We have previously reported that α4β1 (but not α2β1) integrin-mediated melanoma cell adhesion is inhibited by removal of cell surface chondroitin sulfate glycosaminoglycan (CSGAG), suggesting that melanoma chondroitin sulfate proteoglycan plays a role in modulating the adhesive function of α2β1 integrin. In the current study, we demonstrated that α4β1 integrin binds to CSGAG. We have identified a peptide from within α2 integrin termed SG1 (KKEDIKMKKT) that binds to cell surface melanoma chondroitin sulfate proteoglycan, indicating that SG1 represents a CSGAG binding site within the α2 integrin subunit. Soluble SG1 inhibits α2β1 integrin-mediated human melanoma cell adhesion to CS1. Polyclonal antibody generated against the peptide inhibits melanoma cell adhesion to CS1, and the inhibition is reversed by Mn2+ and an activating monoclonal antibody anti-β1 (8A2). Additionally, pretreatment of cells with anti-SG1 IgG inhibits the expression of the monoclonal antibody 15/7 epitope in the presence of soluble CS1 peptide, suggesting that anti-SG1 IgG prevents ligand binding by α2β1 integrin. These results demonstrate that α2β1 integrin interacts directly with CSGAG through SG1 site, and that this site can affect the ligand binding properties of the integrin.

Tumor cells must adhere to a variety of extracellular matrix (ECM) proteins and molecules on other cells as they invade and metastasize. These interactions of tumor cells have a profound effect on their phenotype (1, 2). Integrins are a family of receptors that are fundamentally important for mediating cell adhesion to ECM proteins (3). α2β1 integrin is important in tumor cell invasion and metastasis, although its exact role is complex and not completely understood. This integrin is expressed on many hematopoietic malignancies and also on non-hematopoietic tumors, such as melanomas (4, 5). It is unusual among integrins in that it binds to both ECM components (e.g. fibronectin (FN)) and the Ig superfamily adhesion receptor VCAM-1, which is expressed on activated endothelial cells and other cell types (4). α2β1 integrin has been implicated in tumor cell arrest and/or extravasation, because agents that interfere with α2β1 integrin can inhibit tumor cell/endothelial cell interactions in vitro or experimental metastasis and pulmonary retention of injected tumor cells in mice (6, 7). α2β1 integrin also binds to itself and promotes homotypic cell adhesion (8, 9).

Various monoclonal antibodies (mAbs), divalent cations, or phorbol 12-myristate 13-acetate can regulate α2β1 integrin-mediated cell adhesion in lymphoid cell systems (10–13). This regulation of integrin function results from conformational alterations induced by the direct binding of these reagents to integrin subunits or by indirect (so called inside-out) effects of signaling pathways on integrin conformation. Distinct conformational changes associated with α2β1 integrin activation or ligand binding have been identified by using specific mAbs. One mAb, 15/7 (anti-β1 integrin), detects an epitope that is induced by ligand binding or is exposed on activated integrins in lymphoid cells (14). Although a role for α2β1 integrin has clearly been established in influencing various aspects of tumor cell biology, the mechanisms by which tumor cells control the functional activity of this integrin merits further consideration.

Cell surface proteoglycans (PGs) are important in modulating cell adhesion and motility (15, 16). Many cell adhesion-promoting components in ECM or on cell surfaces (e.g. CD31 or PECAM) can bind PGs and support adhesion as a result of this interaction (17). PGs act as co-receptors for integrins and play a role in transducing signals. Cell surface proteoglycans interact with ECM proteins and often colocalize with integrins and/or may stimulate integrin-mediated signaling through outside-in/inside-out mechanisms. Syndecan-4 localizes in focal contacts (18–21), whereas syndecan-1 associates with stress fibers (22). Recently, it has also been reported that Raji cells transfected with syndecan-1 spread on substrata coated with fibronectin, whereas mock transfectants failed to spread (23). Furthermore, the transfectedants adhered and spread on substrata coated with an anti-syndecan-1 core protein monoclonal antibody (23). These results suggest that the core protein of certain proteoglycans can modulate integrin-mediated cell spreading as a result of intracellular signaling events that are stimulated by clustering of the core proteins.

Previous studies have demonstrated that melanoma chondroitin sulfate proteoglycan (MCSP) might play a role in melanoma cell adhesion and invasion. For example, certain mAbs against MCSP will partially inhibit melanoma cell adhesion to...
cultured endothelial cells (24). Harper et al. (25, 26) have reported that mAb 9.2.27 inhibits human melanoma cell adhesion and cytoplasmic spreading on ECM proteins, such as collagen, or on collagen-fibronectin complexes and that this mAb can also inhibit anchorage-independent cell growth. Additionally, at least one anti-NG2 mAb (NG2, the rat homologue of human MCSP (27)) has been demonstrated to inhibit melanoma cell invasion through reconstituted basement membranes in vitro (28). Recently, human MCSP was cloned by Pluschke et al. (29). We have also demonstrated that clustering of MCSP stimulates α5β1 integrin-mediated cell spreading and focal contact formation in melanoma cells (30), implying a role for the MCSP core protein in modulating integrin function by an inside-out signaling mechanism. Furthermore, we have previously shown that removal of cell surface chondroitin sulfate glycosaminoglycan (CSEG) by chondroitinase ABC or inhibition of synthesis of chondroitin sulfate proteoglycan (CSPG) by p-nitrophenyl β-d-xyloside inhibits α5β1 but not αβ5 integrin-mediated cell adhesion, suggesting an important role for cell surface CSEG in enhancing α5β1 integrin function (31).

In the current study, we demonstrated that α5β1 integrin can bind to CSEG. Furthermore, we used a synthetic peptide approach for identifying potential CSEG binding site(s) within the α5β1 subunit. One site, defined by synthetic peptide SG1 (KEKKDIMKKTI), directly binds to MCSP proteoglycan, and when used as a soluble antagonist, it inhibits melanoma cell adhesion to CS1. Polyclonal antibody generated against this peptide (anti-SG1 IgG) also inhibits α5β1 integrin-mediated melanoma cell adhesion, and the inhibition can be reversed by Mn2+ and mAb 8A2. Furthermore, anti-SG1 IgG prevents the CS1-induced exposure of the mAb 15/7 epitope. These results demonstrate that α5β1 integrin interacts directly with CSEG through the SG1 site and that this interaction can regulate the ligand binding properties of the integrin.

MATERIALS AND METHODS

Cell Culture—Highly metastatic human melanoma cells (A375SM), which were selected by in vivo experimental metastasis assays of parental A375 cells in nude mice, were kindly given by Dr. I. J. Fidler (M. D. Anderson Cancer Center, University of Texas, Houston, TX) (32). SKMEL-2 was purchased from the American Type Culture Collection (Rockville, MD). The cells were maintained in Eagle’s minimum essential medium supplemented with 10% fetal calf serum, 50 μg/ml gentamicin, vitamin solution, and 1 mM sodium pyruvate. Ramos cells were kindly given by Dr. Tucker Lebien (Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. These cells were routinely used after fewer than 15 passages from frozen stocks.

mAbs—P4C2 (anti-α4), P1D6 (anti-α5), and P5D2 (anti-β1) were provided by Dr. Elizabeth A. Wayner (University of Washington, Seattle, WA). The specificity of these mAbs was demonstrated elsewhere (33–36). mAbs 15/7 (anti-β1) and 8A2 (anti-β1) were also used (14, 37). Anti-MCSP core protein antibody (9.2.27) was kindly given by Dr. Ralph Reisfeld (The Scripps Research Institute, San Diego, CA) (38). Anti-CD44 mAb (clone P1G12) was purchased from Chemicon International (Temecula, CA).

Protein Isolation—Human plasma FN was purified as a by-product of factor VIII production by sequential ion-exchange and gelatin affinity chromatography as described previously (6). The tryptic/catheptic 33- kDa heparin binding fragment of FN A-chains was purified according to methods previously reported (6). Purity of FN or a tryptic, RGD-containing 75-kDa fragment was verified by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

Peptide Synthesis and Characterization—All of the peptides used in this study were synthesized at the Microchemical Facility of the University of Minnesota (Minneapolis, MN) using a Beckman System 990 peptide synthesizer. The procedures used were based on the Merrifield solid phase synthesis as described previously (30). Lyophilized crude peptides were purified by preparative reverse-phase high performance liquid chromatography on a C-18 column using an elution gradient of 0–60% acetonitrile with 0.1% trifluoroacetic acid in water. The purity and composition of the peptides were verified by high performance liquid chromatography analysis of hydrolysates prepared by treating the peptides under nitrogen in 6 N HCl overnight at 110 °C (30).

Generation and Purification of Polyclonal IgG against Peptides—Synthetic peptides were keyhole limpet hemocyanin (Sigma) using 9-mer α-(3-aminopropyl) carbodiimide hydrochloride (EDC) (Sigma) as a coupling reagent. The conjugate was mixed with Freund’s adjuvant and then used to immunize New Zealand White rabbits. IgG was purified from pooled immune sera by precipitation with ammonium sulfate followed by DEAE anion exchange chromatography as described previously (39). Purity of the IgG was determined by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining of the gels. Specificity of the purified IgG was then determined by enzyme-linked immunosorbent assay as described previously (31). Fab fragments of the anti-SG1 IgG or normal rabbit IgG were obtained by incubating the purified IgG with immobilized papain (Pierce) for 5 h at 37 °C following the manufacturer’s instructions. The digestion products were examined by SDS-polyacrylamide gel electrophoresis and were found to be free of intact IgG heavy chain.

Conjugation of the Peptide to Ovalbumin (OVA)—Peptide was chemically conjugated to OVA using EDC because previous studies have shown that the coupling of synthetic peptides to larger carrier proteins resulted in enhanced cell adhesion activities (30, 31). Briefly, equal amounts (by weight) of peptide CS1 and OVA were solubilized in water and mixed with a 10-fold (by weight) excess of EDC in water. The sample was then mixed overnight at 4 °C on a circular rotator. The coupled peptide was then dialyzed extensively against PBS to remove the excess EDC and uncoupled peptide (Spectrapore, 10-kDa exclusion, Spectrum Medical Industries, Los Angeles, CA). The conjugates were stored at 20 °C until use. The specificity of cell adhesion to CS1/OVA was verified by using mAbs against α5 and β1, as well as soluble peptide CS1 as described under “Results.”

CSEG-affinity Chromatography—Aggrecan was prepared as described previously (40). The purified aggrecan (dry weight, 50 mg) was digested with both 1 mg of trypsin (Sigma) and 1 mg of protease K (QIagen) in 50 mM Tris-HCl containing 100 mM sodium acetate (pH 7.5) overnight at 37 °C and then mixed with an equal volume of 50 mM Tris-HCl containing 8 mM guanidine. The sample was added to the solution at a ratio of 6:10 (w/w) to give a density of 1.56 and then centrifuged at 40,000 rpm for 48 h. The bottom of the solution, in which CSEG was enriched, was collected and extensively dialyzed against 0.25 M sodium acetate following by PBS. The purified CSEG was then coupled to CNBr-activated sephorese (Sigma) as described in manufacturer’s protocol. Under these experimental conditions, 1 mg (as uronic acid) of CSEG was immobilized on the matrix. Ramos cells were biotinylated and lysed in 50 mM Tris-HCl (pH 7.3) containing 50 mM β-mercaptoethanol, 15 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM Mn2+, 1 mM phenylmethylsulfonyl fluoride, and 1 mM N-ethylmaleimide (NEM) (lysis buffer) and then centrifuged at 36,500 rpm for 1 h at 4 °C. The lysates were precleared on a mock column and then applied to the CSEG column. The column was extensively washed with the lysis buffer, and then the bound CSEG was eluted by 1 M NaCl in the above buffer containing 0.4 M NaCl (41). The proteins were separated by SDS-polyacrylamide gel consisting of 7.5% running with 5% stacking gels and then transferred onto an Immobilon-P membrane. Nonspecific binding sites on the membranes were blocked with PBS containing 3% bovine serum albumin (BSA) overnight at 4 °C. The membranes were then incubated with convidex-coupled streptavidin for 1 h at room temperature. Immunoreactivity was visualized by using an enhanced chemiluminescence systems (Boeringer Mannheim). Elutions from the affinity column were immunoprecipitated with mAb anti-α4 (P4C2) or isotype-matched control antibody (FLOPC21). The proteins were separated, transferred onto Immobilon-P membrane, and detected as described above.

Cell Adhesion Assays—Cell adhesion assays were performed as described, with minor modifications (30). Briefly, ligands were diluted in PBS, and 50-μl aliquots were dispersed in triplicate into Immulon-1 polystyrene microtiter wells. The wells were incubated at 37 °C overnight, and nonspecific binding sites on plastic were then blocked by treating the wells with 200 μl of PBS containing 2% BSA. Subconfluent A375SM human melanoma cells that had been radiolabeled overnight with [3H]thymidine (specific activity, 6.7 Ci/mmol; NEN Life Science Products) were harvested by rinsing with 1 ml EDTA, washed two times with Eagle’s minimum essential medium/BSA (Eagle’s minimum essential medium containing 2 mg/ml BSA and 0.15 mM Hepes, pH 7.2) and adjusted to a concentration of 105 cells/ml in the same medium. Aliquots of 100 μl of the cell suspension were dispensed into the wells, and the cells were incubated at 37 °C for 30 min. The assays were...
terminated by aspirating loosely bound and unbound cells from the wells, washing the wells three times, and solubilizing the bound cells in 0.5 N NaOH containing 1% SDS. Bound radioactivity, determined in a Beckman model 3801 liquid scintillation counter, was used to calculate the percentage of cells that remained adherent to each substratum. Unless otherwise indicated, the data represent the means of triplicate determinations.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—A375SM and SKMEL-2 cells were lysed in 80 mM Tris-HCl (pH 6.8) containing 3% SDS, 15% glycerol, and 0.01% bromphenol blue. The same amounts of proteins were separated by 7.5% running with 5% stacking gels and then transferred onto an Immobilon-P membrane. Nonspecific binding sites on the membranes were blocked with PBS containing 3% BSA overnight at 4°C. The membranes were then incubated with primary IgGs (1 μg/ml) for 4 h at room temperature followed by an incubation with peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. Immunoreactivity was visualized by using an enhanced chemiluminescence system (Boeringer Mannheim).

Affinity Chromatography—Peptide-affinity chromatography was performed according to the methods described previously (31). Peptide SG1 was immobilized on activated CH-Sepharose 4B (Pharmacia Biotech Inc.) and blocked according to the methods described in manufacturer's instructions. Human melanoma cells were surface-radioiodabeled with 125I using lactoperoxidase and extracted as described previously (30) containing 50 mM β-octylglucoside, 15 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM MnCl2, 1 mM PMSF, and 1 mM NEM (extraction buffer) and then centrifuged at 36,500 rpm for 1 h at 4°C. The cell lysates were first precleared by incubating with a mock column, on which no peptide was immobilized, and then applied to an SG1 column. The column was extensively washed with the extraction buffer, and then bound proteins were eluted by a linear gradient of sodium chloride (15 mM to 1 M). Radioactivity in each fraction was quantified using a γ counter, and positive fractions were pooled and immunoprecipitated as described below.

The samples were first precleared with normal mouse IgG/rabbit anti-mouse IgG/protein A-agarose for 2 h at 4°C. A 1 μg of 9.2.27 or anti-CD44 mAb was immobilized on activated mouse IgG/protein A-agarose for 4 h at 4°C. Equal amounts (based on cm) of the (breached) cell lysates were incubated with the 9.2.27 rabbit anti-mouse IgG/protein A-agarose or the CD44/rabbit anti-mouse IgG/protein A-agarose for 4–6 h at 4°C. The immune complexes were extensively washed with 50 mM Tris-HCl (pH 7.3) containing 50 mM β-octylglucoside, 15 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM MnCl2, 1 mM PMSF, and 1 mM NEM. For enzyme treatment, the beads were washed two times with the digestion buffer (0.1 M Tris-HCl, pH 7.2, containing 5 mM sodium acetate, 0.1 M EDTA, 10 mM NEM, 5 mM PMSF, and 0.36 mM pepstatin) and then incubated with 0.01 unit/ml of protease-free chondroitinase ABC (Seikagaku America, Inc., Rockville, MD) in the digestion buffer for 4 h at 37°C. For heparitinase treatment, digestion buffer (0.1 M Tris-HCl, pH 7.2, containing 5 mM sodium acetate, 0.01 mM EDTA, 0.01 mM NEM, 1 mM PMSF, 0.36 mM pepstatin) was used with 10 milligrams of heparitinase (Seikagaku America). The immune complexes were released from the beads with boiling in SDS-sample buffer and then analyzed on SDS-polyacrylamide gel electrophoresis. The gels were dried and exposed on x-ray films at –80°C.

RESULTS

αβ1 Integrin Interacts with CSGAGs—To evaluate potential interactions between αβ1 integrin with CSGAGs, we first prepared a CSGAG-affinity column as described under “Materials and Methods.” Aggrecan was used as a source of CSGAG chain because the proteoglycan contains CSGAG as the major GAG. Biotinylated cell lysates were applied to the CSGAG column and washed, and the bound proteins were eluted with 0.4 M NaCl as described by Diamond et al. (41). The proteins were separated on SDS-polyacrylamide gel electrophoresis and transferred onto an Immobilon-P membrane. Although several proteins were eluted from the CSGAG column under these conditions, two prominent proteins migrated at 140 and 120 kDa (Fig. 1, CSGAG). These proteins were not eluted from mock column (Fig. 1, Mock), indicating that they specifically interact with the GAG chain. Anti-α4 mAb (P4C2) specifically immunoprecipitated αβ1 integrin from the CSGAG column eluants, whereas no detectable immunoprecipitated proteins were detected from the mock column eluant, indicating that αβ1 specifically bound the CSGAG column.

Peptide SG1 Derived from α4 Integrin Inhibited Melanoma Cell Adhesion to CS1—In previous reports, we demonstrated that removal of cell surface CSGAGs inhibits αβ1 integrin-mediated but not αβ2 integrin-mediated cell adhesion (31). These results suggest that cell surface CSGAGs can enhance the adhesive function of αβ1 integrin, possibly as a result of directly interacting with the α4 integrin subunit. To further explore this possibility, we selected several positively charged, relatively hydrophilic synthetic peptides from the α4 integrin subunit sequence (Fig. 2A). One of these peptides, termed SG1, is uniquely contained within the α4 integrin subunit as described by Takada et al. (42), when comparing integrin sequences that are aligned for maximum homology using CLUSTAL W algorithm (43) (Fig. 2B).

In the first set of experiments, each of the peptides was tested for the ability to inhibit cell adhesion to substrata coated with CS1. Cells were preincubated with each peptide for 15 min at 37°C and then incubated on substrate coated with CS1 for 30 min. Cell adhesion to CS1 was inhibited by soluble CS1, as expected (Fig. 3A). Although the synthetic peptides were similarly positively charged and possess similar hydrophathy indices, only peptide SG1 inhibited cell adhesion in a concentration-dependent manner (Fig. 3A). The inhibitory effects of CS1 and SG1 are not due to their toxic activity on the cells, as assessed by trypan blue exclusion (not shown). The inhibitory effect of SG1 was also not due to the blocking of CS1 binding sites by the soluble SG1 because preincubation of CS1-coated plates with soluble SG1 prior to cell adhesion assays had no effect on cell adhesion (not shown). An all-D form of peptide SG1 (d-SG1) was also tested for the ability to inhibit cell adhesion to evaluate the specificity of inhibitory effect of soluble SG1. This all-D-SG1 has a sequence and charge distribution exactly the same as that of an all-L-SG1, except that the conformations of the residues within each pepptide are mirror images of each other. Although both CS1 and all-L-SG1 significantly inhibited cell adhesion to CS1, all-D-SG1 did not affect cell adhesion, indicating that the inhibitory effect of SG1 is dependent upon the chirality of the peptide and is not due to a simple charge effect of the peptide (Fig. 3B).
against this and the other synthetic peptides and tested the ability of these antibodies to inhibit α4β1 integrin-mediated melanoma cell adhesion. Cells were preincubated with 100 μg/ml of anti-SG1 IgG, anti-A4–107 IgG, or anti-A4–110 IgG for 15 min at room temperature prior to cell adhesion to substrata coated with 5 μg/ml CS1. Anti-SG1 IgG completely inhibited cell adhesion to CS1, however, neither anti-A4–107 nor anti-A4–110 polyclonal antibodies had any effect on cell adhesion to the same substrata (Fig. 4), demonstrating that the SG1 site plays a specific role in α4 integrin-mediated cell adhesion to CS1. As controls, mAbs anti-α5 integrin (but not anti-α2 integrin) inhibited cell adhesion to CS1 (Fig. 4). Monovalent Fab fragments of anti-SG1 IgG were also generated and tested for the ability to inhibit cell adhesion to CS1. Fab fragments of anti-SG1 IgG inhibited cell adhesion to CS1 in a concentration-dependent manner (Fig. 5), suggesting that the inhibitory effect of anti-SG1 IgG does not occur because of antibody-induced cross-linking of αβ2 integrin. Furthermore, anti-SG1 IgG did not cross-react with peptide CS1 as determined by enzyme-linked immunosorbent assay (not shown), indicating that anti-SG1 IgG did not inhibit cell adhesion by binding to peptide-coated surfaces.

To further demonstrate the specificity of anti-SG1 IgG, we tested its ability to inhibit cell adhesion to FN-C/H-III and 75 kDa fragments. As reported previously (31), cell adhesion to FN-C/H-III is mediated by CSPG, whereas adhesion to the 75-kDa fragments utilizes αβ1 integrin. Cell adhesion to CS1 was totally sensitive to anti-SG1 IgG; however, cell adhesion to FN-C/H-III was completely resistant (Table I). Furthermore, anti-SG1 IgG did not affect cell adhesion to the 75-kDa fragment (Table I), demonstrating that anti-SG1 IgG specifically inhibits αβ1 integrin function.

To demonstrate that anti-SG1 IgG recognized α4 integrin, two melanoma cell lines (A375SM, α4 integrin-positive, and SKMEL2, α4 integrin-negative) cells were lysed and then separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto Immobilon-P membrane and blotted with anti-SG1 IgG following by peroxidase conjugated-goat anti-rabbit IgG. As shown in Fig. 6, the antibody specifically recognized α4 integrin migrating at 140 kDa, indicating that the anti-SG1 IgG recognizes the α4 integrin subunit.

**Anti-SG1 IgG Inhibits αβ1 Integrin Interactions with CS1**—To further evaluate the mechanism of anti-SG1 IgG-mediated inhibition of αβ1 integrin function, we first tested the ability of agents that activate integrin-mediated cell adhesion to counteract the inhibitory effects of anti-SG1 IgG (Fig. 7). When anti-SG1 IgG was used in the presence of Mn2+, it was ineffective in inhibiting melanoma cell adhesion to CS1, suggesting that anti-SG1 IgG may affect ligand binding by the integrin. Furthermore, the addition of mAb 8A2, which activates β1 integrin-mediated cell adhesion by stimulating high affinity ligand binding (37), also reverses the inhibitory effects of anti-SG1 IgG on melanoma cell adhesion.

**Table I.** Localization of synthetic peptides derived from the α4 integrin subunit used in this study (A) and alignment of the SG1 site with other integrin α subunits (B). A, column a, synthetic peptides used in this study using the single letter amino acid code. Column b, residue numbers were shown according to the previous report by Takada et al. (42). Column c, the sum of all charged residues, where K and R residues are positively charged (+1) and E and D are negatively charged (−1) at neutral pH. H is assumed to be uncharged at neutral pH. Column d, hydropathy indexes were calculated by the methods of Kyte and Doolittle (56). Using this method, hydrophilic regions of a protein have a negative hydropathy index. B, the alignment was analyzed by using the CLUSTALW program with default settings. The SG1 sequence is underlined. * indicates identical amino acid residues.

| Primary sequence (a) | Residue(b) | Net charge(c) | Net hydropathy(d) |
|----------------------|------------|---------------|-------------------|
| LHMKOKSGLS           | 261-271    | +2            | −8.8              |
| NWQAEFSFRFY          | 491-501    | +2            | −14.2             |
| SSREANCORTXQAM       | 518-537    | +2            | −20.7             |
| KKKQDMMKKT          | 570-583    | +3            | −14.4             |
| SKDTKRLLLY           | 848-856    | +2            | −6.0              |

**Fig. 2.** Localization of synthetic peptides derived from the α4 integrin subunit used in this study (A) and alignment of the SG1 site with other integrin α subunits (B). A, column a, synthetic peptides used in this study using the single letter amino acid code. Column b, residue numbers were shown according to the previous report by Takada et al. (42). Column c, the sum of all charged residues, where K and R residues are positively charged (+1) and E and D are negatively charged (−1) at neutral pH. H is assumed to be uncharged at neutral pH. Column d, hydropathy indexes were calculated by the methods of Kyte and Doolittle (56). Using this method, hydrophilic regions of a protein have a negative hydropathy index. B, the alignment was analyzed by using the CLUSTALW program with default settings. The SG1 sequence is underlined. * indicates identical amino acid residues.

**Fig. 3.** SG1 specifically inhibits A375SM melanoma cell adhesion to CS1/OVA. A, microtiter wells were coated with 5 μg/ml of CS1/OVA and blocked as described under “Materials and Methods.” A375SM cells were preincubated with the indicated concentrations of each of the peptides for 15 min and then cultured on the substrata coated with CS1/OVA for 30 min. B, plates were coated as described above. Cells were preincubated with 1 mg/ml of the peptides for 15 min and then cultured on the substrata coated with CS1/OVA for 30 min. *, p < 0.001 (by Student's two-tailed t test).
We next studied the effect of anti-SG1 IgG on the expression of the mAb 15/7 ligand-induced epitope in the presence of soluble peptide CS1. This mAb detects an epitope on b1 integrin that correlates with the high affinity activation state of b1 integrins (14). It also detects ligand bound a4b1 integrin (14).

In contrast to hematopoietic cells, melanoma cells express detectable levels of the 15/7 epitope in the absence of soluble ligand. Preincubation with anti-SG1 IgG had little effect on basal staining intensity of 15/7 (Fig. 8). We also evaluated CS1-induced expression of the 15/7 epitope in the presence and absence of anti-SG1 IgG because this antibody can be used as an indicator of ligand binding by integrin (14). Cells were first preincubated with anti-SG1 IgG (or normal rabbit IgG as a negative control) in the presence or absence of 1 mM peptide CS1 and then incubated with mAb 15/7 (Fig. 8). Anti-SG1 IgG, but not normal rabbit IgG, prevented the CS1-induced expres-
sion of the 15/7 epitope. These results suggest that anti-SG1 IgG inhibits integrin function by preventing ligand binding, despite the fact that the SG1 site is located outside the region of the molecule that has been implicated in ligand binding (Fig. 2B).

MCSP Proteoglycan Interacts Directly with Peptide SG1—To evaluate possible interactions between peptide SG1 and melanoma CSPG, we next performed affinity chromatography using immobilized SG1. Iodinated total cell extracts were applied to an SG1 affinity column, and bound proteins were eluted by a linear gradient of NaCl (Fig. 9A). Almost all radioactivity (over 97%) was eluted from the SG1 peptide affinity column at 0.13–0.15 M NaCl. These fractions were pooled and immunoprecipitated using specific anti-MCSP or anti-CD44 mAbs because these cells express both core proteins as cell surface chondroitin sulfate proteoglycans. Furthermore, these cells express large amount of unmodified MCSP and CD44 core proteins (26, 30, 44). Chondroitinase ABC treatment of immunoprecipitates demonstrated that chondroitin sulfate-modified MCSP was the main core protein eluted from the SG1 column eluates (Fig. 9B). In contrast, CD44 chondroitin sulfate proteoglycan was not detected in the pooled fractions (Fig. 9B). To further characterize the nature of interaction between MCSP and the SG1 peptide, 35SO4-labeled MCSP/CSPG was purified according to the methods described previously (31) and applied to the SG1 affinity column. Purified MCSP was eluted at 0.13–0.15 M NaCl, indicating that MCSP and peptide SG1 interact directly (Fig. 9C). When purified MCSP/CSPG was treated with chondroitinase ABC, the core protein did not bind the SG1 peptide column (not shown), demonstrating that the interaction is mediated through CSGAG.

DISCUSSION

Integrin-mediated adhesion to ECM components and to other cells is tightly controlled by regulating functional properties of specific integrin heterodimers. We have previously shown that MCSP cell surface chondroitin sulfate proteoglycan can enhance the activity of α4β1 integrin on melanoma cells. The effect of MCSP on integrin function is complex, involving contributions from the core protein, as well as the glycosaminoglycans (45). The current study focuses on understanding the role of CSGAG in the functional activity of α4β1 integrin. The results show that α4β1 integrin binds directly to CSGAG and that the α4 integrin subunit contains at least one CSGAG binding site, defined by synthetic peptide SG1. Furthermore, this interaction directly affects α4β1 integrin adhesive function. These results are consistent with a model in which cell surface MCSP interacts directly with α4β1 integrin and enhances α4β1 integrin-mediated adhesion and signaling.

Among several synthetic peptides tested from α4 integrin, only peptide SG1 or antibodies against SG1 significantly inhibit α4β1 integrin-mediated (but not α5β1 integrin-mediated) cell adhesion. This inhibition could be reversed by the addition of Mn2+ or mAb 8A2, indicating that anti-SG1 IgG inhibition is reversible and is related to the ability of the antibody to inhibit ligand binding by α4β1 integrin. These results suggest that anti-SG1 IgG may act by changing integrin conformation, leading to a direct inhibition of ligand binding, although it is also possible that anti-SG1 IgG may act by mechanisms related to integrin clustering. It is of interest that the SG1 site is not within the ligand binding site of α4 integrin. Furthermore, SG1 is a unique site among other α chains when sequences of α integrin subunits are aligned and compared (Fig. 2B). Thus, SG1 may represent a specific GAG binding site that promotes the interaction between cell surface proteoglycans and α4β1 integrin.

Although this study demonstrates that MCSP but not CD44/CSPG binds SG1, these results should be interpreted with caution. A375SM melanoma cells express almost 50% of their MCSP as CSPG, whereas levels of CD44 expressed as a CSPG are much lower (only approximately 5% of total CD44 is expressed as CSPG). In light of this discrepancy, it is possible that the apparent specificity of MCSP binding to SG1 may reflect the large excess of MCSP in the samples. Previous studies in hematopoietic progenitor cells have indicated that cell surface CD44, expressed as a CSPG, may influence the adhesive function of α4β1 integrin (46), suggesting that α4β1 integrin interactions may involve more than one type of cell surface proteoglycan. Further work is necessary using MCSP deficient cell lines to determine whether CD44/CSPG on melanoma cells can similarly effect α4β1 integrin-mediated adhesion.

GAGs have been implicated in modulation of integrin function, although the exact mechanism by which they do this is not known. Earlier work has demonstrated that heparan sulfate plays a role in formation of focal contacts in fibroblasts (20, 21,

2 J. Iida, unpublished data.

3 J. Iida, unpublished observations.
Circles represent radioactivity in each fraction, and the bar represents concentrations of NaCl in each fraction.

MCSP was eluted by a linear gradient of NaCl. In this study, we demonstrated that α4β1 integrin interacts with CSGAGs by affinity chromatography and that removal of CS reduces α4β1 integrin-mediated melanoma cell adhesion and ligand binding. Furthermore, we have observed that clustering of α4β1 integrin by CS1-coated beads causes accumulation of MCSP in close proximity with α4β1 integrin on human melanoma cells. Importantly, this accumulation is prevented by pretreating cells with chondroitinase ABC, indicating that CSGAG chains may promote the colocalization of MCSP with α4β1 integrin that has been clustered with substratum-bound ligand. These results indicate that CSGAGs may be important for bringing cell surface CSPG and α4β1 integrin into close proximity on the cell surface and that binding of ligand by integrin may help to facilitate codistribution of the two receptors.

It remains to be determined whether α4β1 integrin/GAG interactions are specific for chondroitin sulfate. Although CSGAGs are the primary GAG expressed by these melanoma cells, previous work has shown that other melanoma cells can also express heparan sulfate (51, 52). Although there are many examples of protein binding GAGs with a high degree of selectivity for a specific GAG or even a sequence within a GAG, the same is not true for several cell surface proteins. Many of the receptors on the cell surface that bind polyanionic molecules recognize, although with different affinity, members of several different GAG families. For example, CD44 binds both chondroitin sulfate and hyaluronic acid (53). L-Selectin, which binds several variants of sialyl Lewis antigen, including fucosylated or sulfated forms in mucin and glycolipids, will recognize related sulfated lactosaminoglycan structures of keratan sulfate (54) and unrelated structures in heparan sulfate (55). Because there is no clear precedent, this leaves open the discussion of specificity, which will be pursued in studies using quantitative assay systems.

Collectively, regulation of integrin function on cell surfaces has been studied to understand the molecular mechanisms of inflammation and tumor metastasis. In this regard, it is important to realize that cell surface proteoglycans act not only as a cell surface receptor but also as an important modulator of integrins. The fact that PG binding sites are often expressed in close proximity to integrin binding domains within ECM proteins or cell surface adhesion molecules suggests that cellular recognition of the ECM or counter-adhesion receptors on opposing cells might involve the formation of a receptor cluster on the plasma membrane that includes both PGs and integrins (45). Understanding the nature of such interactions may help to explain cell type-specific behavior on ECM proteins that are often observed for integrins. Studying the molecular mechanisms of the cooperation between these two distinct receptors will lead to a better understanding of the complexity of integrin functions in the processes of migration and extravasation, which occur during development and in tumor invasion, angiogenesis, and diseases associated with inflammation.

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FIG. 9. Peptide SG1 interacts with MCSP proteoglycan. A, peptide SG1 was immobilized on activated CH-Sepharose 4B beads as described under “Materials and Methods.” Detergent lysates from 125I-labeled melanoma cells were preclear and then applied to the peptide column. The specifically bound proteins were eluted with a linear gradient of NaCl. B, the peak fractions were collected and immunoprecipitated with mAbs anti-MCSP (lanes 1–3) or anti-CD44 (lanes 4–6). The immunoprecipitated proteoglycans were then treated with chondroitinase ABC (lanes 2 and 5) or heparitinase (lanes 3 and 6). Nontreated glycosaminoglycan-lyase controls were shown in lanes 1 and 4. A molecular mass marker (kDa) is shown on the right. C, MCSP was purified from melanoma cells, preclear, and applied to the peptide SG1 column. MCSP was eluted by a linear gradient of NaCl. In A and C, open circles represent radioactivity in each fraction, and the solid line represents concentrations of NaCl in each fraction.
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