Post-translational Modifications of $\alpha_5\beta_1$ Integrin by Glycosaminoglycan Chains

THE $\alpha_5\beta_1$ INTEGRIN IS A FACULTATIVE PROTEOGLYCAN*

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Cell-fibronectin interactions, mediated through several different receptors, have been implicated in a wide variety of cellular properties. Among the cell surface receptors for fibronectin, integrins are the best characterized, particularly the prototype $\alpha_5\beta_1$ integrin. Using [125I]iodine cell surface labeling or metabolic radiolabeling with sodium $[^35S]$sulfate, we identified $\alpha_5\beta_1$ integrin as the only sulfated integrin among $\beta_1$ integrin heterodimers expressed by the human melanoma cell line Mel-85. This facultative sulfation was confirmed not only by immunoprecipitation reactions using specific monoclonal antibodies but also by fibronectin affinity chromatography, two-dimensional electrophoresis, and chemical reduction. The covalent nature of $\alpha_5\beta_1$ integrin sulfation was evidenced by its resistance to treatments with high ionic, chaotrophic, and denaturing agents such as 4 M NaCl, 4 M MgCl$_2$, 8 M urea, and 6 M guanidine HCl. Based on deglycosylation procedures as chemical $N$- and $O$-glycosylation increase the complexity of these molecules (for review see Refs. 1 and 2).

Proteoglycans are complex molecules formed by a core protein to which one or more glycosaminoglycan (GAG) chains are linked. This basic definition, although true, hides the molecular complexity shown by these molecules. They encompass an exceptionally large range of structures involving different core proteins, different classes of GAGs, and different numbers and lengths of individual GAG chains. Other post-translational modifications such as $N$- and $O$-glycosylation increase the complexity of these molecules (for review see Refs. 1 and 2).

The biological functions of proteoglycans are numerous. They have been involved in several biological effects (1, 3–5), such as extracellular matrix (ECM) assembly (6) and cell surface-ECM receptors for growth factors and hormones (2, 5, 7) or have had a role in biological processes such as cell-cell recognition (8) and control of cell growth (9). The fact that several ECM proteins, such as fibronectin (10), laminin (11), thrombospondin (12), vitronectin (13), type IV collagen (14), and tenascin (15), have GAG binding sites adds credence to the postulated multiple roles of proteoglycans. Supporting the idea of proteoglycans as ECM receptors, syndecan type I binds fibronectin, thrombospondin, collagen (5), and tenascin (16); the heparan sulfate proteoglycan of Schwann cells binds laminin (17); a cell surface chondroitin sulfate proteoglycan is apparently involved in cell adhesion to laminin (18); and a cell surface phosphatidyl inositol-anchored heparan sulfate proteoglycan mediates melanoma cell adhesion to fibronectin (19). Strong corroborations for these proteoglycan-ECM interactions comes from the presence of a heparan sulfate proteoglycan that co-localizes with $\beta_1$ integrins as a widespread component of focal adhesion (20).

Among the several ECM molecules that bind proteoglycans, the role of fibronectin should be emphasized not only because of its GAG binding domains but also because of the adhesive properties conferred to this molecule by these domains together with the RGD cell-binding fragment (21–23). Cells devoid of proteoglycans or bearing proteoglycans with altered GAG chains have a reduced capacity of adhesion to fibronectin and have a defective focal adhesion plaque formation in response to this molecule (24, 25).

The best studied receptors for fibronectin that bear adhesive- and focal adhesion plaque formation are integrins that are $\alpha\beta$ heterodimers widely expressed by almost all animal cells (26, 27). Integrins represent good examples of how post-translational modifications can alter the structure of a molecule, thus modulating its biological activity. Integrin glycosylations represent a kind of regulation by which a wide variety of these receptors have their specificity and affinity modulated in several cell lines (28–30). However, the versatility of cells to modulate the binding properties of integrins is not restricted to glycosylation. Integrin functions can be modulated by acylation of membrane lipid (31), by divalent metal binding (32), and, for the cytoplasmic domain, by tyrosine phosphorylation, which is the best understood example of this type of biological modification, especially in leukocytes and platelets (27, 33).

In the present study, we characterize $\alpha_5\beta_1$ integrin as a part-time proteoglycan containing both heparan and chon-
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EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Human fibronectin was purified from fresh plasma (obtained from Hospital A. C. Camargo, São Paulo, Brazil) by gelatin affinity chromatography as described (34). Monoclonal antibodies that recognize the β1 integrin subunit (35) and B-SG10 that react with the α5 integrin subunit (36) were provided by Dr. Martin E. Hemler (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). Monovalent antibody II-F5, which specifically recognizes the α5 integrin subunit (37), was a gift from Dr. Renata Pasqualini (The Burnham Institute, San Diego, CA). Monoclonal antibodies against αβ1 integrin subunit CLB-thromb/4 and α5 integrin chain SAM-1 were purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). Rabbit polyclonal antibody against the cytoplasmic domain of the α5 integrin subunit (38) was a gift from Dr. Stephen J. Kaufman (Department of Cell and Structural Biology, University of Illinois, Urbana, IL), and rabbit polyclonal antiserum against the αβ1 integrin molecule (RB3847) that in immunoblotting reacts only with the β1 integrin subunit was provided by Dr. Kenneth M. Yamada (National Institute for Dental Research, Bethesda, MD). Monoclonal antibody against chondroitin sulfate chains (CS-56) was purchased from Sigma.

Cell Culture—A human melanoma cell line (Mel-85) was provided by Dr. Stephan Carrel (Ludwig Institute for Cancer Research, Lausanne, Switzerland). A human osteosarcoma cell line (MG-63) was given by Dr. Evandro Caplan (Interdisciplinary Program in Molecular, Cell, and Tumor Biology, San Diego, CA), and a human colon adenocarcinoma cell line (HCT-8) was given by Dr. M. M. Brennai (Department of Oncology, School of Medicine, São Paulo University, Brazil). All cells were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Cultilab, Campinas, Brazil) and gentamicin (50 μg/ml) at 37 °C, 5% CO2 in humidified conditions. Cells were harvested using divalent cation-free phosphate-buffered saline containing 2 mM EDTA. For [35S]sulfate incorporation, cells were labeled in the presence of sodium [35S]sulfate (240 μCi/ml of medium) for 24 h.

Immunoprecipitation Reactions—Cell surface expression of β1 integrin heterodimers in Mel-85 cells was probed through immunoprecipitation reactions of cells that were surface labeled using [35S]iodine by the lactoperoxidase-H2O2 method as described previously (39). After washing, cells were solubilized by lysis buffer (50 mM Tris-HCl, pH 7.3, 1% Triton X-100, 50 mM NaCl, 5 mM CaCl2, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml of aprotinin) for 15 min at 4 °C. The extract was clarified by centrifugation for 10 min at 13,000 × g, and the supernatant was preincubated with either normal mouse or rabbit serum followed by precipitation with protein A-Sepharose (Sigma). Mel-85 extract (at the same mass of protein, 1 mg) was incubated respectively with antibodies against different integrin subunits (as shown above), and for B-SG10 (an IgG molecule), rabbit IgG was preincubated against mouse IgG followed by protein A-Sepharose. Affinity beads were washed with lysis buffer, and the immunoprecipitates were eluted by boiling for 5 min with Laemmli buffer.

[35S]Sulfate-labeled Mel-85 cell extracts were immunoprecipitated using the same mono- or polyclonal antibodies as above. Immunoprecipitates were analyzed by 7.5% SDS-PAGE (40) followed by electrotransfer onto nitrocellulose membranes (41) and exposure to x-ray films (Kodak, Rochester, NY). The same procedure was used to study the specific pattern of glycosylation of α5 and β1 integrin subunits, [35S]sulfate-labeled Mel-85 cell lysate was immunoprecipitated using a monoclonal antibody against the α5 integrin subunit as already described, and the precipitate was submitted to a preparative 7.5% SDS-PAGE under nonreducing conditions using precast prestained β-galactosidase (116 kDa) that comigrates with the β1 integrin subunit as a standard. Autoradiography of separated α5 and β1 subunits was done in identical conditions as above and used as a guide. Gel pieces were then cut off in the positions of separated α5 and β1 subunits, and proteins were excised and eluted from the gel by incubation in 50 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100 overnight at 4 °C. The mixtures were then filtered through 0.45-μm filters (Nalgene, Rochester, NY) to remove polycrylamide, and the solutions containing extracted proteins were dialyzed against water and concentrated 20-fold. Purified α5 and β1 integrin subunits were then submitted to β-elimination reaction to release free GAG chains, which were incubated with chondroitinase ABC, heparinase I and II, or a mixture of these enzymes (see below), and the digest was analyzed by agarose gels.

Immunoblotting reactions using Rb3847 (a rabbit polyclonal antibody that only reacts with the β1 integrin subunit but not with the denatured α5 integrin subunit) and a monoclonal antibody against chondroitin sulfate chains (CS-56) were performed as described previously (30).

Chemical β-Elimination and Enzyme Digestions—The GAG chains from [35S]sulfate-labeled α5β1 integrin, purified by immunoprecipitation using a monoclonal antibody against the α5 integrin subunit, were liberated by digestion of the protein core using excess proteinase-K (50 μg; Sigma) at 58 °C overnight or by β-elimination reaction (treatment overnight at 37 °C with 0.1 M NaOH in the presence of 2 M NaBH4; Sigma). The products obtained were analyzed by agarose gel electrophoresis.
represents the negative controls. The immunoprecipitates were reduced by a [35S]sulfate were immunoprecipitated by monoclonal antibodies against transferred onto nitrocellulose membranes that were exposed to x-ray lanes 6. Immunoprecipitates were separated by 7.5% SDS-PAGE and captoethanol containing buffer, separated by 7.5% SDS-PAGE, and transferred to a nitrocellulose membrane that was exposed to an x-ray film. EDTA-phoresis. β-Eliminated materials were submitted to digestion with chondroitinase ABC from Proteus vulgaris (Seikagaku, Kogyo Co, Tokyo, Japan), heparitinases from Flavobacterium heparinum (45), or all enzymes and analyzed by agarose gel electrophoresis.

RESULTS

Sodium [35S] Sulfate Labeling of αβ, Dimer Integrin—Because integrins are substrates for several different post-translational modifications, we decided to determine whether they could function as substrates for sulfation. We decided to address this question using [35S]sulfate labeling of the cells, immunoprecipitation, and blotting experiments. As shown in Fig. 1, Mel-85 cells in culture efficiently incorporate [35S]sulfate. The cell lysate was submitted to immunoprecipitation with a monoclonal antibody against the β1 integrin subunit, and a [35S]sulfated αβ1 integrin molecule dimer was detected. This suggests that β1 integrin is a substrate for post-translational sulfation.

αβ1 Is the Only Sulfated Integrin in Mel-85 Cells—After the demonstration that αβ1 dimer integrin is a sulfated molecule, our next experimental procedure was to identify the α subunit complementing the β1 subunit in this particular integrin heterodimer. To investigate this, Mel-85 cells were surface radio-labeled with [125I]iodine by the lactoperoxidase method or metabolically labeled with [35S]sulfate. Both cell lysates were immunoprecipitated with antibodies against different integrin subunits. We can see in the [125I]iodine-labeled immunoprecipitates (Fig. 2A) the presence of β1, α2, α3, α4, α5, α7, and probably α1 subunit, a 200-kDa signal (Fig. 2A, lane 1) that could be precipitated with the β1 subunit. Neither cell flow cytometry nor immunoprecipitation showed detectable levels of the α6 integrin subunit in Mel-85 cells (data not shown). Interestingly, Fig. 2B shows that only α5 and the corresponding β1 subunit are sulfated. These findings suggested that αβ1 integrin is a facultative sulfated β1 integrin molecule because none of the other β1 integrin molecules incorporated [35S]sulfate.

It is known that after reduction of the disulfide bonds by β-mercaptoethanol (chemical reduction), the α5 integrin subunit comigrates with the β1 subunit (36). The immunoprecipitates obtained using monoclonal antibodies to β1 and α5 integrin subunits or a polyclonal antibody against the α5β1 integrin dimer from a [35S]sulfate-labeled Mel-85 cell lysate were then subject to chemical reduction. As shown in Fig. 2C, after chemical reduction the immunoprecipitates reveal just one band in the gel, confirming that α5β1 integrin is a sulfated molecule. It is also known that the α5β1 integrin has fibronectin as the only ECM ligand (27, 46). Fig. 2D shows that after elution from a fibropectin affinity chromatography αβ1 integrin is detected as a sulfated molecule.

αβ1 Integrin Is the Only Sulfated Molecule; No Other Sulfated Molecule Co-precipitates with Integrin—Data in the literature describe proteoglycans as αβ1 integrin-associated molecules that complement the requirements involved in cell adhesion to fibropectin. In addition, in melanoma cells a heparan sulfate proteoglycan of 150/175 kDa has been described to bind fibropectin (19, 22, 47, 48). We cannot therefore discard the possibility of a physical association between a third molecule that comigrates with αβ integrin subunits, masking the sulfated signals in the autoradiograms. To rule out this possibility the same [35S]sulfate-labeled Mel-85 cell extract was again immunoprecipitated by specific monoclonal antibodies to α5 and β1 integrin subunits and now submitted to a two-dimensional electrophoresis (Fig. 3, A and B). We can observe that the immunoprecipitation reactions using either anti-β1 antibody or anti-α5 antibody show only a sulfated signal of α5β1 dimer.

To corroborate the findings described above and demonstrate that the sulfated groups in αβ1 integrin are covalently linked and not adsorbed to this molecule or to the beads during immunoprecipitation, this integrin was immunoprecipitated from a [35S]sulfate-labeled Mel-85 cell. After washing with phosphate-buffered saline, the beads were submitted to different conditions of elution such as high ionic strength (4 M NaCl) and chaotropic agents (6 M guanidine HCl, 4 M MgCl2, 8 M urea). After boiling, the precipitates were dialyzed against water, and patient subjected to electrophoresis.
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Fig. 3. No other sulfated molecule co-precipitates with α5β1 integrin. Sulfate groups in α5β1 integrin are covalently linked. Cell extracts from [35S]sulfate-labeled Mel-85 cells were immunoprecipitated with monoclonal antibodies against the β1 integrin subunit (A) or the αs integrin subunit (B); the precipitates were separated by two-dimensional electrophoresis (isoelectric focusing and 7.5% SDS-PAGE) and electrotransferred onto nitrocellulose membranes that were exposed to x-ray film. The arrows represent the β1 integrin subunit, and the arrowheads represents the αs subunit. pH gradient is shown on the top. C, immunoprecipitates obtained using monoclonal antibodies against αs integrin subunit from a Mel-85 cell extract labeled as described above were incubated for 2 h in the presence of 4 M NaCl (lane 1), 4 M MgCl2 (lane 2), 8 M urea (lane 3), and 6 M guanidine HCl (lane 4). After elution and dialysis, precipitates were separated by 7.5% SDS-PAGE and transferred onto nitrocellulose strips that were exposed to x-ray films. Molecular mass standards are on the left.

Fig. 4. The α5β1 integrin is a hybrid proteoglycan. A, lysates from Mel-85 cells labeled with sodium [35S]sulfate were immunoprecipitated with a monoclonal antibody to the αs integrin subunit. The precipitates were submitted to β-elimination reaction (lane 2) or digested with proteinase-K (lane 3), and the obtained materials were submitted to agarose gel electrophoresis in 0.05 M 1.3 diminoanopropanoic buffer, pH 9.0. GAGs were precipitated in the gel with 0.1% cetavlon. After drying and staining, the gel was exposed to x-ray film. Lane 1 represents glycosaminoglycan standards. CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate. B, [35S]-labeled αsβ1 integrin obtained as above was submitted to β-elimination reaction, and the obtained GAG chains were digested with chondroitinase ABC (lane 3), heparitinases I and II (lane 4), or a mixture of the enzymes (lane 5). Lane 2 represents β-eliminated material before enzymic digestion, and lane 1 represents glycosaminoglycan standards as above. The incubation mixtures were analyzed by agarose gel electrophoresis and were dried and exposed to x-ray film.

Submitted to 7.5% SDS-PAGE, and transferred onto nitrocellulose filters, which were then exposed to an x-ray film (Fig. 3C). These results show that the αsβ1 integrin bears covalently linked sulfate groups.

αsβ1 Integrin Is a Hybrid Proteoglycan—Proteoglycans represent the best characterized sulfated molecules containing serine-linked sulfated GAG chains as a result of post-translational modifications of the protein core (2, 7, 10). To determine the site of sulfate substitution in the αsβ1 integrin dimer, [35S]sulfate-labeled Mel-85 cell lysates were immunoprecipitated using antibody against the αs subunit and subjected to β-elimination. This procedure cleaves GAG chains from the protein core. As shown in Fig. 4A (lane 2), αsβ1 integrin after β-elimination showed two sulfated bands that comigrate electrophoretically with chondroitin and heparan sulfate standards. An identical result was obtained when [35S]sulfated αsβ1 integrin was submitted to proteinase-K digestion (a serine protease of broad specificity) (Fig. 4A, lane 3). These experiments suggest that αsβ1 integrin is a hybrid chondroitin/heparan sulfate proteoglycan. This was further investigated by degradation of the β-eliminated material from αsβ1 integrin with specific enzymes that degrade chondroitin sulfate (chondroitinase ABC) and heparan sulfate (heparitinases type I and II). We can see that under these conditions, both sulfated bands were completely degraded by the specific enzymes (Fig. 4B), demonstrating that αsβ1 integrin is in fact a hybrid chondroitin/heparan sulfate proteoglycan.

Both αs and β1 Integrin Subunits Have Chondroitin and Heparan Sulfate Chains—Because in Mel-85 cells αs and β1 subunits of integrin contain sulfate, our next goal was to determine the specific pattern of glycosylation, that is, to which subunit chondroitin and heparan sulfates are linked. Two complementary approaches based on immunologic specificities were used. First, αsβ1 integrin was immunoprecipitated from a [35S]-labeled Mel-85 cell extract as described above. After separation by polyacrylamide gel electrophoresis, the immunoprecipitate was exposed to an x-ray film, blotted, and reacted with a monoclonal antibody specific for chondroitin sulfate chains (Fig. 5A). We can see that both integrin subunits bear chondroitin sulfate chains. Also, Mel-85 cell lysate was immunoprecipitated with an anti-chondroitin sulfate monoclonal antibody and blotted with a polyclonal antiserum against the β1 integrin subunit (Fig. 5B). Interestingly, we can see that only the 116-kDa β1 integrin subunit, which corresponds to the completely glycosylated form, has chondroitin sulfate chains. In contrast, the pre-β1 integrin chain (100 kDa), which corresponds to a high mannose structure, has no chondroitin sulfate chains. Pre-β1 integrin shows the glycosylation profile of a protein that has not crossed the Golgi. Because synthesis of GAG occurs in the Golgi, the absence of chondroitin sulfate in the pre-β1 integrin should be expected (49). This finding was also substantiated by results shown in Fig. 1 in which the pre-β1 integrin subunit, although coprecipitated, is not sulfated. These results demonstrate that the integrin dimer αsβ1 is a proteoglycan and that in Mel-85 cells both integrin subunits have covalently linked chondroitin sulfate chains.

As a second approach we have used successive immunoprecipitation reactions to isolate αsβ1 integrin from a [35S]-labeled
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obtained from this last set of experiments support the concept that α5β1 integrin is a proteoglycan. It shows that both subunits contain chondroitin sulfate and heparan sulfate, thereby demonstrating that α5β1 integrin is a hybrid chondroitin/heparan sulfate proteoglycan.

Sulfate Incorporation in α5 Integrins Is a Conserved Phenomenon—Because all experiments described so far were performed using the human melanoma cell line Mel-85, which has a neuro-ectodermic origin, we decided to investigate whether this α5β1 integrin post-translational modification was also present in cell lines of endodermic (HCT-8 cells) and mesodermic (MG-63 cells) origin. Cells were labeled with [35S]sulfate, and lysates were immunoprecipitated with monoclonal antibodies against the α5 integrin subunit and analyzed by SDS-PAGE followed by electroblotting onto nitrocellulose. A polyclonal antibody against the β1 integrin subunit (Fig. 6A) was used to detect the integrin. We can see that both cells display the [35S]sulfate incorporation into the α5 integrin subunit. The results indicate that GAG substitution of α5β1 integrin has been conserved, suggesting its biological significance. However, in the case of the MG-63 cells, only the α5 subunit was labeled; the β1 subunit found in MG-63 cells did not contain sulfate. To corroborate the results described in Fig. 6A and provide more evidence that the glycosylation of integrin α5β1 integrin as a proteoglycan is maintained in different tissues, we submitted [35S]sulfate-labeled α5β1 integrin obtained from HCT-8 and MG-63 cell extracts to a β-elimination reaction. Products were analyzed by agarose gel electrophoresis. As shown in Fig. 6B, we can see that heparan and chondroitin sulfates are present in α5β1 integrins of endodermic and mesodermic origins, as observed for neuro-ectodermic cells. The results not only confirm the conservative proteoglycan nature of α5β1 integrin from different origins but also indicate a similar glycosylation pattern.

DISCUSSION

Working with the human melanoma cell line Mel-85, we have described α5β1 integrin as a hybrid chondroitin/heparan sulfate proteoglycan. Based on immunoprecipitation reactions from cell lysates that were cell surface labeled with [125I]iodine or metabolically labeled with [35S]sulfate, we were able to detect α5β1 integrin as the only sulfated integrin compared with other α5β1 heterodimers present in Mel-85 cells. Sulfation of α5β1 integrin was confirmed not only by immunological methods but also by fibronectin affinity chromatography, two-dimensional electrophoresis, and reduction of disulfide bonds of the α5β1 heterodimer leading to comigration of both α5 and β1 integrin subunits, characteristic of this integrin as described (36). Based on different procedures such as chemical deglycosylation by β-elimination, proteinase-K digestion, immunological methods, and susceptibility to chondroitinase ABC and heparitinases, we were able to confirm this integrin as a proteoglycan. These results raise the important question of which mechanisms determine α5β1 as the only sulfated integrin. Why are β1 subunits not sulfated in other αβ1 heterodimers? The existence of alternative splicing for the β1 integrin subunit as described (50) (reviewed in Ref. 27) could explain in part such differences. However, because glycosylation of cell surface proteoglycans is restricted to extracellular domains (2) and the β1 integrin subunit has only alternatively spliced cytoplasmic domains (27) this mechanism does not explain our findings. Oligomerization of αβ integrin heterodimers is an event that occurs during transit through the endoplasmic reticulum (28) and precedes glycosaminoglycan biosynthesis, which occurs during transit through the Golgi (2). Perhaps the best explanation for the part-time proteoglycan nature of α5β1 integrin is that the conformation of the heterodimer exposes the serine

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**Fig. 5.** Both α5 and β1 integrin subunits have chondroitin and heparan sulfate. A, α5β1 integrin from [35S]sulfate-labeled Mel-85 cell lysate was immunoprecipitated using a monoclonal antibody to the α5 integrin subunit. The immunoprecipitates were separated by electrophoresis in 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was exposed to x-ray film (lane 1) and reacted with a monoclonal antibody against chondroitin sulfate chains (lane 2) or normal mouse serum as a negative control (lane 3). The arrow shows the position of β1 integrin subunit, and the arrowhead shows the α5 integrin subunit. B, Mel-85 cell extract was submitted to an immunoprecipitation reaction using normal mouse serum as a negative control (lane 2) or monoclonal antibody against chondroitin sulfate chains (lane 3). In lane 1 Mel-85 lysate before immunoprecipitation is shown. Immunoprecipitates were separated by 7.5% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and immunoblotted with a rabbit polyclonal antiserum against the β1 integrin subunit. The open arrow depicts the position of pre-β1 integrin subunit, and the closed arrow depicts complex glycosylated β1 integrin subunits. Molecular mass markers are on the left. C, [35S]sulfate-labeled Mel-85 cell lysate was depleted of α5β1 integrin by successive immunoprecipitation reactions using an anti-α5 integrin subunit monoclonal antibody. The immunoprecipitates were electrophoresed in preparative 7.5% SDS-PAGE under nonreducing conditions using prestained molecular mass standards. Separated α5 and β1 integrin subunits were excised and eluted from polyacrylamide gels and β-eliminated as described under “Experimental Procedures.” β-Eliminated materials from α5 or β1 subunits (lane 2) were treated with chondroitinase ABC (lane 3), heparitinases I and II (lane 4), and a mixture of the enzymes (lane 5) and electrophoresed in an agarose gel as described in the legend to Fig. 4. Lane 1 shows standard glycosaminoglycan. CS, chondroitin sulfate; DS, dermanan sulfate; HS, heparan sulfate.

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Mel-85 cell lysate. The purified α5β1 integrin was submitted to preparative polyacrylamide gel electrophoresis. Using an autoradiogram of the gel and prestained molecular mass standards as guides, separated α5 and β1 subunits were removed from the gel and subjected to β-elimination to obtain GAG free chains. These chains were analyzed by agarose gel electrophoresis before and after treatment with chondroitinase ABC, heparitinases, and a mixture of these enzymes (Fig. 5C). The result
residues that are acceptors for the GAG chains, which does not happen with other $\alpha\beta$ heterodimers. This conformational hypothesis is consistent with the lack of a consensus sequence for proteoglycan biosynthesis initiation (1, 2) and by the experiments performed with decorin, a proteoglycan in which the primary structure of the protein core surrounding the sugar acceptor serine residue can be changed without appreciable modification in the glycosaminoglycan (51). Considering the findings described above, it is possible to assume that the same conformational folding of $\alpha_5\beta_1$ that makes this integrin capable of recognizing the RGD peptide only in fibronectin among several other ECM molecules (52) is also responsible for a specific GAG interaction that complements the molecular requirements involved in the interaction of this integrin with fibronectin.

The possibility that integrins can also be sulfated is not ruled out by the present study because we could not detect $\alpha_5\beta_1$ integrin in Mel-S5 cells. This is an integrin that binds laminin, a molecule with a GAG binding domain spatially close to the ES domain corresponding to the $\alpha_5\beta_1$ integrin binding site (53). Furthermore, the structural relationship of the $\alpha_5$ chain with $\alpha_1\alpha_2$ and $\alpha_3$ (reviewed in Ref. 27) could suggest other $\alpha\beta$ integrin heterodimers as putative acceptors for GAG addition. Interestingly, Hayashi, Madri, and Yurchenco (54) have shown that endothelial cell interaction with the basement membrane proteoglycan (perlecan) occurs between the core protein of perlecan and $\beta_1$ and $\beta_3$ integrins, an interaction partially RGD-independent and modulated by GAGs. The $\beta_1$ integrin heterodimer involved in this adhesion resembles the $\alpha_5\beta_1$ molecule and the $\alpha_5\beta_3$ vitronectin receptor (54).

The present study suggests for the first time that integrins such as $\alpha_5\beta_1$ may have two extracellular binding sites that play a role in fibronectin binding. Previous studies have implicated a specific involvement of the heparin binding site of fibronectin with cell adhesion. These data were based on the fact that the purified fibronectin fragment containing only the heparin binding domain without the RGD peptide can promote adhesion in several different cell models (21, 23). Because our work describes $\alpha_5\beta_1$ integrin as a part-time proteoglycan compared with other $\alpha_5\beta$ dimers, we can postulate that the fibronectin-$\alpha_5\beta_1$ integrin interaction, which occurs primarily through the RGD peptide in fibronectin, is complemented and stabilized by the secondary interactions of $\alpha_5\beta_1$ chondroitin or heparan sulfate chains with the fibronectin heparin binding domains. The possibility that $\alpha_5\beta_1$ integrin, an integrin that binds only fibronectin, has chondroitin and heparan sulfate chains interacting with the fibronectin heparin binding domains is suggested by the facts that during ECM assembly the fibronectin heparin binding domain can also bind chondroitin sulfate or dermatan sulfate proteoglycans (10) and that soluble proteoglycans can inhibit cell adhesion to fibronectin (reviewed in Ref. 22) and by the existence of nonintegrin fibronectin receptors like CD44 (a chondroitin sulfate proteoglycan) and a heparan sulfate proteoglycan (19, 48) as well as by the recent finding that monoclonal antibodies raised against the fibronectin heparin binding domain (Hep II/IIICS) inhibit cell adhesion and also partially inhibit integrin binding to fibronectin (55). A model is postulated in which the RGD and heparin binding sites in fibronectin, although linearly separated, are spatially close due to fibronectin folding. It is thus possible to assume that cell surface proteoglycans and integrin cooperativity during cell adhesion can really be achieved in the case of $\alpha_5\beta_1$ integrin by two binding sites in the integrin molecule that bind RGD peptide and GAG binding domains in fibronectin.

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Post-translational Modifications of $\alpha_5\beta_1$ Integrin by Glycosaminoglycan Chains: THE $\alpha_5\beta_1$ INTEGRIN IS A FACULTATIVE PROTEOGLYCAN
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