: differential effects of eliciting and activating agents. *J. Reticuloendothel. Soc.* 34:253–269.

Woda, B. A., and M. B. Woodin. 1984. The interaction of lymphocyte membrane proteins with the lymphocyte cytoskeletal matrix. *J. Immunol.* 133:2767–2772.

Zhou, D. F. H., J. F. Ding, L. J. Picker, R. F. Bargatze, E. C. Butcher, and D. V. Goeddel. 1989. Molecular cloning and expression of Pgp-1. The mouse homolog of the human H-CAM [hermnes] lymphocyte homing receptor. *J. Immunol.* 143:3390–3395.