CD48 Binds to Heparan Sulfate on the Surface of Epithelial Cells*

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CD48 is a member of the immunoglobulin superfamily whose cell surface expression is strikingly up-regulated on the surface of Epstein-Barr virus-infected B cells. To date, no ligand for human CD48 has been characterized. In this study, we show that human recombinant CD48 binds to the glycosaminoglycan heparan sulfate on the surface of human epithelial cells. We have produced a monoclonal antibody (615) against epithelial cell surfaces that blocks this binding and show that it too recognizes heparan sulfate. The specific epitope on heparan sulfate that is recognized by the antibody and is involved in binding is also expressed in vivo on the basolateral surfaces of mucosal epithelium and lamina propria.

CD48 is a cell surface molecule that is expressed by B lymphocytes and other hematopoietic cells. Its expression is up-regulated in response to activation signals (1). CD48 is a 40–45-kDa glycoprotein member of the immunoglobulin superfamily, which is composed of a single polypeptide chain that has a high degree of homology to CD58 (LFA-3) and to a lesser extent CD2, the CD58 ligand. Despite intensive efforts, no ligand for human CD48 has been found, although it is now known to replace CD58 as the ligand for CD2 in rodents, which lack CD58 (2–5). It has been reported that CD48 is an alternate ligand for CD2 in humans (6, 7); however, this was not confirmed by surface plasmon resonance studies, which failed to detect binding of human CD48 to CD2 (K < 0.5 mM). We have recently presented evidence that there is a ligand for human CD48 on epithelial cells (8). In this study, we show that recombinant CD48 binds to the glycosaminoglycan (GAG) heparan sulfate, which is expressed on epithelial cells in vitro and in vivo.

CD48 expression is up-regulated when B cells are driven by Epstein-Barr virus (EBV) infection to become activated, proliferating lymphoblasts (9, 10), and an EBV-responsive element has recently been mapped within the upstream region of the CD48 gene (10). In comparison, we have shown that EBV-infected cells in the peripheral blood are all resting cells (11).

We hypothesize that EBV specifically up-regulates CD48 expression on lymphoblastoid B cells in order to cause them to be preferentially retained in the mucosal epithelium through the interaction of CD48 with heparan sulfate, thus explaining the absence of infected lymphoblasts from the peripheral blood.

MATERIALS AND METHODS

Cell Lines and Culture—ER and JY are EBV-immortalized B lymphoblastoid cell lines and were derived in this laboratory. Jurkat, Mol4, HI-60, and HeLa were obtained from the ATCC. EBV was a kind gift from Dr. Brian Seed. Wild type CHO K1 cells and the mutant derivative pgsA-745, pgsB-650, pgsD-677, and pgsE-606 were kindly supplied by Dr. Jeff Esko and have been characterized in detail elsewhere (12). Lymphoid and HeLa S3 cells were grown in RPMI 1640 supplemented with 10% FCS, 2 mM sodium pyruvate, 55 μM β-mercaptoethanol, and 50 μg/ml gentamicin. The remaining epithelial cells were grown in Dulbecco’s modified Eagle’s/Ham’s F-12 medium (3:1) supplemented with 10% FCS, 100 units/ml penicillin G, 100 units/ml streptomycin, 5 μg/ml insulin, 1.1 μM hydrocortisone, 1.64 μM epidermal growth factor, 5.5 μg/ml epinephrine, 5 μg/ml transferrin, 2 mM triiodothyronine, 18 μM adenine. Adherent cells were removed for analysis using 0.5 mM EDTA/PBS. HeLa S3 cells were grown in suspension in spinner flasks (Belco).

Antibodies—The monoclonal antibodies used in this work, CJ250, 451, and 615, were produced as follows. Female Balb/cAnN mice (Charles River Laboratories, 8–10 weeks old) were immunized intraperitoneally with whole live HeLa S3 cells (2 × 10⁷ cells) on days 1, 31, and 73 and with a crudely purified membrane preparation from 1.25 × 10⁶ HeLa S3 cells on days 253 and 275, all without adjuvant. Five days after the final injection, splenocytes were fused to the non-secreting mouse myeloma cell line X63-Ag8.653. Hybridoma supernatants were screened for antibodies capable of blocking the binding of HeLa S3 cells to immobilized sCD48 (see below). The antibodies were purified from ascites fluid using immobilized protein L (Kappapack, Zymed Laboratories Inc.) and concentrated to 4 mg/ml by spin filtration using a Centricon-30 concentrator (Amicon).

FACS® Analysis—Purified antibodies were conjugated by standard methods to fluorescein isothiocyanate (FITC) for direct staining or biotin for indirect staining. FITC-conjugated secondary reagents to detect primary antibodies were: FITC-conjugated F(ab')₂ fragment of rabbit anti-human μ chain (Dako), FITC-conjugated F(ab')₂ fragment of goat anti-mouse Ig (Dako), FITC-conjugated rabbit anti-mouse Ig (Dako), and FITC-conjugated streptavidin (Dako). Analysis was performed on a FACScan® flow cytometer (Becton-Dickinson).

Production of Monomeric sCD48—The cDNA 2A1.sec, encoding a novel secreted form of human CD48, was generated by oligonucleotide-directed mutagenesis as described previously (13, 14). The cDNA 2A1.sec was excised from the CDMS vector by digestion with XhoI and the resulting 0.9-kilobase fragment was inserted into the unique XhoI site of the eukaryotic glutamine synthetase (GS) expression vector pEE14 (Celltech) to create pEE2A1.sec. The GS gene on the plasmid confers resistance to the GS inhibitor methionine sulfoximine and was used as a selection marker. Exponentially growing monolayers of CHO K1 cells (1 × 10⁷) were transfected with 25–30 μg of pEE2A1.sec DNA, previously linearized with Drd-1, using the ProFection® calcium phosphate transfection system (Promega). Following transfection, CHO K1 monolayers were “glycerol-shocked” for 2 min using 15% (v/v) glycerol/ HBSS and returned to culture. Thirty-six hours following transfection, cells were harvested and transferred to 96-well plates at 5 × 10⁴ cells/well in medium containing methionine sulfoximine at a final concentration of 25 μM. After 14 days, supernatants from wells containing growing colonies were harvested and screened for the presence of sCD48 protein by sandwich ELISA. To induce amplification of the plasmid, cells were incubated in 1–100 μM methionine sulfoximine.

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‡ The abbreviations used are: GAG, glycosaminoglycan; EBV, Epstein-Barr virus; FACS, fluorescence-activated cell sorting; CHO, Chinese hamster ovary; BSA, bovine serum albumin; FCS, fetal calf serum; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; GS, glutamine synthetase; HBSS, Hank’s buffered saline solution; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

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Cells producing the highest levels of sCD48 were retained, and large amounts of sCD48 protein were generated by bulk culture of these cells. Upon culture exhaustion, supernatants were harvested and supplemented with protease inhibitors: AEBSF (100 μM), phenylmethylsulfonyl fluoride (500 μM), leupeptin (1 μg/ml), aprotonin (2 μg/ml), and pepstatin A (0.1 μg/ml). Each solution was sterilized by 0.22 μm chromato- graphic purification using mAb 6.28 immobilized on Affi-Gel-10. Purified sCD48 was concentrated to 1 mg/ml by spin filtration using a Centriprep-10 concentrator (Amicon) and stored in aliquots at −70 °C until use.

Adhesion Assays Using Monomeric sCD48—Purified sCD48 was diluted to 100 μg/ml with NaCl, 0.02% (w/v) Tween 20, and 0.2% (v/v) d-mannose and dispensed into the wells of 96-well polystyrene microtiter plates (Linbro) at 75 μl/well and incubated for 16 h at 4 °C. Following immobilization, wells were filled with 0.1% (w/v) BSA/HBSS, pH 7.4, and incubated for 2 h at 25 °C to block unbound sites. Coated, blocked wells were washed three times each with 200 μl of 1% FCS/HBSS, pH 7.4 (binding buffer), and then filled with binding buffer and kept at 4 °C until use. Cells were washed twice with ice-cold HBSS and resuspended in 50 μg/ml calcein-AM (Molecular Probes)/HBSS at ~1 × 10^5 cells/ml and incubated for 30 min at 25 °C in total darkness to label the cells. Following this, labeled cells were washed twice and resuspended in the same buffer at 1 × 10^5 cells/ml for loading into coated wells. 1 × 10^4 labeled cells, in a volume of 100 μl, were loaded into each well and incubated for 1 h. Following incubation, unbound cells were removed by inverting the plates and allowing them to float for 60 min in tanks of ice-cold 1% (v/v) FCS HBSS, 0.5 mM MgCl₂, pH 7.4. After 60 min, the intensity of fluorescence remaining in the well was measured using the CytoFluor 2300 system (Millipore).

Indirect ELISA with Glycosaminoglycan-coated Plates—The wells of 96-well flat bottom plates (Linbro) were treated with a solution of 1% glutaraldehyde for 5 min and washed three times with PBS, pH 7.4, at 25 °C. Immediately following the removal of glutaraldehyde, the wells were coated for 2 h at 25 °C with 100 μl of serially diluted GAGs (heparin (Sigma), chondroitin sulfate C (Sigma), and hyaluronic acid (Sigma)) in PBS or PBS alone. Following coating, wells were filled with blocking buffer, consisting of 0.25% (w/v) BSA/PBS, pH 7.4, incubated for 2 h and then washed three times with blocking buffer. Culture supernatants containing either mAb 615 or mAb 1117 were added to each well, and wells were incubated at room temperature for 1 h.

Following incubation, the wells were washed three times with blocking buffer and incubated for 1 h at room temperature with alkaline phosphatase-conjugated rabbit anti-mouse Ig (Dako). Wells were again washed three times with blocking buffer, and 100 μl of substrate solution, consisting of 3 mg/ml p-nitrophenyl phosphate (Sigma), 0.5 mg/ml MgCl₂, 50 mM NaCl, 100 μM Na₂HPO₄, pH 9.6, were dispensed into each well. Wells were incubated for 30 min at room temperature, and color development was stopped by the addition of 25–50 μl of 1 N NaOH to each well. The absorbance of each reacted well was measured at 405 nm using a DynaTek MRT700 ELISA plate reader.

GAG-degrading Enzyme Digestions—Briefly, HeLa S3 cells were harvested from culture and washed twice with ice-cold HBSS by centrifugation at 200 × g for 10 min at 4 °C. Approximately 1.6 × 10^7 washed cells were resuspended in 4 ml of reaction buffer alone or reaction buffer containing one of the following GAG-degrading enzymes: chondroitinase ABC (1.25 units/ml, EC 4.2.2.4, Sigma), hyaluronidase (25 units/ml, EC 3.2.1.35, Calbiochem), keratanase (1.8 units/ml, EC 3.2.1.103, Sigma), heparin lyase I (3.125 units/ml, EC 4.2.2.7, Sigma), heparin lyase II (3.125 units/ml, Sigma), and heparin lyase III (2.5 units/ml, EC 4.2.2.8, Sigma). Reaction buffer consisted of 1% (w/v) BSA, 1 μg/ml leupeptin, 10 units/ml aprotinin, 1 μg/ml antipain, 100 μM AEBSF/PBS, pH 7.4. Cells were incubated with enzyme for 60 min at 35 °C with occasional agitation. Following this incubation, cells were washed three times with 2.5% (v/v) FCS, 0.025% (v/v) NaN₃, HBSS by centrifugation and stained with antibodies for FACS analysis as described above.

To monitor the efficacy of each enzyme, the above digestions were performed in parallel using radiolabeled HeLa S3 cells. For labeling of GAGs, cultures of HeLa S3 cells were established in complete SO₄-/glucose-deficient RPMI growth medium supplemented with p-[3H]glucosamine and p-[3H]glucosamine chloride (40 Ci/mmol, NEN Life Science Products) and Na₂SO₄ (563 mCi/mmol, NEN Life Science Products) and incubated for 48 h. Following digestion, the cells were pelleted and supernatants were collected for scintillation counting with HydroFluor scintillation fluid (National Diagnostics) and an LKB RackBeta scintillation counter.

Immunohistochemistry—Degraffinated sections of human small intestine, fixed previously in 2% paraformaldehyde, were incubated for 30 min at 25 °C with either mAb 615 or 1117 (culture supernatants) and then rinsed in HBSS containing 1% BSA (w/v) for 10 min. Specific staining was detected using biotinylated rabbit anti-mouse immuno- globulin and peroxidase conjugated avidin-biotin complexes (Vector Laboratories, Burlingame, CA) as directed by the vendor. Visualization of stained sections was accomplished using 0.05% 3,3′-diaminobenzeno- dine (w/v) and 0.01% hydrogen peroxide (w/v). Prior to antibody staining in some experiments, deparaffinated sections were first treated with either heparin lyase III (2.5 units/ml, EC 4.2.2.8) or chondroitinase ABC (1.25 units/ml, EC 4.2.2.4) for 30 min at 25 °C followed by three rinses in HBSS containing 1% BSA (w/v) for 5 min each. Reaction buffer for both enzyme digestions consisted of 1% (w/v) BSA, 1 μg/ml leupeptin, 10 units/ml aprotinin, 1 μg/ml antipain, 100 μM AEBSF/PBS, pH 7.4.

RESULTS

The Ligand for CD48 Is Highly and Preferentially Expressed on Epithelial Cells—We have screened a large panel of cell lines for their ability to bind sCD48 immobilized on plastic dishes. A representative set of binding results for the most informative human cell lines is shown in Fig. 1A. Of all the cell lines tested only, epithelial cells bound consistently and avidly as exemplified by the HeLa S3 and tsA201 cells. Not shown is that epithelial cell lines from other species including monkeys and rodents bound equally well as the human lines. We produced a panel of monoclonal antibodies against the surface of HeLaS3 cells and used the binding assay to test for their ability to block the interaction between epithelial cells and CD48. Three mAbs, CJI250, 451, and 615, were derived that bind to the surface of epithelial cells and proved useful for further study. All three antibodies recognize the same or overlapping epitopes as demonstrated by competitive RIA where each mAb specifically blocked the binding of the others. As shown in Fig. 2, all three antibodies specifically block the interaction of epithelial cells with CD48. The antibodies were then used to stain the panel of cell lines, tested in the binding assay, for expres- sion of the putative CD48 ligand. FACS analysis for the same, most informative, human cell line is shown in Fig. 1 (B–H) adjacent to the results obtained with the same cell lines in binding assays. All three antibodies gave the same staining pattern so only the results for CJI250 are shown. Strong staining was observed for all epithelial cell lines tested irrespective of their tissue or species origin. In contrast, the antibodies bound weakly to human B cell lines, including EBV-transformed B cell lines, and did not stain any of the human T cell or myeloid lines tested with the exception of Jurkat which stained weakly. In sum, the FACS staining profiles obtained with the blocking antibodies on the complete panel of cell lines corre- lated precisely with the results of the adhesion assays (Fig. 1, A–H, and Table I).

Analysis of the CD48 Ligand with CHO Cell Lines Mutated in Their GAG Synthetic Pathways—Despite intensive biochemical efforts, we have been unable to identify a specific protein entity to account for the CD48 ligand. Furthermore, we noted that all three of the blocking monoclonal antibodies were of the IgM isotype, raising the possibility that the ligand may be carbohydrate in nature and therefore unable to elicit a secondary antibody response. As there is evidence suggesting that members of the immunoglobulin superfamily can interact with carbohydrate ligands, especially the complex carbohydrates repre- sented precisely with the results of the adhesion assays (Fig. 1, A–H, and Table I).

Analysis of the CD48 Ligand with CHO Cell Lines Mutated in Their GAG Synthetic Pathways—Despite intensive biochemical efforts, we have been unable to identify a specific protein entity to account for the CD48 ligand. Furthermore, we noted that all three of the blocking monoclonal antibodies were of the IgM isotype, raising the possibility that the ligand may be carbohydrate in nature and therefore unable to elicit a secondary antibody response. As there is evidence suggesting that members of the immunoglobulin superfamily can interact with carbohydrate ligands, especially the complex carbohydrates repre- sented by the GAGs, we decided to test for a possible role for GAGs in epithelial cell binding to CD48. To do this, we took advantage of the fact that there are available a number of mutant variants of the CHO epithelial cell line that are defec- tive in various aspects of GAG synthesis. Each mutant cell line has been well characterized (12) and is described briefly in Table II. Mutants pgS-A745 and pgS-B650 are unable to add any GAGs to proteins because they lack the enzymes needed to synthesize the core proximal tetrasaccharide upon which the
GAGs are synthesized. Mutant pgsD-677 fails specifically to synthesize heparan sulfate, and pgsE-606 is defective in the sulfation of GAGs.

The wild type and mutant CHO cell lines were first examined by FACS® analysis after staining with the three blocking mAbs. Fig. 3 shows the result of staining wild type CHO K1 cells (gray histograms) in comparison to the various mutant cell lines (white histograms), whereas an isotype-matched control mAb 1117 did not. None of the anti-

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**TABLE I**

A summary of all of the cell lines tested in the CD48 adhesion assays and by FACS staining with the blocking monoclonal antibodies

For data see Figs. 1, 3, and 4.

| Cell line   | Binding assay | FACS staining | Cell type       |
|-------------|---------------|---------------|-----------------|
| HeLa S3     | ++           | +++          | Epithelium human|
| tsA201      | ++           | +++          | Epithelium human|
| JY          | +            | ++           | B cell human    |
| ER          | +            | +            | B cell human    |
| Molt-4      | /−           | /−           | T cell human    |
| Jurkat      | +/−          | /−           | T cell human    |
| HL-60       | /−           | /−           | Myeloid human   |
| CHO-K1      | ++           | +++          | Epithelial hamster|
| CHOpgsA-745 | −            | −            | mt epithelial hamster|
| CHOpgsB-650 | −            | −            | mt epithelial hamster|
| CHOpgsD-677 | −            | −            | mt epithelial hamster|
| CHOpgsE-606 | ++           | +++          | mt epithelial hamster|

* Binding is denoted as follows: +++ > 80%, ++ = 20–40%, +/− < 10%, = undetected.

* FACS staining is denoted as follows: +++ = strong (e.g. HeLaS3), ++ = moderate (e.g. JY), +/− weak (e.g. Jurkat), = undetected e.g. Molt-4.

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**FIG. 1.** Binding assays and flow cytometry with blocking antibodies demonstrate the presence of a ligand for CD48 on epithelial cell lines. A selected group of all the lines tested is shown. They are the human epithelial lines HeLa S3 and tsA201, the human hematopoietic cell lines JY and ER (B cells), Jurkat and Molt-4 (T cells), and HL-60 (myeloid). The lines shown were chosen to demonstrate the range of binding, from undetectable to strong, seen in the adhesion assay and intensity of staining observed by FACS analysis with the blocking antibodies. They are representative of all the cell lines tested. Thus, for example, all epithelial cell lines, irrespective of their tissue or species of origin, bound strongly in the assay and stained brightly with the antibody. A, binding assay. Cells loaded with an intracellular fluorescent dye (either BCECF-AM or calcein-AM, Molecular Probes) were allowed to settle to the bottom of wells in a flat bottomed microtiter plate precoated with sCD48 or BSA at a concentration 10 μg/ml. The plates were then submerged and inverted in a tank of 1 mM MgCl₂, 0.5 mM CaCl₂, 1% (v/v) FCS/PBS, pH 7.4, to remove unbound cells from the well (force = 1 g). The intensity of fluorescence in the well before and after inversion was measured and used to calculate the fraction of cells bound as described under “Materials and Methods.” Each measurement shown represents the mean of triplicate wells. B–G, FACS analysis of the same cell lines as were used in A. Cells were stained with the blocking antibody CJI250 (white histograms) or the isotype control 1117 (gray histograms). The same staining pattern was obtained with the other two blocking monoclonal antibodies, 451 and 615, except 615 consistently stained more strongly (see for example Fig. 3).

**FIG. 2.** Three monoclonal antibodies that block the binding of epithelial cells to sCD48. Blocking of HeLa S3 binding to immobilized sCD48. Each assay was performed in the presence of undiluted culture supernatant from the isotype control antibody 1117 or one of the blocking IgM antibodies. Additional controls include the addition of binding buffer alone or a polyclonal mouse anti-CD48 serum (1:100). For details, see the legend to Fig. 1 and “Materials and Methods.”
bodies stained the mutant CHO lines pgsA-745 or pgsB-650, demonstrating that all three antibodies recognize a GAG or GAG-associated structure. In addition, the antibodies failed to stain the mutant line pgsD-677. The data from assays with CHO line pgsD-677 were most revealing, as these cells lack heparan sulfate, but tend to overexpress chondroitin sulfates by a factor of 2-3, suggesting that the GAG recognized by the mAbs CJI250, 451, and 615 is heparan sulfate. When mutant pgsE-606 was examined by FACS®, the staining with mAbs 451 and CJI250 increased slightly, whereas that with mAb 615 decreased by about a factor of 5-10. This result suggested that the N-sulfation of heparan sulfate is most important for mAb 615 binding and that mAbs 451 and CJI250 recognize overlapping but distinct epitopes from 615.

To directly demonstrate interaction of sCD48 with cell surface heparan sulfate, adhesion assays were performed using the various CHO cell mutants. The results of these assays are shown in Fig. 4. Only the wild type CHO K1 and CHO pgsE-606 bound significantly to immobilized sCD48, both at levels comparable to those seen with HeLa S3.

Thus, the binding assay results are in agreement with the FACS analysis providing strong evidence that the antibodies recognize structural elements of GAGs that are directly involved in mediating the binding of epithelial cells to CD48.

#### Table II

| CHO strain | Biochemical defect | Glycosaminoglycan production |
|------------|--------------------|-----------------------------|
| K1-wt      | None               | Heparan sulfate, Chondroitin sulfate |
| pgsA-745   | Xylosyltransferase | No                          |
| pgsB-650   | Galactosyltransferase | No                          |
| pgsD-677   | N-Acetylglucosaminyl/glucuronosyltransferase | No                          |
| pgsE-606   | N-Sulfotransferase | Yes⁴                   |

⁴ Chondroitin sulfate accumulates to levels 2–3 times higher than in wild-type cells.

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**CHO Cell Line**

![Flow cytometric analysis of wild type CHO cells versus GAG-deficient CHO cell mutants with antibodies that specifically block epithelial cell binding to sCD48. Each panel presents the data for a different CHO cell mutant (white histograms) overlaid upon the data for the wild type CHO K1 (gray histograms). The CHO cell mutants analyzed were CHO pgsA-745, CHO pgsB-650, CHO pgsD-677, and CHO pgsE-606. Cells were harvested for assay using EDTA only. For each experiment, both wild type and mutant cells were incubated with either the blocking monoclonal antibodies CJI250, 451, and 615, or the isotype control 1117. The defects in the CHO cell mutants are summarized in Table II.**

**FIG. 3.** Flow cytometric analysis of wild type CHO cells versus GAG-deficient CHO cell mutants with antibodies that specifically block epithelial cell binding to sCD48. Each panel presents the data for a different CHO cell mutant (white histograms) overlaid upon the data for the wild type CHO K1 (gray histograms). The CHO cell mutants analyzed were CHO pgsA-745, CHO pgsB-650, CHO pgsD-677, and CHO pgsE-606. Cells were harvested for assay using EDTA only. For each experiment, both wild type and mutant cells were incubated with either the blocking monoclonal antibodies CJI250, 451, and 615, or the isotype control 1117. The defects in the CHO cell mutants are summarized in Table II.

**TABLE II**

| CHO mutant cell lines used and their defects in glycosaminoglycan synthesis |
|-----------------------------|-----------------------------|
| CHO strain | Biochemical defect | Glycosaminoglycan production |
|------------|--------------------|-----------------------------|
| K1-wt      | None               | Heparan sulfate, Chondroitin sulfate |
| pgsA-745   | Xylosyltransferase | No                          |
| pgsB-650   | Galactosyltransferase | No                          |
| pgsD-677   | N-Acetylglucosaminyl/glucuronosyltransferase | No                          |
| pgsE-606   | N-Sulfotransferase | Yes⁴                   |

⁴ Chondroitin sulfate accumulates to levels 2–3 times higher than in wild-type cells.

**CHO Cell lines**

![Analysis of wild type and mutant CHO cell binding to sCD48 by adhesion assay. Wild type CHO K1, CHO pgsE-606, CHO pgsD-677, and CHO pgsA-745 were analyzed by adhesion assay for the ability to bind to immobilized sCD48. Cells were harvested for the assay using EDTA only. For each cell line tested, sCD48 from a stock at a concentration of 1 mg/ml was diluted as indicated and immobilized to polystyrene surfaces. The adhesion assays were performed as described in Fig. 1 and “Materials and Methods.” Each measurement shown represents the mean of triplicate wells. Error bars represent the standard deviation of the mean.**

**FIG. 4.** Analysis of wild type and mutant CHO cell binding to sCD48 by adhesion assay. Wild type CHO K1, CHO pgsE-606, CHO pgsD-677, and CHO pgsA-745 were analyzed by adhesion assay for the ability to bind to immobilized sCD48. Cells were harvested for the assay using EDTA only. For each cell line tested, sCD48 from a stock at a concentration of 1 mg/ml was diluted as indicated and immobilized to polystyrene surfaces. The adhesion assays were performed as described in Fig. 1 and “Materials and Methods.” Each measurement shown represents the mean of triplicate wells. Error bars represent the standard deviation of the mean.

Furthermore, the FACS® and cell binding data with the CHO cell mutants suggest that the GAG involved in the binding of epithelial cells to CD48 is heparan sulfate.
Analysis of the Ligand for CD48 by Enzyme Digestion—Well characterized mutant human epithelial cell lines that are defective in GAG production are not available. To confirm the role of GAGs in the binding of human epithelial cell lines to CD48 and more precisely define the nature of the GAG, we have employed digestion with GAG specific enzymes. Specifically, we looked at the ability of HeLa S3 cells to bind to CD48 before and after digestion with heparin lyase I, heparin lyase II, heparin lyase III, chondroitinase ABC, hyaluronidase, and keratanase. The specific structures cleaved by these enzymes are shown in Table III. For heparin lyases I, II, and III, the enzyme activity varies depending on the particular modifications present in the heparin/heparan sulfate chain. As shown in Table III, digestion with these enzymes can distinguish heparin from heparan sulfate, because lyase I preferentially digests heparin, whereas lyase III preferentially digests heparan sulfate. Each lyase removed the epitopes recognized by monoclonal antibodies CJI250, 451, and 615, with heparin lyase I being the least effective and heparin lyase III being the most effective (Fig. 5). Lyase III was so effective that staining could no longer be detected with mAbs CJI 250 and 451 and staining with CJI250 was reduced more than 10-fold. A protein epitope recognized by a control antibody BU75, specific for CD44, was not affected by treatment with these enzymes. Treatment of the Hela S3 cell surface with enzymes specific for the GAGs chondroitin sulfate, hyaluronic acid, and keratin sulfate did not affect staining with any of the antibodies (Fig. 5).

We have also analyzed the ability of proteases to destroy the ligand structure on the surface of HeLa cells. As shown in Fig. 6, staining of HeLa S3 cell with the blocking monoclonal antibodies could be drastically reduced upon digestion with trypsin.

Taken together, the enzyme digestion data further support...
the idea that heparan sulfate is the ligand for CD48 on epithelial cells and suggest that protein is required for its presentation.

**Heparin and Heparan Sulfate Specifically Compete for the Binding of Epithelial Cells to CD48 or the Blocking mAb 615**—An alternate interpretation to all of the studies presented above is that the ligand is not heparan sulfate, but instead a protein that associates with heparan sulfate. To exclude this possibility, we have attempted to directly demonstrate the binding of CJI250, 451, and 615 to GAGs either directly or by competitive inhibition assays. As shown in Fig. 7, heparin, heparan sulfate, and dextran sulfate each inhibited binding of mAb 615 to HeLa S3 cells, but had no effect on the binding of monoclonal antibodies CJI250 and 451. As expected, chondroitin sulfate C, hyaluronic acid, and dextran did not have any effect on the binding of mAb 615. The same results were obtained when the GAGs were used to directly block binding of HeLa S3 cells to sCD48 (Fig. 8). Only heparin, heparan sulfate, and dextran sulfate could block the binding. It was striking that in competition assays for mAb 615 or sCD48 binding to epithelial cells heparin was much more efficient than heparan sulfate. This suggests that the epitope structure recognized by CD48 and the blocking antibody 615 is much more densely present on the preparations of heparin we have used than on the heparan sulfate. This was confirmed in indirect ELISA assays where it was possible to demonstrate direct binding of mAb 615 to heparin. We were unable to detect direct binding to chondroitin sulfate C or hyaluronic acid (Fig. 9). Heparin is not present on the surface of epithelial cells. However, heparin and heparan sulfate are structurally the same, the only difference being that heparin tends to be more extensively modified through sulfation and epimerization. However, the modifications on each are heterogeneous; therefore, there is extensive structural overlap between the two. We conclude therefore that 615 specifically recognizes an epitope on epithelial cell-associated heparan sulfate that mediates the binding of sCD48 to epithelial cells. This structure is more abundant on the preparations of heparin that we have used than on the preparations of heparan sulfate. The data are inconclusive about the precise nature of the structures recognized by CJI250 and 451.

**Expression of the CD48 Ligand in Tissues**—Heparan sulfate is broadly expressed throughout the tissues. We wanted to ascertain if the form of heparan sulfate recognized by CD48 and the blocking antibody 615, was localized to specific tissues, particularly epithelial tissue. Demonstration of specificity would support the functional significance of this structure. As shown in Fig. 10A, mAb 615 clearly stains distinct structures within sections of small intestine, compared with the isotype-matched control 1117 (Fig. 10B). Although staining of the apical surfaces of epithelial cells in these sections is minimal, the basolateral surfaces of these cells, as well as the underlying basement membranes are stained. Significant staining can also be seen within the lamina propria underlying the epithelium, a common site for resident B lymphocytes. No staining was observed outside of the mucosal epithelium and the lamina propria (data not shown). The specificity of the staining was confirmed by predigestion of the tissue slice with heparin lyase III, which resulted in a dramatic reduction in the staining compared with a control digestion with chondroitinase (Fig. 10, C and D). After digestion with the lyase, there did still remain some focal areas of epithelium which stained weakly. It is not clear whether these represented structures that were resistant to staining or areas to which the enzyme failed to gain access.

**DISCUSSION**

The results presented here demonstrate that, in humans, the GAG heparan sulfate, expressed on the surface of epithelial cells, binds recombinant sCD48. Several different approaches have been taken to prove this. Wild type CHO cell lines bind to sCD48 and are recognized by three monoclonal antibodies (CJI250, 451, and 615), which specifically block the binding, whereas mutant CHO cells lacking heparan sulfate fail to bind either CD48 or the antibodies. Digestion of HeLa S3 cells with heparin lyase III, which is specific for heparan sulfate, inhibits binding of the cells both to CD48 and the blocking monoclonal antibodies. Finally, the blocking antibody 615 binds directly to heparan sulfate and heparan sulfate specifically competes for the binding of CD48 and antibody 615 to epithelial cells. The third point suggests that there is a direct CD48-heparan sulfate interaction. This is important because GAGs typically dominate the physical characteristics of the proteoglycans they decorate, principally due to their large size and charge density, and could affect the conformation of the core protein. Therefore, the possibility existed that CD48 binds to a protein whose conformation is dependent on heparan sulfate. However, if this were true, heparan sulfate would not directly compete for binding. Therefore, we can conclude that CD48 binds directly to heparan sulfate.

The discovery that CD48 binds to a heparan sulfate structure that is expressed in mucosal epithelium and lamina propria is particularly interesting in the context of the relationship between CD48 and EBV. EBV infects resting B cells and causes
them to become activated, immortalized, proliferating lymphoblasts. In parallel, it induces a 10-fold increase in the surface expression of CD48 through the action of an EBV-responsive element in the upstream region of the CD48 gene (10). Thus, CD48 was the first cell surface activation marker shown to be activated by EBV (9). This raises the possibility that EBV specifically up-regulates CD48 expression during the lymphoblastoid stage of latency to cause the cells to be preferentially retained in the mucosal epithelium or lamina propria. This would account for the observation that only infected, resting B cells but no lymphoblasts are detected in the peripheral circulation (11, 15). By extension, the role of CD48 in the human may be to cause activated B lymphocytes to be retained in the lamina propria, a known location for mucosal specific B cells.

There is increasing precedence for complex carbohydrates interacting with adhesion molecules (16) including members of the immunoglobulin superfamily (17–21). Indeed, the specific interaction of L-selectin with mucin like molecules carrying sulfated Lewis antigens is known to play a central role in the homing of lymphocytes to lymph nodes, via high endothelial vescules, and to areas of inflammation in peripheral and mucosal locations (reviewed in Refs. 22 and 23). Thus, there is precedence for the interaction of CD48 with GAGs and the possibility that this interaction could be important in the retention of activated B cells in the lamina propria and the mucosal epithelium.

GAGs are carbohydrate polymers defined by specific combinations of a repeating disaccharide unit, which consists of a uronic acid and a hexosamine joined by various glycosidic bonds. The four major classes (heparin/heparan sulfates, chondroitin/dermatan sulfates, keratin sulfates, and hyaluronic acid) are each described in Table III. With the exception of

![Fig. 7. Competitive inhibition by purified glycosaminoglycans of HeLa S3 staining by the blocking monoclonal antibodies. The ability of heparin, heparan sulfate, dextran sulfate, chondroitin sulfate, hyaluronic acid, and dextran to competitively inhibit the binding of mAb 615 (middle and bottom) to HeLa S3 cells was assessed by FACS®. In each panel, white histograms, representing antibody staining in the presence of competitor, are overlaid on gray histograms, which represent antibody staining in the absence of competitor. No blocking of CJ1250 (top: heparin, heparan sulfate, and dextran sulfate only shown) or 451 (data not shown) was observed with any GAG.](image1)

![Fig. 8. Competitive Inhibition of HeLa S3 binding to sCD48 with purified GAGs. Adhesion assays were performed in the presence of increasing concentrations of the GAGs dextran, dextran sulfate, hyaluronic acid, chondroitin sulfate A, heparan sulfate, or heparin as described under “Materials and Methods.” For details see the legend to Fig. 1 and “Materials and Methods.”](image2)

![Fig. 9. mAb 615 binds directly to heparin in an indirect ELISA assay. The GAGs heparin, chondroitin sulfate C, and hyaluronic acid were coupled to microtiter plate wells as serial 10-fold dilutions, as indicated by the wedges, starting at 1 mg/ml. Bound antibody was assessed by indirect ELISA. The antibodies tested included CJ1250, 451 (data not shown), 615, and the isotype control 1117. Binding was only observed with 615.](image3)
hyaluronic acid, biosynthesis of all GAGs is initiated upon a core protein to which the GAG may or may not remain attached. Proteins that remain decorated by GAG are referred to as proteoglycans. In each case, the GAG is attached to a serine residue in the core protein through the following characteristic tetrasaccharide sequence: GlcUA-1,3-Gal-1,4-Gal-1,4-Xyl-1,3-O-Ser (24, 25). The trypsin sensitivity of the CD48 ligand is consistent with the GAG being associated with a proteoglycan but the identity of this structure remains unknown. It is quite likely that several different proteoglycans can express the GAG. This conclusion is based on our observations that certain mAbs to the homing proteoglycan CD44 can block the binding of epithelial cells to CD48 yet CD44-negative epithelial cell lines still express the GAG (8). This suggests that CD44 may be one of several proteins capable of expressing the ligand. Another likely candidate is syndecan-1 as a syndecan-like proteoglycan has been reported to be the major proteoglycan expressed by CHO cells (26). More importantly, syndecan is also one of the major proteoglycans expressed on the basolateral surface of epithelial cells where it is decorated with heparan sulfate GAGs which are structurally variant depending on the tissue location (27, 28). Syndecan is also found to a limited extent on the surface of B lymphocytes at different stages of differentiation. Moreover, T lymphocytes do not express significant amounts of this proteoglycan and, for that matter, typically do not express heparan sulfate appreciably on their surfaces. This pattern of expression is completely consistent with the binding data presented including the restricted expression in tissue sections and suggests that syndecan may also carry the CD48-interacting heparan sulfate chains.

Heparin and heparan sulfate are the most structurally diverse of the GAGs being extensively modified by O- and N-sulfation and selective epimerization of glucuronic acid residues to iduronic acid. The differences between heparin and heparan sulfate are subtle and revolve primarily around the extent to which sulfation and epimerization occur throughout the chain. Within a single heparin or heparan sulfate chain, there can be significant compositional heterogeneity, referred to as microheterogeneity, whereby one stretch of disaccharides may be extensively modified by both N- and O-sulfation and epimerization, whereas a contiguous stretch of the same GAG chain may not be modified at all and simply remain N-acetylated (24, 29). Exactly how this clustering of sulfation and epimerization occurs has not been resolved, but it is clearly capable of generating a great variety of epitope structures as reflected in the tissue specific heparan sulfate structure of the syndecans (30, 31). This no doubt explains why the blocking antibody 615 stains restricted regions in tissue sections, even though heparan sulfate is widely expressed.

Although heparin was consistently more effective at blocking the binding of both sCD48 and mAb615 to epithelial cells, we believe that heparan sulfate, as opposed to heparin, is mediating CD48 interactions with epithelial cells. This is because none of the cells which bind in the adhesion assays produces heparin and, furthermore, heparin is not usually found attached to the cell surface, even in cells that do produce it. This does not rule out the possibility that under certain circumstances heparin could serve as a ligand for CD48. Many proteins that interact with heparan sulfate also interact with heparin. The fact that heparin competitively blocks the binding is probably due to the presence of disaccharide modifications common to both GAGs as similar motifs of sulfation and epimerization can be found between the two GAGs (32). Examination of the primary sequence of CD48 reveals the presence of clusters of positively charged groups that could account for the interaction with heparan sulfate. Specifically, the distribution of basic amino acids within these sequences bears a striking similarity to consensus sequences previously reported for both heparin and hyaluronic acid binding (33, 34).

Epitope mapping indicated that all three blocking antibodies recognize overlapping epitopes (data not shown), but we only have definitive information on 615 as it was the only one of the three that could be shown to bind GAGs directly. Most interesting was that the three GAGs that bind 615 are the same ones that block binding of epithelial cells to sCD48 and with the same hierarchy heparin > dextran sulfate > heparan sulfate. These considerations suggest that CJI 250 and 451 recognize a different structure from 615. The explanation we favor is that CJI 250 and 451 recognize a more specific structure on heparan sulfate, which in turn is part of a more generic structure recognized by 615. The epitopes recognized by 250 and 451 would not then be frequently expressed in the commercial, non-human preparations of heparin and heparan sulfate that we have tested, and therefore these preparations would not
bind to or block the antibodies

In conclusion, we have demonstrated that heparan sulfate binds CD48 and produced highly specific antibodies against the carbohydrate structure. The specific structure is restricted in its expression in vivo to the basolateral surfaces of mucosal epithelium and lamina propria leading to the speculation that EBV up-regulates CD48 expression to allow preferential retention of EBV-infected B lymphoblasts in the mucosal epithelium. This also raises the possibility that CD48/heparan sulfate interactions could be important in interactions between lymphocytes and specific tissues. It should now be extremely informative to perform an extensive immunohistological study to identify sites that express the ligand and see if the expression correlates with any of the known patterns of behavior and migration of hematopoietic cells.

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