αvβ5 Integrin Sustains Growth of Human Pre-B Cells through an RGD-independent Interaction with a Basic Domain of the CD23 Protein

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CD23 is a type II transmembrane glycoprotein synthesized by hematopoietic cells that has biological activity in both membrane-bound and freely soluble forms, acting via a number of receptors, including integrins. We demonstrate here that soluble CD23 (sCD23) sustains growth of human B cell precursors via an RGD-independent interaction with the αvβ5 integrin. The integrin recognizes a tripeptide motif in a small disulfide-bonded loop at the N terminus of the lectin head region of CD23, centered around Arg172, Lys173, and Cys174 (RKC). This RKC motif is present in all forms of sCD23 with cytokine-like activity, and cytokine activity is independent of the lectin head, an “inverse RGD” motif, and the CD21 and IgE binding sites. RKC-containing peptides derived from this region of CD23 bind αvβ5 and are biologically active. The binding and activity of these peptides is unaffected by inclusion of a short peptide containing the classic RGD sequence recognized by integrins, and, in far-Western analyses, RKC-containing peptides bind to the β subunit of the αvβ5 integrin. The interaction between αvβ5 and sCD23 indicates that integrins deliver to cells important signals initiated by soluble ligands without the requirement for interactions with RGD motifs in their common ligands. This mode of integrin signaling may not be restricted to αvβ5.

CD23 is a 45-kDa type II transmembrane glycoprotein that functions as the low affinity receptor for IgE and negatively regulates IgE production by B lymphocytes (1–5). CD23 is cleaved by membrane-associated metalloproteases (6, 7) to yield soluble CD23 (sCD23)6 proteins with molecular masses ranging from 37 to 16 kDa, all of which retain the capacity to regulate IgE synthesis; human sCD23 proteins exhibit pleiotropic cytokine-like activities (1–3). In the B cell compartment, sCD23 inhibits apoptosis of germinal center centrocytes and promotes their differentiation into plasmablasts (8), at least in part by binding to CD21 (9), and sCD23 also inhibits apoptosis in pre-B cell lines (10) through, as we report here, an interaction with the αvβ5 integrin. In association with interleukin-1α, sCD23 promotes differentiation of monocytes and early thymocyte precursors (11) and, via binding to the αvβ2 (CD11b-CD18), αvβ2 (CD11c-CD18) (12), and αvβ3 integrins (13), stimulates tumor necrosis factor-α and interleukin-1α production by monocytes.

The structures of the derCD23 protein, a naturally occurring sCD23 fragment generated by action of the derp1 protease from Dermatophagoides pteronyssinus, and of a 25-kDa sCD23 (residues 150–321), have recently been solved by heteronuclear nuclear magnetic resonance spectroscopy (14) and x-ray crystallography (15), respectively. Although there are pronounced differences between the structures derived by the two methods, both show a generally consistent overall structure for the C-terminal lectin head domain comprising eight β sheets and two α helices (14, 15). The NMR structure reveals a striking distribution of acidic and basic residues on opposite faces of the lectin head domain and demonstrates unequivocally that the interaction surfaces for IgE and CD21 binding are spatially distinct (14); the data also support unequivocally earlier studies that suggested that these binding sites and the structures responsible for expression of cytokine-like activities are distinct (16). The crystal structure confirms that CD23 contains only a single calcium binding site in the lectin head and that conformational changes in the CD23 structure accompany calcium coordination in this site (15). The binding site for integrins was not identified in either study, and, as reported here, this resides at the N-terminal region of the C-terminal lectin domain.

The αvβ5 integrin is a member of the vitronectin receptor (VnR) family. VnRs are heterodimers of the αv integrin chain in association with any one of five β subunits (17) and have well...
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documented roles in cell attachment, spreading, and migration, rescue from apoptosis, and angiogenesis (18–21). VnRs bind to the extracellular matrix proteins vitronectin (Vn) and fibronectin (Fn) by recognition of an Arg-Lys-Asp (RGD) motif in target proteins (22); both the α and β integrin subunits contribute to ligand binding (23, 24). Integrins are known to bind to proteins via other peptide motifs (22), usually resulting in enhancement of ligand binding (23, 24). Integrins are known to bind to proteins (22); both the αv5 basic domain compared with an equivalent Vn domain (25) suggests that ligands other than Vn could interact with the αv5 basic domain binding site and could have distinct signaling functions.

This report demonstrates that the αv5 integrin interacts with sCD23 using a site distinct from the RGD binding pocket and that recognition of a basic motif on CD23 that is distinct from its interaction sites for IgE, CD21, or itself, enhances survival of human pre-B cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-CD47 (BRIC 126, IgG2b), anti-αvβ3 (LM609, IgG1), anti-αvβ5 (P1F6, IgG1; 15F11, IgG2a), biotinyl-murine IgG1, rabbit polyclonal anti-peptide antibodies specific for integrin αv and β5 subunits and purified αvβ5 protein were obtained from Chemicon, UK. Anti-αv/CD51 (AMF7, IgG1) was obtained from Beckman Coulter, High Wycombe, UK; BU38 anti-CD23 was purchased from AMS Biotechnology, Abingdon, UK. Fluorescein isothiocyanate (FITC)–anti-mouse IgG1 and FITC- and phycoerythrin (PE)-labeled murine IgG1 proteins were supplied by DAKO Ltd., Denmark. Radiochemicals and materials for enhanced chemiluminescence (ECL) were obtained from Amersham International plc, Amersham, England, and fine chemicals, including streptavidin-Quantum Red, Fn, HRP-coupled protein A, and octyl-β-D-glucopyranoside (OGP), were supplied by Sigma. Vn was obtained from Invitrogen. Recombinant 25–kDa sCD23, encompassing residues Met151–Ser231 with an N-terminal His6 tag, was expressed in Escherichia coli and affinity-purified by nickel chelate chromatography, or was purchased from R&D Systems. The derCD23 protein was a generous gift from Dr. J. McDonnell, University of Oxford, UK, a CD23-GST fusion protein preparation comprising residues Asp48–Gly248 of CD23 (referred to as sCD23248–248) was obtained from Bio-supplies Ltd., Bradford, UK. C1 sensor chips were obtained from BIAcore SpA, Uppsala, Sweden, and siRNA constructs were purchased from Qiagen.

**Cell Culture, siRNA Procedures, and Flow Cytometry**—The SMS-SB cell line was derived from a female patient presenting with acute lymphoblastic leukemia (26); Nalm-6 and Blin-1 cell lines were from laboratory stocks. Cell lines were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM fresh glutamine, and penicillin and streptomycin, at 37 °C in a 5% CO2 in air in a humidified atmosphere. Human telomerized fibroblasts were cultured as described previously (27). Cytokines, obtained from R&D Laboratories, were used at 5–10 ng/ml, but had no effect over a wide dose-response range. SMS-SB cells were also propagated in protein-free hybridoma medium-II (PFHM, Invitrogen), at 2.5–5 × 105 cells/ml (“normal cell density”). In stimulation experiments, SMS-SB cells were cultured at 2500 cells/100-μl culture (low cell density (LCD)) a seeding density at which the cells are prone to apoptosis (10). NALM-6 and Blin-1 cells were washed extensively in PFHM prior to culture at 2500 cells/100-μl culture. Cultures were propagated in the presence or absence of cytokines, mAbs or peptides, at 37 °C for 72 h followed by addition of 0.3 μCi/well tritiated thymidine ([3H]TdR) for 18 h prior to harvest; incorporation was determined by liquid scintillation spectrometry.

Integrin αv knockdown was achieved using siRNA targeting the sequence, 5′-AGCAACCTTATTAGATTTA-3′ (Hs_ITGAV_1-HP siRNA), whereas the control non-silencing siRNA (cat. no. 1022076) targeted the sequence, 5′-AATTCTCCGAACGTGTCACTG-3′. 89 μl of 2 μM siRNA was diluted in 2.9 ml of Dulbecco’s modified Eagle’s medium (serum-free) then 44.5 μl of HiPerFect (Qiagen) was added prior to 10-min incubation at room temperature. This solution was added to a 10-cm plate that had been seeded 2 h previously with 6 × 105 cells in 12 ml of Dulbecco’s modified Eagle’s medium containing 20% (v/v) fetal calf serum. The medium was replaced 48 h later, and further control and silencing siRNA molecules were added as above. Cells were harvested after a further 24 h and analyzed for binding of CD23, peptides, and anti-αv integrin mAbs.

For flow cytometry, 5 × 105 cells were stained with either FITC-conjugated or unlabeled primary mAb for 30–60 min; unlabeled primary antibody was visualized using a secondary FITC-conjugated anti-mouse IgG or, in the case of biotinylated anti-αvβ5, using streptavidin-Quantum Red. Cells were analyzed on a FACScan flow cytometer, using CellQuest software.

**Affinity Isolation and Analysis of Cellular Proteins**—108 SMS-SB cells were harvested, washed twice in ice-cold phosphate-buffered saline, suspended in 1.5 ml of ice-cold OGP extraction buffer (1% (w/v) OGP in 50 mM HEPES/KOH, pH 7.4, 5 mM CaCl2, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin), and lysed with 40 strokes of a chilled glass homogenizer. The homogenate was centrifuged at 1,000 for 45 min at 4 °C, and the resulting supernatant further was centrifuged at 35,000 for 45 min at 4 °C. Cellular extracts were added to bovine serum albumin-Affi-Gel pre-equilibrated with OGP extraction buffer and incubated at 4 °C for 6 h. The matrix was pelleted, and unbound proteins were recovered, added to pre-equilibrated sCD23-Affi-Gel, and incubated at 4 °C overnight. The sCD23-Affi-Gel was pelleted, and the unbound fraction was retained. Both matrices were exhaustively washed, and specifically bound material eluted by boiling in sample buffer and subjected to SDS-PAGE under reducing conditions on a 10% (w/v) acrylamide gel. Eluates were transferred to nitrocellulose membranes and probed with anti-VnR-component antibodies, followed by an HRP-labeled secondary antibody and ECL. For far-Western analysis, purified integrin proteins were subjected to SDS-PAGE under non-reducing conditions and transferred to nitrocellulose membranes. The blots were probed with biotinylated CD23-derived peptides, in the presence or absence of competing peptides, and
binding was visualized using HRP-conjugated streptavidin and ECL. Areas of the peaks visualized by ECL/autoradiography were determined using ImageJ software. Lanes containing β5 bands were scanned, and identical peak widths were selected for calculation of areas under the selected β5 peaks.

Peptide Biochemistry and Surface Plasmon Resonance Studies—A library of 83 overlapping nonapeptides encompassing residues 151–321 of the 25-kDa sCD23 sequence was synthesized by Mimotopes Inc. (Chester, UK); biotinylated and non-biotinylated peptides and peptides with specific substitutions were synthesized by the same firm. For screening experiments, each peptide was synthesized as tridecapeptide comprising a unique CD23-derived nonapeptide sequence, plus a common N-terminal tetrapeptide extension (SGSG) to which a biotin moiety was attached (i.e. biotin-SGSG-X9 where X9 is the specific CD23-derived nonapeptide sequence). The C-terminal residue was amidated. Each unique nonapeptide sequence had a two-residue C-terminal offset relative to its immediate neighbor. The specific nonapeptide sequences were #9, KWINFQRKC; #10, INFQRFKCY; #11, FQRKCYFYG; #12, RKCYFYGKG; #9AA, KWINFQAAAC; and #12AA, AACYYFGKG; the LP long peptide sequence are KWINFQRKCYYFGKG.

Biotinylated peptides were captured on 96-well streptavidin-coated enzyme-linked immunosorbent assay trays and blocked with 1% (w/v) casein. Purified αβ5 integrin (0.2 μg) was added to each well, and binding was quantitated by use of P1F6 mAb and HRP-anti-mouse IgG followed by tetramethylbenzidine as substrate. Binding of biotinylated peptides to cells was visualized with fluorochrome-conjugated streptavidin and flow cytometry; mean fluorescence intensity data were derived using Cell Quest program. In cell stimulation experiments, peptides were used in the 10 nM to 6 μM concentration range, and appropriate solvent vehicle controls (e.g. acetonitrile and Me2SO) were always performed. Biotinylated and non-biotinylated peptides provoked identical responses in functional assays.

Interactions between CD23-derived peptides and αβ5 integrin were assessed at 25 °C using a BIACore 2000 instrument (Biacore AB). The αβ5 integrin was immobilized at 2 μg per 3500 response units on a C1 chip, and an underativated reference cell was employed as a control surface. CD23-derived peptides in HBS-EP buffer (0.01 M HEPES-KOH, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) Surfactant P20) were injected over the test and control chip surfaces at a flow rate of 40 μl/min with a 3-min association phase followed by a 6-min dissociation phase. In all experiments, peptides bound exclusively to the test cell, there was no nonspecific binding to the control reference cell, and regeneration of a stable baseline (using 0.2 M glycine-HCl, pH 2.5, where necessary) was readily achieved. Data were standardized according to standard double referencing data subtraction methods (28), using control and blank injections, prior to kinetic analysis using BIAevaluation 3.0.2 software.

RESULTS

The αβ5 Integrin Is a CD23-binding Protein—Recombinant sCD23 rescues cultures of an acute lymphoblastic leukemia-

FIGURE 1. The αβ5 Integrin is a CD23 receptor in SMS-SB cells. A, low cell density (LCD, 2,500 cells/10 μl or 25,000 cells/ml) cultures of SMS-SB in PFHM were established with the indicated concentrations of recombinant 25-kDa sCD23 (panel i) or other cytokines (panel ii) and cellular proliferation (panel i) or ratio of viable to apoptotic cells (panel ii) determined. Apoptosis was determined by staining harvested cells with propidium iodide and Hoechst 33342 for 1 min prior to analysis on a Coulter Elite flow cytometer as previously described (10). Normal cell density cultures (2.5–5×105 cells/ml) or other cytokines (panel ii) determined. Apoptosis was determined by staining harvested cells with propidium iodide and Hoechst 33342 for 1 min prior to analysis on a Coulter Elite flow cytometer as previously described (10).
derived human pre-B cell-like cell line, SMS-SB (26), from low cell density-induced apoptosis in a dose-dependent manner (10) (Fig. 1A, panel i); sCD23 is the only cytokine tested that rescues SMS-SB cells from apoptosis (Fig. 1A, panel ii). SMS-SB cells do not express CD23 itself or any of its known receptors (10) (Fig. 1C, and data not shown), and this suggested that the interaction of sCD23 with acute lymphoblastic leukemia-derived pre-B cells was mediated via a hitherto undefined receptor. CD23-Affi-Gel affinity chromatography of lysates of derived human pre-B cells was mediated via a hitherto undefined receptor of sCD23 with acute lymphoblastic leukemia-derived pre-B cells (Fig. 1D). The demonstration that SMS-SB cells express αβ5 and that it interacts with CD23 suggests that this integrin is linked to pre-B cell growth and survival. To test this proposal, SMS-SB cells were incubated with αβ5-specific monoclonal antibodies (mAb) or with Vn and Fn, the normal ligands for αβ5. Incubation with the αV-specific mAb AMF7 stimulated proliferation of SMS-SB cells in response to increasing concentrations of mAb, whereas an isotype-matched control mAb was without effect (Fig. 2A). Importantly, a second pre-B cell line, NALM-6, also showed enhanced proliferation when stimulated by the AMF7 mAb (Fig. 2B). The 15F11 mAb that recognizes fully assembled αβ5 heterodimers but does not impede αβ5 binding to Vn (31), stimulated SMS-SB proliferation (Fig. 2C). However, the P1F6 mAb, which recognizes a distinct epitope on assembled αβ5 heterodimers and does inhibit αβ5 binding to Vn (30), failed to stimulate proliferation (Fig. 2C). These data are reminiscent of those of Hermann and colleagues (13) who demonstrated that an anti-αβ3 reagent directed to the RGD binding site could not impede CD23-driven production of cyto-

**FIGURE 2.** Ligand-selective responses regulated by αβ5. LCD cultures (2500 cells/well) of SMS-SB (A) or Nalm-6 cells (B) were propagated with the indicated concentrations of AMF7 anti-αV mAb (black bar) or IgG1 isotype control mAb (gray bar); proliferation was measured by [3H]thymidine incorporation. In B, AMF7 and IgG1 were used at 5 μg/ml. C, effect of assembled heterodimer-specific anti-αβ5 mAbs P1F6 (dark gray bar) and 15F11 (light gray bar) anti-αβ5 mAbs with corresponding IgG1 and IgG2a isotype controls on SMS-SB cellular proliferation. D illustrates the effect of culture with bovine serum albumin (white bar) Vn (dark gray bar) or Fn (light gray bar). All experiments were performed a minimum of three times.
that this response seems to be both ligand-selective and potentially independent of the integrin binding site for RGD. The lack of an RGD sequence in sCD23 suggests that the motif on CD23 recognized by the integrin is not an RGD-related sequence (see Fig. 3B; there is an inverse RGD sequence of DGR but this is, of course, stereochemically different to RGD). One precedent for such recognition is the non-RGD-dependent interaction of αβ5 with a basic motif of the HIV Tat protein (25).

αβ5 Binds a Basic Motif in CD23—The sequence of 25-kDa human CD23 (Met^{151} to Ser^{321}) is shown in Fig. 3A together with a schematic representation of CD23 showing the major functional regions of the protein (Fig. 3B). A library of 83 biotinylated overlapping nonapeptides was constructed based on the 25-kDa sCD23 sequence (Fig. 3A) and probed with purified αβ5 to identify binding sites for the integrin. Purified αβ5 binds specifically and consistently to a series of four adjacent peptides (peptides #9 –#12) encompassing residues Lys^{166}–Gly^{180} (data not shown), and the sole feature common to these four peptides is a tripeptide of sequence Arg-Lys-Cys (RKC) beginning at Arg^{172} in the CD23 sequence. The structural data for sCD23 (14, 15) demonstrate that the RKC motif resides in a small disulfide-bonded loop at the N-terminal end of the C-type lectin domain. The interaction between the four CD23-derived RKC-containing peptides and αβ5 was assessed by surface plasmon resonance (SPR) analysis. This analysis demonstrates that the four CD23-derived, RKC-containing peptides bind to immobilized αβ5 integrin in a rank order of #11 > #12 > #9 > #10, whereas peptide #58, which does not have an RKC motif, failed to bind to the integrin (Fig. 3C). A longer RKC-containing, CD23-derived pentadecapeptide (the “long peptide” or LP), spanning all 15 amino acids encompassed by peptides #9 to #12, displayed greater binding to αβ5 than the RKC nonapeptides (Fig. 3D). The slow dissociation of the LP suggests that, although the RKC motif may be essential for the CD23-αβ inte-

kine release by mononuclear cells. Moreover, finding that neither Vn nor Fn stimulated proliferation of SMS-SB cells (Fig. 2D) supports the contention that the binding sites for RGD-containing ligands and sCD23 are distinct. These data show that αβ5 regulates a pro-survival response in pre-B cell lines and containing, CD23-derived pentadecapeptide (the “long peptide” or LP), spanning all 15 amino acids encompassed by peptides #9 to #12, displayed greater binding to αβ5 than the RKC nonapeptides (Fig. 3D). The slow dissociation of the LP suggests that, although the RKC motif may be essential for the CD23-αβ inte-

FIGURE 3. Definition of CD23 sequences bound by purified αβ5 integrin in vitro. A, illustrates the sequence of 25-kDa sCD23, from Met^{151} to Ser^{321}, used as the parent sequence for construction of the library of 83 synthetic biotinylated tridecapeptides, of the form biotinyl-SGSG-X$_X$$_X$$_X$$_X$, where X$_X$$_X$ is the unique nonapeptide sequence based on the CD23 sequence that was probed with purified αβ5 integrin. The black lines denote the RKC-containing peptides #9–#12 that bind the αβ5 integrin. A schematic diagram of the CD23 protein is illustrated in B showing the lectin head and stalk domains; the bolded loop represents the region of the protein where the RKC motif recognized by the αβ5 integrin resides. Purified αβ5 integrin was immobilized on a CM1 sensor chip, and different concentrations of the indicated peptides were injected at room temperature. C, binding sensograms for peptides #9–#12 and, as a control, peptide #58; D, illustrates the binding of the pentadecapeptide LP in comparison with peptides #9 and #58; the traces shown are for injections of 20 μM peptide. E, sensogram for binding of derCD23, at the indicated concentrations; F, measured affinities (± S.D.), assuming a simple linear 1:1 association model, for derCD23 and each of peptides #9–#12 and LP for immobilized αβ5 integrin. G, illustrates binding of CD23 to SMS-SB cells in the absence (gray-filled area) or presence of an excess of peptide #11 (solid line) or peptide #11AA (dotted line); H, binding of biotinylated peptide #11 in the absence (gray-filled area) or presence (solid line) of excess sCD23; binding of streptavidin-PE alone is shown in the dotted line.
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CD23 interaction, the complete binding site may extend well beyond the small common RKC tripeptide motif. Fig. 3E illustrates a representative sensorgram for the binding of derCD23 to immobilized αvβ5 integrin; the affinity of this interaction (6.04 × 10⁻⁶ M) is of the same order of magnitude as those for binding of derCD23 to CD21 (8.7 × 10⁻⁷ M) and to the Fc region of human IgE (1.3 × 10⁻⁶ M), respectively (14). Fig. 3F states the affinities of each of peptides #9 to #12 and long peptide for immobilized αvβ5 integrin. LP had the highest affinity, consistent with the sensorgrams shown in Fig. 3 (C and D), whereas the remaining peptides had affinities for the integrin similar to that of derCD23. There was no consistent correlation between affinity of a peptide for αvβ5 and its biological activity. A similar trend of data was observed when purified αvβ3 integrin was immobilized on the sensor surface (data not shown). The SPR data demonstrate that CD23-derived, RKC-containing peptides bind to αvβ5 integrin. Fig. 3G demonstrates that the binding of CD23 to SMS-SB cells was greatly attenuated by inclusion of excess peptide #11 but not by a variant of peptide #11 where the arginine and lysine have been replaced by alanines (peptide #11AA). Excess LP also inhibited binding of CD23 to the cells (data not shown). Fig. 3H shows the reciprocal experiment, where excess CD23 reduced the binding of peptide #11 to background levels. These data indicate that CD23 and peptides derived from it compete for binding to the same sites on SMS-SB cells.

CD23-derived, RKC-containing Peptides Bind to Cells and Are Biologically Active—The data of Fig. 3 indicate that CD23-derived RKC-containing peptides bind to αvβ5 in vitro. The binding of biotinylated CD23-derived peptides to SMS-SB cells was detected by flow cytometry, and all four RKC-containing peptides bound to SMS-SB cells (Fig. 4A and B), and to other αvβ5⁺ pre-B cell lines such as NALM-6 (data not shown). CD23-derived peptides immediately adjacent to peptides #9 and #12, namely #8 and
that have the inverse RGD (DGR) sequence, also did not bind to αvβ5 or to cells (data not shown). Binding of biotinylated peptide #9 to SMS-SB cells was significantly inhibited by the presence of excess non-biotinylated peptides #10 and #12, but not peptide #8 (Fig. 4C, panels i and ii): a similar result was obtained for NALM-6 cells (data not shown). The LP inhibited binding of the αvβ5 heterodimer-specific mAb P1F6 to Bln1 pre-B cells (Fig. 4D), indicating that binding of this peptide to the integrin on the cell surface perturbs the conformation of the integrin such that the integrity of the epitope recognized by the antibody is compromised. Finally, the contribution of such that the integrity of the epitope recognized by the antibody on the cell surface perturbs the conformation of the integrin was unaffected by either siRNA (Fig. 4V). 

Confirming that for NALM-6 cells (data not shown). The LP inhibited binding of the αvβ5 integrin was lost (Fig. 4A), but peptides #10 and #11, which bound to SMS-SB cells, did not stimulate proliferation, raising the possibility that these peptides might act as inhibitors of the stimulatory activity of peptides #9 and #12 (Fig. 5A). Accordingly, SMS-SB cells were incubated with an excess of peptides #10 or #11 and then challenged with either peptide #9 or #12. For both peptide #9 and #12, peptides #10 and #11 suppressed proliferation, whereas the irrelevant peptide #57 did not (Fig. 5B, panels i and ii). These data indicate that peptides #10 and #11 can indeed inhibit the action of stimulatory peptides #9 and #12. Moreover, because the fibroblasts also express the effects on expression of αv integrins and binding of CD23 and CD23-derived peptides to the cells was determined. The αv subunit was targeted because successful attenuation of αv expression will reduce expression of all αv-containing integrins due to the requirement for α and β chains to assemble in the endoplasmic reticulum prior to transport to the cell surface. Moreover, because the β5 subunit pairs only with α(17), a successful knockdown of αv expression will also diminish αvβ5 cell surface levels. Fig. 4E shows that exposure of telomerized fibroblasts to siRNAαv, but not the control siRNA, significantly reduced staining by the AMF7 and P1F6 reagents (Fig. 4E, panels i and ii, respectively), showing both total αv and αvβ5 expression at the cell surface were reduced; the background level of staining with the isotype control IgG1 was unaffected by either siRNA (Fig. 4E, panel iii). Similarly, binding of biotinylated CD23 (Fig. 4E, panel iv) or peptide #9 (Fig. 4E, panel v) to siRNAαv-treated cells was reduced by a substantial amount, whereas the binding of a negative control peptide (#41, Fig. 4E, panel vi) was unaffected. These data indicate that loss of αv integrin expression in general, and αvβ5 in particular, correlates with a dramatic reduction of binding of CD23 and RKC-containing CD23-derived peptides to the cells, confirming that αv integrins are the target for CD23 binding. Because the fibroblasts also express β1 and β2 integrin subunits, the expression of which will be unaffected by siRNAαv, it is clear that none of these integrins, and in particular the β1 integrin subunit, can substitute for αv in binding CD23. These data are entirely consistent with the SPR data of Fig. 3, which show that CD23 binds the αvβ5 integrin.

The biological activities of CD23-derived peptides were assessed in standard low cell density proliferation assays. Peptides #9 and #12 stimulated thymidine incorporation by SMS-SB cells, demonstrating that CD23-derived peptides that bind αvβ5 via their RKC motif can mimic the effect of CD23 (Fig. 5A), but peptides #10 and #11, which bound to SMS-SB cells, did not stimulate proliferation, raising the possibility that these peptides might act as inhibitors of the stimulatory activity of peptides #9 and #12 (Fig. 5A).

sCD23 Cytokine Activity Is Independent of the IgE and CD21 Binding Sites—The hypothesis that αvβ5 integrin recognizes the dibasic peptide region in CD23 was tested by substituting Arg177 and Lys178 in peptides #9 and #12 with alanine (peptides #9AA and #12AA, respectively). Peptide #9AA shows a severely impaired ability to sustain growth of SMS-SB cells compared with wild-type peptide #9, being comparable to that achieved with peptide #58 (Fig. 6A, panel i). Biotinylated peptide #9AA displayed greatly reduced binding to SMS-SB cells (Fig. 6A, panel ii), and SPR analysis demonstrated clearly that essentially all peptide #9AA binding to immobilized αvβ5 integrin was lost when the two basic residues of peptide #9 were substituted with alanine (Fig. 6A, panel iii). Similar data were obtained for peptide #12AA, which induced SMS-SB proliferation at levels identical to those seen with control peptide #58 (Fig. 6B), and which also showed reduced binding to SMS-SB cells (data not shown). Peptide #11 showed the highest level of binding to immobilized αvβ5 (see Fig. 3D) and to cells (see Fig. 4B), and the peptide #11AA double-alanine mutant failed to completely bind to immobilized αvβ5 as determined by SPR analysis (Fig. 6C).

Finally, a CD23-GST fusion protein comprising residues 48–248 of CD23 (scCD2348–248), which contains the RKC motif but lacks the inverse RGD motif, the CD21 and IgE interaction surfaces, and all four protein residues that participate in coordination of calcium binding in the lectin head (15), sustained growth of SMS-SB cells in a dose-dependent manner (Fig. 6D). The approximate EC50 for the scCD2348–248 construct, in the 5–10 nM range, is broadly similar to that observed for 25-kDa sCD23 (10) (Fig. 1A, panel i). The LP, comprising the RKC motif flanked by six residues on either side, also promoted growth of SMS-SB cells (Fig. 6E) supporting the conclusion that

FIGURE 4. RKC-containing, CD23-derived peptides bind to pre-B cells. Binding of biotinylated peptides to pre-B cell lines was detected by addition of PE-streptavidin and flow cytometry; mean fluorescence index data were derived using CellQuest software. The flow cytometric plots for binding of peptides #9 (black line), #11 (gray line), and #57 (shaded area) are illustrated (A). Mean fluorescence index data for binding of the indicated peptides to SMS-SB cells are shown together with the unique amino acid sequence of each biotinylated tridecapeptide; the RKC motif shared by αvβ5 binding peptides is displayed in bold (B). C, panel i illustrates competition of binding of biotinylated peptide #9 to SMS-SB cells; negative control (biotinyl-peptide #76 in this case) is shown in the filled area, and binding of biotinylated peptide #9 in the absence (black line) or presence of a 10-fold excess of named competitor peptide (gray line) showed for peptides #6, #10, and #12. Panels ii and iii show percentage inhibition of binding of biotinyl peptide #9 to SMS-SB and NALM-6 cells, respectively. D, binding of the P1F6 mAb (1 μg/ml) to Bln1 cells in the presence (shaded area) or absence (thick black line) of 1 μg/ml LP. Isotype-control staining is shown by the dotted line. E, illustrates the binding of the indicated mAbs (panels i–iii) and biotinylated CD23 (panel iv) or peptides #9 or #41 (panels v and vi) derived from CD23 to telomerized human fibroblasts cultured for 96 h with either control siRNA (gray-filled area) or siRNAαv (solid line) prior to staining; note that the dotted line in panel iv represents staining with streptavidin-PE alone.
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FIGURE 5. CD23-derived peptides containing the RKC motif are biologically active. A, LCD cultures of SMS-SB cells (2500 cells/well) were established in the presence of the indicated concentrations of peptides #9–#12 (black bars), and pulsed for 16 h with [3H]TdR after 72 of culture. The effect of stimulatory peptide alone is shown in the white bars, or, as a negative control, peptide #57 (lane 2). In each case, the effect of excess individual competitor shown as light gray bar; [3H]TdR incorporation values in pg/ml RGDS. LCD cultures were established with no stimulus (lane 1). By lane 5 and 3, inclusion of excess peptide #10 (lane 3) inhibited binding of biotinylated peptide #9 to the separated β5 subunit by ~90% (Fig. 7B, panel ii). These data are completely consistent with those of Fig. 5 showing that peptide #10 inhibited peptide #9-driven proliferation of SMS-SB cells. The data of Fig. 7 indicate that CD23-derived, RKC-containing peptides bind to a structure present on the β chains of the α integrins.

DISCUSSION

This study identifies the αvβ5 integrin as a CD23 receptor, defines the recognition site for the integrin on the CD23 protein, and demonstrates a new role for CD23 in regulation of human B cell precursor growth. The data demonstrate that survival signaling via the integrin is ligand-selective and is mediated via a structure on the integrin that is distinct from the RGD binding site and recognizes a basic domain on CD23; this second site appears to reside on the β subunit. Importantly, given the protein-free conditions of the proliferation assays and the sizes of the ligands used, the data demonstrate that integrins send important signals to cells without the need for ligation of the RGD binding site. This property may not be limited to αvβ5. The data illustrate further the complex roles of CD23 in regulating human B cell function and show clearly that the pleiotropic functions of the RKC motif is necessary and sufficient for expression of sCD23 cytokine activity in SMS-SB cells. The data are consistent with the interpretation that the cytokine activity of CD23 mediated via the αvβ5 integrin in pre-B cells does not require any contribution from the inverse-RGD motif or from the IgE and CD21 binding sites.

RKC-containing Peptides Derived from CD23 Bind Integrin β Chains—The data of Fig. 6 indicate that the RKC-containing CD23-derived peptides do not bind to the RGD binding site of the α integrins, and this is fully supported by the x-ray crystallographic data for αvβ3 in association with an RGD-containing cyclic pentapeptide that suggest that the lysine is too large to be accommodated in the RGD binding site (24). As a first step in seeking the binding site for RKC-containing peptides in the αvβ5 integrins, a far-Western approach was adopted in which purified αvβ5 and αvβ3 integrins were electrophoresed under non-reducing conditions and the separated subunits were transferred to nitrocellulose and probed with biotinylated RKC-containing peptides. Fig. 7A (lanes 1 and 2) illustrates that peptide #9 bound strongly to the β5 and β3 chain but only very weakly, if at all, to the α subunit; treatment of identical blots with an irrelevant biotinylated peptide (#76), gave no signal (Fig. 7A, lanes 3 and 4). Fig. 7B (panel i) demonstrates that binding of peptide #9 to the β5 subunit is not impeded by the presence of excess, negative control peptide #58 (lane 2), where no reduction of peak areas of the β5 bands was evident (Fig. 7B, panel ii); inclusion of excess peptide #10 (lane 3) inhibited binding of biotinylated peptide #9 to the separated β5 subunit by ~90% (Fig. 7B, panel ii). These data are completely consistent with those of Fig. 5 showing that peptide #10 inhibited peptide #9-driven proliferation of SMS-SB cells. The data of Fig. 7 indicate that CD23-derived, RKC-containing peptides bind to a structure present on the β chains of the α integrins.

FIGURE 7. a. Peptide binding experiments were established as in Fig. 4A. Panel i shows mean fluorescence index values for peptide binding determined in the absence (dark gray bar) or presence (light gray bar) of 10 μg/ml RGDS. LCD cultures were established with no stimulus (white bar), with 10 μg/ml RGDS peptide (light gray bar), peptide #9 or #12 alone (dark gray bars), or either peptide #9 or #12 together with 10 μg/ml RGDS (mid-gray bars); [3H]TdR uptake was assessed as noted in Fig. 2. Panel ii shows panels

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CD23 are controlled by discrete structural motifs on the protein.

The RKC motif recognized by the αvβ5 integrin is located at positions 172–174 of the human CD23 protein and is preserved in all forms of sCD23 that possess cytokine activity (1). Fig. 8A places the RKC motif in the context of its general position in the CD23 protein and demonstrates that the RKC-centered integrin binding site is completely distinct from those used to cap-
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Arg\textsuperscript{172} and Lys\textsuperscript{173} are clearly exposed to the solvent in the derCD23 structure (Fig. 8D, gray lines), whereas the sCD23 crystal structure shows Lys\textsuperscript{173} occupying a less solvent-available position close to the α helix and the arginine in a different but still exposed position (Fig. 8D, blue lines, and Fig. 8B, panels ii and iv). Finally, although binding of calcium causes notable conformational changes in the structure of sCD23 as a whole (15), there is relatively little effect on the orientations and relative positions of the RKC residues in the calcium-free and bound states (Fig. 8E). The NMR structure can accommodate a direct binding model for the αvβ5-CD23 interaction, based on having both Arg\textsuperscript{172} and Lys\textsuperscript{173} available for binding, but the crystal structure does not preclude an induced-fit model in which Arg\textsuperscript{172} provides an initial point of contact that allows the two protein structures to alter such that a larger interaction surface becomes available. A comparison of the human and murine sequences in the context of the αvβ5 binding site defined in this report offers a partial explanation for the apparent lack of cytokine-like activity in murine CD23 mediated via the α integrins. The human CD23 sequence between residues 169 and 181 is NFKRCYFNGKT while the equivalent murine sequence is HFKQKYYFKGQS; importantly, the RKC motif that may be the primary determinant of binding and activity is altered to QKC in the murine CD23 sequence. In contrast to the large, mobile, and basic arginine side chain, glutamine may lack both sufficient length and flexibility to enter a binding cleft and/or may lack the charged termini required to bind securely to the target integrin in a conformation capable of signaling to the cell interior. Although Arg\textsuperscript{172} alone is unlikely to be the sole determinant of cytokine activity, it is clearly a key residue for binding and, by extension, biological activity.

The data of Fig. 2 demonstrate that different αvβ5 ligands (CD23, Vn, and Fn) and mAbs directed to different epitopes of αvβ5 itself elicit characteristic responses in pre-B cell lines by acting via distinct, RGD-dependent and RGD-independent, binding sites on αvβ5. There are precedents for ligand-selective signaling via αvβ3 in monocytes where CD23 promotes pro-inflammatory cytokine synthesis, while Vn drives cell spreading but no cytokine production (13). The importance of adhesion interactions between pre-B cells or acute lymphoblastic leukemia cells with stromal cells for survival is widely appreciated (e.g., the VLA-4-VCAM-1 interaction (34–36)), and, in the bone marrow environment where several αv ligands are present (i.e., Fn and Vn (37) and CD23 (38)), the availability of ligand-selective responses, mediated via two distinct binding sites on the same integrin, may be important. If engagement of the RGD binding site was linked to cell survival, then Vn and Fn in the bone marrow would sustain growth of precursor cells in a non-selective manner, resulting in failure to eliminate redundant or malignant precursors. However, the αvβ5 integrin may allow B cell precursors to adhere to the stromal matrix via Vn in an interaction that is neutral with respect to cell survival. Signals for inhibition of apoptosis and promotion of cell growth would then be delivered via the second, non-RGD binding site on the αvβ5 integrin. Whether the αvβ5 integrin delivers a growth-sustaining signal to cell types other than lymphoid precursors remains to be established.

**FIGURE 7. RKC-containing peptides bind to integrin β chains.** A, purified αvβ3 and αvβ5 integrins (0.5 μg) were electrophoresed under non-reducing conditions, and separated subunits were transferred to nitrocellulose filters and probed with 4.5 μg/ml biotinylated peptides #9 (lanes 1 and 2) or #76 (lanes 3 and 4). Peptide binding was visualized with streptavidin-HRP and ECL. Blots of separated αvβ5 integrin chains, generated as described above, were probed with biotinylated peptide #9 (lane 1) in the presence of a 2-fold molar excess of peptide #58 (lane 2) or #10 (lane 3) (B, panel i). Peak area analysis was performed using ImageJ software (B, panel ii).
In our system, RGD-containing peptides do not sustain cell growth themselves, and fail to inhibit either peptide #9 or #12 binding to cells or their ability to sustain cell growth (Fig. 5), and far-Western data (Fig. 7) suggest that RKC-containing peptides bind separated β3 and β5 chains. The x-ray crystallographic model of αvβ3 integrin in association with an RGD-containing cyclic pentapeptide fully supports the interpretation that αvβ5 binds CD23 using a structure distinct from the RGD binding site. The RGD-peptide arginine is secured by a bidentate salt link with Asp218 and a second such link with Asp150 from the αv chain (24), whereas the aspartate side chain makes contacts with Tyr122, Arg214, and Asn215 from the β3 chain, plus a Mn$^{2+}$ ion (24). The peptide glycine residue resides at the interface between the αv and β3 domains making multiple hydrophobic interactions, including a dominant contact with the carbonyl oxygen of Arg216 of αv (24). The long side chain of the RKC peptide lysine would collide with this carbonyl moiety and so preclude stable insertion of the RKC motif into the RGD binding site.

Integrins recognize sequences other than RGD (22). Thus, the α2β1 integrin binds a basic tetrapeptide sequence (RKKH) in the snake venom metalloprotease, jararhagin, a potent inhibitor of platelet binding to collagen (39), and the I-domain of the integrin is known to be the binding site. However, it is becoming increasingly apparent that small groups of basic residues, such as described in this report, are often targets for integrin binding, and in many cases recognition of such motifs by integrins is linked to, or cooperates with, binding to the RGD binding site in facilitating adhesion reactions. For example, both binding of the angiogenic factor CCN3 to αvβ5 and CCN3-induced migration of fibroblasts is inhibited by RGD-containing peptides even though the protein does not possess an RGD, or related, sequence (40). The C-terminal domain of the closely related CCN1 protein has two distinct regions, each containing a pair of adjacent lysine residues, that bind the αMβ2 integrin and support monocyte adhesion (41). CCN3 contains equivalent sequences in its C-terminal domain, although whether these are bound by αvβ5 remains to be formally proven. The binding of the non-RGD-containing cobra venom cardiotoxin A5 to αvβ3 and its ability to inhibit osteoclastogenesis is inhibited by RGD-containing peptides (42). Interestingly, the related A6 toxin binds only weakly to αvβ3 and is not cardiotoxic, and this is explained by replacement of Lys28 and Lys29.

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of the A5 molecule with valine and alanine, respectively (42). The αvβ5 integrin recognizes the HIV Tat protein via a non-RGD motif that is basic in character (25). Studies of binding of αIIbβ3 to peptide fragments of fibrinogen demonstrated that the integrin recognizes a cluster of basic residues located between residues 370 and 381 of the γC domain that is devoid of an RGD sequence (43). Intriguingly, the structure of the γC domain (32) reveals two pairs of basic residues on exposed surfaces of the protein that are crucial for the interaction with the integrin. In particular, substitution of either Lys380 or Lys381 of γC peptides with alanine reduces peptide binding to αIIbβ3, and a double substitution completely abolishes binding (43).

The data from other systems are completely consistent with our data showing loss of binding and activity of CD23-derived peptides when Arg772 and Lys773 are substituted with alanine.

A range of integrin-mediated responses may be driven by recognition of basic domains on other extracellular proteins, and such responses may be independent of occupation of the RGD binding site or may show cross-talk with RGD-specific binding events. Indeed, the cellular response initiated following ligation of the basic domain recognition site on αv or other integrins may also be dependent on the integrin heterodimer being engaged. Definition of the precise basic domain binding structure on the β chain of the αv integrins will help address these issues.

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