

Mapping of Potent and Specific Binding Motifs, GLOGEN and GVOGEA, for Integrin $\alpha 1 \beta 1$ Using Collagen Toolkits II and III^{*[S]}

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Background: The collagens contain GxOGER^{''} integrin-binding motifs, whose identity and specificity are poorly defined.

Results: GLOGEN in collagen III is a high affinity ligand, and GVOGEA in collagen II is a specific ligand for $\alpha 1 \beta 1$.

Conclusion: Collagen Toolkits enable such sites to be identified and compared.

Significance: GLOGEN- and GVOGEA-containing peptides can be used to characterize the properties of $\alpha 1 \beta 1$.

Integrins are well characterized cell surface receptors for extracellular matrix proteins. Mapping integrin-binding sites within the fibrillar collagens identified GFOGER as a high affinity site recognized by $\alpha 2 \beta 1$, but with lower affinity for $\alpha 1 \beta 1$. Here, to identify specific ligands for $\alpha 1 \beta 1$, we examined binding of the recombinant human $\alpha 1$ I domain, the rat pheochromocytoma cell line (PC12), and the rat glioma Rugli cell line to our collagen Toolkit II and III peptides using solid-phase and real-time label-free adhesion assays. We observed Mg²⁺-dependent binding of the $\alpha 1$ I domain to the peptides in the following rank order: III-7 (GLOGEN), II-28 (GFOGER), II-7 and II-8 (GLOGER), II-18 (GAOGER), III-4 (GROGER). PC12 cells showed a similar profile. Using antibody blockade, we confirmed that binding of PC12 cells to peptide III-7 was mediated by integrin $\alpha 1 \beta 1$. We also identified a new $\alpha 1 \beta 1$ -binding activity within peptide II-27. The sequence GVOGEA bound weakly to PC12 cells and strongly to activated Rugli cells or to an activated $\alpha 1$ I domain, but not to the $\alpha 2$ I domain or to C2C12 cells expressing $\alpha 2 \beta 1$ or $\alpha 11 \beta 1$. Thus, GVOGEA is specific for $\alpha 1 \beta 1$. Although recognized by both $\alpha 2 \beta 1$ and $\alpha 11 \beta 1$, GLOGEN is a better ligand for $\alpha 1 \beta 1$ compared with GFOGER. Finally, using biosensor assays, we show that although GLOGEN is able to compete for the $\alpha 1$ I domain from collagen IV (IC₅₀ ~3 μ M), GFOGER is much less potent (IC₅₀ ~90 μ M), as shown previously. These data confirm the selectivity of GFOGER for $\alpha 2 \beta 1$ and establish GLOGEN as a high affinity site for $\alpha 1 \beta 1$.

Integrins are glycoprotein cell adhesion molecules that transmit bidirectional signals across the plasma membrane and regulate many physiological functions, including cell differentiation, cell migration, and wound healing (1, 2). They interact either with proteins of the extracellular matrix, such as fibronectin and collagen, or with counter-receptors expressed on other cells, as occurs, for example, in the binding of the

leukocyte integrins to intercellular and vascular cell adhesion molecules. Integrins are unique in that their adhesiveness can be dynamically regulated through a process termed affinity regulation (inside-out signaling). Thus, stimuli delivered by cell surface receptors, such as those for chemokines, cytokines, or foreign antigens, initiate intracellular signals that impinge on integrin cytoplasmic domains and alter adhesiveness for extracellular ligands. Moreover, ligand binding transduces signals from the integrin extracellular domain to the cytoplasm in the classical direction (outside-in signaling).

Integrins contain two noncovalently associated α - and β -subunits, each with a large extracellular domain, a single transmembrane domain, and, typically, a short cytoplasmic domain. The extracellular segment of the α - and β -subunits has up to 1104 and 778 residues, respectively, with the N-terminal portions of each subunit combining to form a globular ligand-binding "head" connected to the membrane by a long (170 Å) stalk. In mammals, 24 integrins have been identified to date, resulting from different pairings among 18 α - and 8 β -subunits (3). Half of the integrin α -subunits (including the collagen-binding subset of $\beta 1$ integrins) contain a domain of ~200 amino acids known as the inserted (I) domain (or the von Willebrand factor A domain). This I domain is the major ligand-binding site in all α I-containing integrins (4, 5), including the collagen-binding integrins, $\alpha 1 \beta 1$, $\alpha 2 \beta 1$, $\alpha 10 \beta 1$, and $\alpha 11 \beta 1$ (3), which all interact with native triple-helical collagen through this domain.

Ligand binding to the integrin I domain requires a divalent cation coordinated within an acidic pocket known as the metal ion-dependent adhesion site (MIDAS).² The ligand invariably uses a solvent-exposed aspartate or glutamate carboxylate anion to bind the MIDAS cation. Glutamate is the major MIDAS-reactive residue within the fibrillar collagens, and a series of GxOGER motifs have been identified and are discussed further below. Two conformations of the $\alpha 2$ I domain were revealed by comparing the crystal structures of the free and ligated domains (6, 7), indicating the presence of two activation states for $\alpha 2 \beta 1$ (8).

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^[S] This article contains supplemental Fig. 1.

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² The abbreviations used are: MIDAS, metal ion-dependent adhesion site; SPBA, solid-phase binding assay; PMA, phorbol 12-myristate 13-acetate.

Integrin $\alpha 1\beta 1$ -binding Sites in Fibrillar Collagens

$\alpha 1\beta 1$ (initially termed very late antigen-1) was identified on the surface of T cells from the joints of patients with rheumatoid arthritis (9). $\alpha 1\beta 1$ is highly expressed in vascular and visceral smooth muscle, hepatic stellate cells (10), pericytes (11), mesangial cells (12, 13), bone marrow mesenchymal stem cells (14, 15), chondrocytes (16), neuronal cells (17), the microvascular endothelium, and many white blood cells (18, 19). During development, there is abundant and dynamic expression of $\alpha 1\beta 1$ in the embryonic tissues. $\alpha 1\beta 1$ expression has been shown to be regulated by cytokines, such as TGF β , interleukin-1 β , TNF α , and interferon- γ , whereas PDGF causes down-regulation in fibroblast cells (20–22). Dysregulation of very late antigen-1 has been shown in melanoma, leiomyosarcoma, and bronchoalveolar carcinoma (23–27).

Based on sequence similarity of the α -subunits, collagen-binding integrins can be subdivided into two pairs: 1) $\alpha 1\beta 1$ and $\alpha 2\beta 1$ and 2) $\alpha 10\beta 1$ and $\alpha 11\beta 1$. Nevertheless, binding studies have indicated that $\alpha 1\beta 1$ and $\alpha 10\beta 1$ preferentially bind collagen types IV and VI over fibrillar collagens, whereas $\alpha 2\beta 1$ and $\alpha 11\beta 1$ preferentially bind fibrillar collagens over the network-forming collagens (28, 29).

A binding site for $\alpha 1\beta 1$ was identified in collagen IV, comprising Asp-441 of the $\alpha 1$ (IV) chain and Arg-458 of the $\alpha 2$ (IV) chain (30, 31). Biophysical studies have identified the most likely register of the chains in collagen IV at this point as $\alpha 1;\alpha 1;\alpha 2$ (32), and modeling of this peptide onto the $\alpha 1$ I domain structure supports Asp-441 being able to coordinate divalent cation in its MIDAS (33). However, a crystal structure of the complex is lacking.

A process of mapping integrin-binding sites within the fibrillar collagens was initiated by Barnes *et al.* (34), culminating in the identification of GFOGER as the high affinity site within collagen I recognized by $\alpha 2\beta 1$ (35, 36). Other related motifs were identified at conserved locations in the fibrillar collagens (37, 38). Among G $\times$ OGER motifs, affinity for $\alpha 2\beta 1$ decreased in the order $x = F, L, M$, and A , and similar but non-canonical motifs GLSGER, GQRGER, and GASGER were of intermediate or low affinity, which increased upon cell activation. Notably, GLOGER had relatively higher affinity for the $\alpha 1$ I domain than for the $\alpha 2$ I domain.

We subsequently developed a systematic approach to receptor mapping by synthesizing the entire triple-helical domain of collagens II and III as a set of overlapping homotrimeric peptides (termed the Collagen II and III Toolkits) (39). Study of $\alpha 2\beta 1$ binding to collagen III led to the first observation of a non-GER integrin-binding motif, GLOGEN, within the fibrillar collagens that was unique to collagen III (39). Very recently, a homolog, GFPGEN, was expressed in *Streptococcus* and described as being $\alpha 1\beta 1$ -selective (40). Here, we used our Toolkit approach to identify high affinity sites for $\alpha 1\beta 1$ in both Collagen II and III Toolkits using recombinant I domains and integrin-expressing cell lines, and we have identified native collagen sites that are specific or selective for $\alpha 1\beta 1$.

EXPERIMENTAL PROCEDURES

Reagents—Unless stated otherwise, all reagents were from Sigma. Collagen fibers were a gift from Ethicon Corp. (Somer-

ville, NJ), and other monomeric pepsin-digested collagens were from Sigma: human I (C-7774), III (C-4407), IV (C-5533), and V (C-3657) and bovine II (C-1188).

Peptides—Toolkit peptides (as C-terminal amides) were synthesized on Tentagel R resin using an Applied Biosystems Pioneer peptide synthesizer as described previously (39). Shorter peptides were made using the same Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry in a CEM Liberty microwave synthesizer. In either case, fractions containing homogeneous product were identified by analytical HPLC on an ACE Phenyl-300 (5 mm) column, characterized by MALDI-TOF mass spectrometry, pooled, and freeze-dried.

Antibodies—Horseradish peroxidase-conjugated mouse anti-GST antibody was from GE Healthcare (catalog no. RPN1236). Mouse anti- $\alpha 2$ I domain monoclonal antibody was from Serotec (product code MCA743), and HRP-conjugated goat anti-mouse IgG was from Dako (catalog no. P0447). Hamster monoclonal antibody to mouse/rat $\alpha 1$ (Ha31/8), and monoclonal antibodies to rat $\alpha 2$ (Ha1/29), mouse $\alpha 2$ (HM $\alpha 2$), and mouse $\beta 1$ (9EG7) were from Pharmingen.

Plasmids—The recombinant human $\alpha 1$ and $\alpha 2$ I domain-encoding plasmids (pGEX-2T- $\alpha 1$ -I and pGEX-2T- $\alpha 2$ -I) (41) were a generous gift from Dr. D. Tuckwell (F2G Ltd., Manchester, United Kingdom). For binding kinetics, the $\alpha 1$ I domain ORF was cloned in the bacterial expression vector pDEST-N110 (a kind gift of Dr. M. Dyson) as described (42).

I Domain Expression and Purification—To express wild-type $\alpha 1$ and $\alpha 2$ I domains, a 100-ml overnight culture of transformants (Origami strain) was used to inoculate 1 liter of Luria broth containing 50 μ g/ml ampicillin. The culture was grown for 2 h at 37 °C and then induced at room temperature for 4 h with isopropyl β -D-thiogalactopyranoside (0.1 mM; catalog no. MB1008, Melford Laboratories Ltd., Suffolk, United Kingdom). Cells were harvested by centrifugation at 4500 $\times g$ for 20 min, and pellets were resuspended in 10 ml of Dulbecco's phosphate-buffered saline containing 1 tablet of protease inhibitor mixture (Roche Applied Science) and 50 mg of lysozyme (Fluka). Suspensions were sonicated, and Triton X-100 was adjusted to 1% (v/v). Suspensions were incubated at room temperature for 15 min on a roller mixer and centrifuged at 18,000 $\times g$ for 20 min, and supernatants were pooled. The lysate was passed down a glutathione-agarose column equilibrated in TBS (20 mM Tris-HCl (pH 7.5) and 150 mM NaCl); the column was washed with 10 volumes of TBS containing 1 M NaCl and 1% (v/v) Triton X-100, and the GST-I domain fusion proteins were eluted with 10 mM glutathione reduced in 50 mM Tris-HCl (pH 8.0). The proteins were then dialyzed against TBS and concentrated using a Microcon-3 system (Amicon, Gloucestershire, United Kingdom). The I domains were checked for purity and degradation by 10% SDS-PAGE and Western blotting. Nitrocellulose blots were probed with horseradish peroxidase-conjugated anti-GST polyclonal antibody (GE Healthcare).

I Domain Binding Assay—I domain adhesion was determined colorimetrically as described (here termed solid-phase binding assay (SPBA)) (43). Peptides were coated at 10 μ g/ml for 1 h at 22 °C on Immulon 2HB 96-well plates (Thermo Scientific) and blocked for 1 h with 200 μ l of TBS containing 50 mg/ml BSA. Wells were washed four times with 200 μ l of adhe-

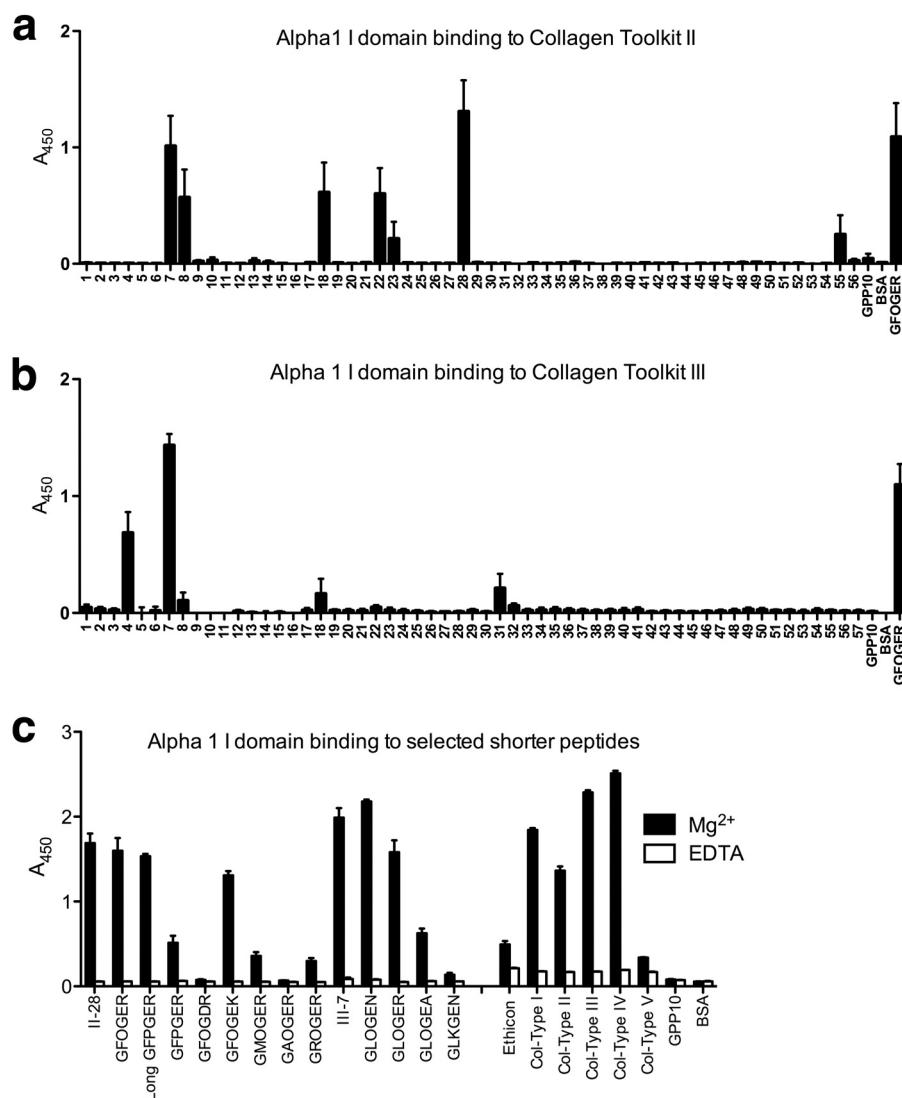


FIGURE 1. Binding of wild-type $\alpha 1$ I domain to Toolkits II and III and to selected shorter triple-helical peptides. The recombinant wild-type $\alpha 1$ I domain was incubated in wells of Immulon 2HB 96-well plates coated with peptides, and adhesion was measured in the presence of either 2 mM Mg^{2+} (black bars) or 2 mM EDTA (white bars) as described under "Experimental Procedures." BSA, GPP10, and GFOGER served as control surface coatings. Experiments were performed in triplicate using 0.1 μ g of protein/well. *a*, adhesion to Toolkit II; *b*, adhesion to Toolkit III; *c*, adhesion to selected shorter peptides, formatted with the indicated sequence flanked by (GPP)₅ host peptides. Long GFPGER has the same sequence as II-28 but with "O" in its GFOGER motif replaced with "P." Data are the mean $\pm$ S.E. of at least six independent experiments. *Col*, collagen.

sion buffer (TBS with 1 mg/ml BSA) before the addition of 100 μ l of adhesion buffer containing 1 μ g/ml recombinant GST-I domains in the presence of either 2 mM $MgCl_2$ or EDTA for 1 h at room temperature. Wells were washed five times with 200 μ l of adhesion buffer containing $MgCl_2$ or EDTA before the addition of 100 μ l of adhesion buffer containing HRP-conjugated anti-GST antibody at 1:10,000 dilution for 1 h at room temperature. After washing, color was developed using a TMB Substrate Kit (Pierce) according to the manufacturer's instructions. In some experiments, mouse anti- $\alpha 2$ I domain monoclonal antibody (38) was used at 1:2000 dilution and incubated at room temperature for 45 min, followed by detection using HRP-conjugated anti-mouse IgG at 1:5000 dilution.

Cell Culture and Adhesion—The rat PC12 cell line was obtained from the European Collection of Animal Cell Cultures (Porton Down, United Kingdom). Parental mouse myoblast C2C12 cells, which lack endogenous collagen-binding integ-

rins, were stably transfected with human $\alpha 2$ cDNA or human $\alpha 11$ cDNA in the pBJ-1 vector as described previously (44). The derivative clones, C2C12- $\alpha 2^+$ (constitutively expressing human $\alpha 2 \beta 1$) and C2C12- $\alpha 11^+$ (constitutively expressing human $\alpha 11 \beta 1$), were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 2.5 μ g/ml amphotericin. Cells were harvested by centrifugation, washed, and suspended at 0.5×10^6 /ml in TBS supplemented with 5.5 mM glucose and either 2 mM EDTA or $MgCl_2$. SPBA was performed as follows: 100 μ l of the cell suspension was added to peptide-coated wells (43) at 20 $^{\circ}C$ for 60 min. Cell adhesion was determined colorimetrically after lysis using a lactate dehydrogenase-based cell detection kit (Roche Diagnostics) according to the supplier's instructions.

Real-time Cell Adhesion Using xCELLigence—The extent of integrin-expressing cell binding to collagen peptides was quan-

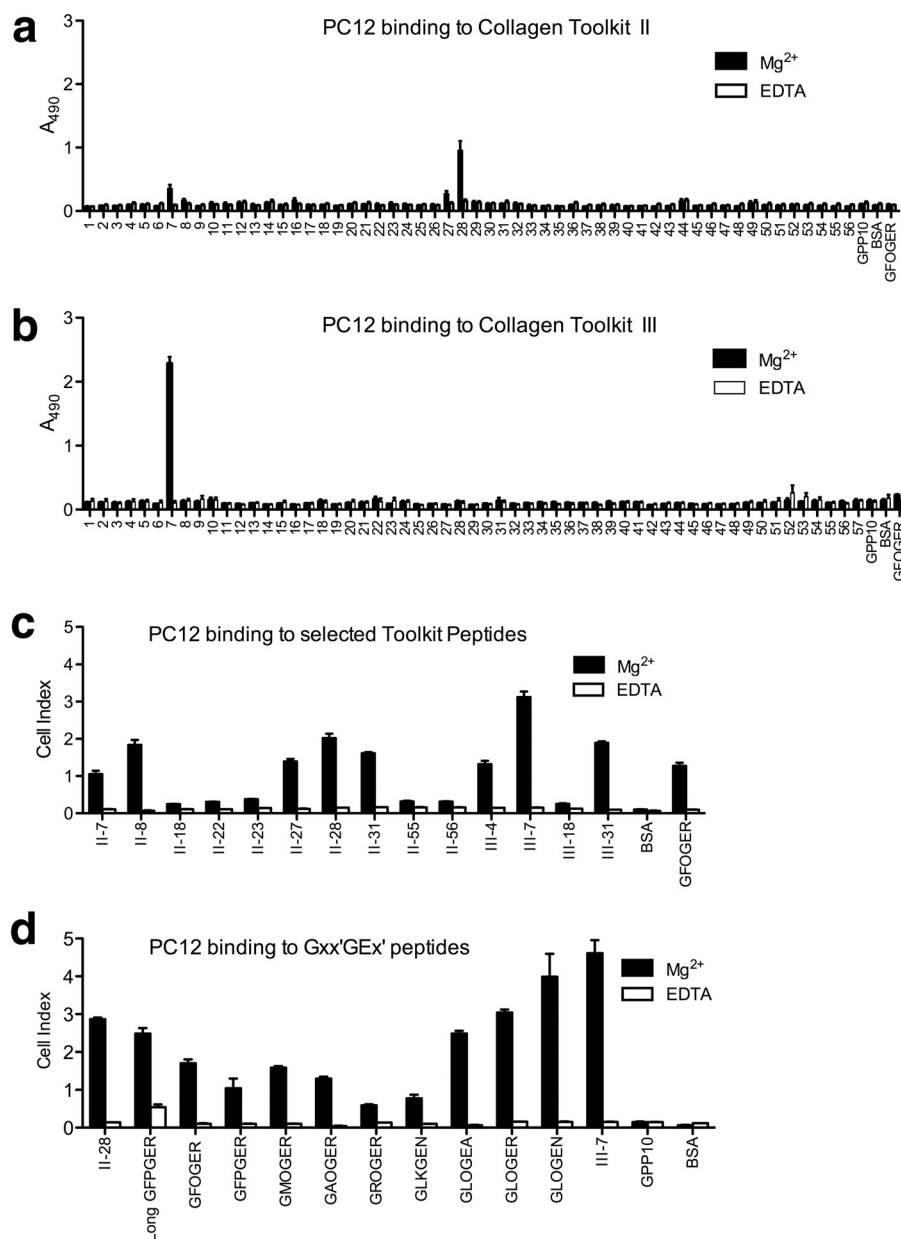


FIGURE 2. Binding of PC12 cells to Toolkits II and III and selected peptides. Immulon 2HB 96-well plates or E-Plates™ for the xCELLigence instrument were coated with the indicated peptides as described under “Experimental Procedures.” *a* and *b*, PC12 cells were seeded at 10^5 /well, and SPBA was performed after 1 h as described using the colorimetric lactate dehydrogenase assay kit. *c* and *d*, PC12 cells were seeded onto E-Plates at 2.5×10^4 cells/well, and the cell index was measured continually. PC12 cell binding to Toolkits II (*a*) and III (*b*) is shown. PC12 cell adhesion to the indicated Toolkit peptides (*c*) and to selected shorter integrin-binding peptides (*d*) is shown as end point data after 2 h of adhesion. All experiments were performed in triplicate in the presence of either 2 mM Mg^{2+} or EDTA as indicated. Data are the mean $\pm$ S.E. of three independent experiments.

titated using the impedance-based xCELLigence system (Roche Applied Science), which allows label-free dynamic monitoring of cell events in real time. The instrument expresses cell adhesion in cell index units, defined as $(R_n - R_b)/15$, where R_n is the impedance of the well when it contains cells, and R_b is the background impedance of the well containing medium alone.

Briefly, PC12 cells were seeded at 25,000 cells/well in a pre-coated E-Plate™ (Roche Applied Science) using the same conditions as described for SPBA. For antibody inhibition, cells were preincubated with the indicated amounts of anti- $\alpha 1$ I domain monoclonal antibody for 30 min and then added to collagen peptide-coated wells of E-Plates. The extent of cell

adhesion was measured as changes in impedance monitored every minute for 2 h, as we have used previously (45).

Competitive Binding of $\alpha 1$ and $\alpha 2$ I Domains to Peptide Coatings—The adsorption of recombinant wild-type I domains onto peptide- or collagen-coated surfaces was measured in the presence of varying concentrations of either GLOGEN or GFOGER using either conventional SPBA, as described above, or the SRU Biosystems BIND Explorer system. This biosensor quantitates the adsorption of biomolecules by measuring the resulting shift in peak wavelength of light reflected from the base of a well. The shift in peak wavelength value (Δ WPV) is directly proportional to the mass of the molecule bound. The

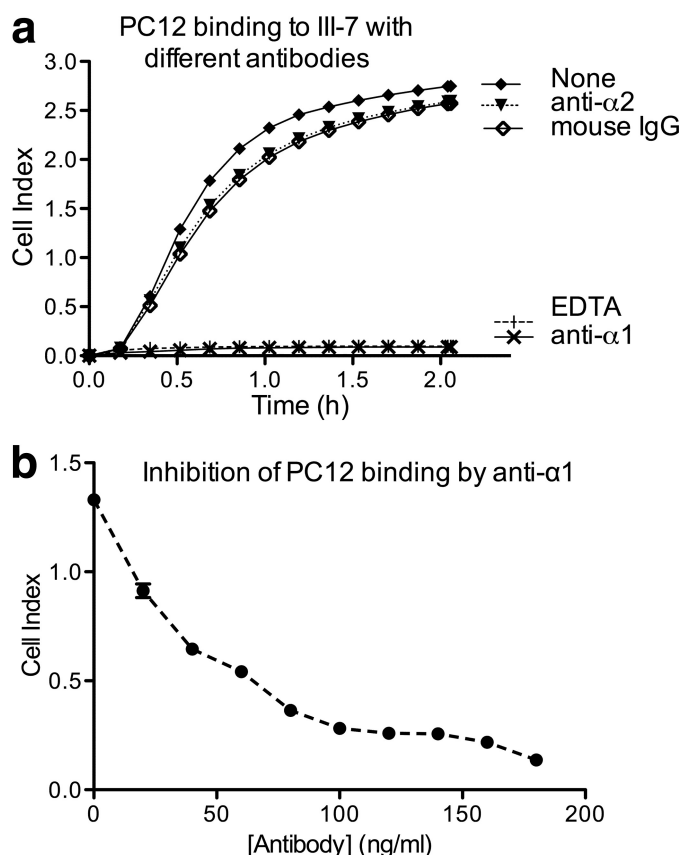


FIGURE 3. Effect of anti-integrin antibodies on PC12 cell binding to peptide III-7. Cells were incubated with anti- $\alpha 1$ or anti- $\alpha 2$ antibodies or controls as indicated for 30 min. Mg^{2+} -dependent label-free binding of PC12 cells was then measured as described under "Experimental Procedures" and in the legend to Fig. 2 (c and d). *a*, time course of adhesion measured at 10-min intervals over 2 h expressed as the mean cell index $\pm$ S.E. of three experiments. *b*, inhibition of PC12 cell adhesion to peptide III-7 in the presence of increasing levels of anti- $\alpha 1$ antibody shown as end point determinations after 2 h. Data are the mean cell index $\pm$ S.E. of five independent experiments. Where not visible, errors lie within the dimensions of the symbols.

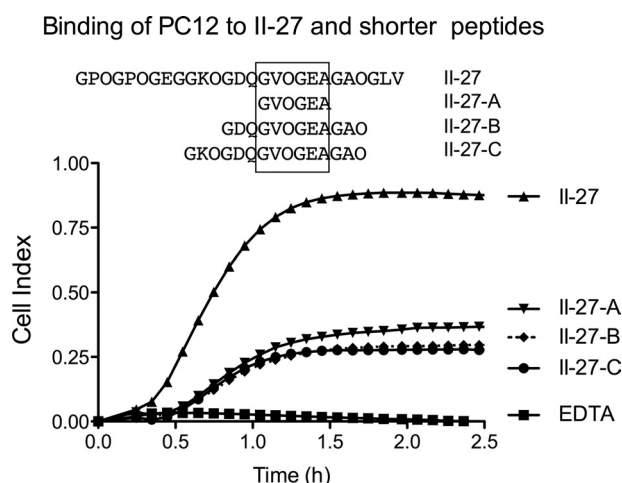


FIGURE 4. Real-time binding of PC12 cells to II-27 and shorter II-27-derived peptides. E-Plate™ wells were coated with the indicated peptides as described in the legend to Fig. 2 (c and d). The sequences of the peptides are shown above the graph. Cell adhesion was monitored for 2 h, and data were collected every minute. Every fifth data point is shown. Experiments were performed in triplicate in the presence of either 2 mM Mg^{2+} or EDTA as indicated, and the mean cell index $\pm$ S.E. is shown from four independent experiments.

96-well TiO Bind biosensor plate was coated with peptide (GLOGEN, GFOGER, or GVOGEA; 50 μ l at 20 μ g/ml in acetate buffer (pH 5.0)). After coating, which was monitored until saturation, the surface was blocked with BSA (2 mg/ml) and then equilibrated in 50 mM Tris-HCl and 140 mM NaCl (pH 7.4) supplemented with 2 mM $MgCl_2$ and 0.5 mg/ml BSA. For the binding step, a fixed concentration of I domain (0.17 μ M) was preincubated in solution with a varying amount of GFOGER or GLOGEN for 45 min and then added to the coated sensor, and the binding of the remaining free I domain was measured as Δ PWV. To estimate binding kinetics, peptide coatings were prepared as described above, increasing amounts of I domain (0–60 μ M) were added, and Δ PWV was measured at saturation. Binding curves were analyzed with GraphPad Prism 5.0d using least-squares fit and constraining B_{max} to the same value for each peptide.

RESULTS

Functional Analysis of $\alpha 1$ I Domain by SPBA—The ligand-binding activity of the recombinant human GST- $\alpha 1$ I domain expressed in bacteria was examined using a conventional colorimetric 96-well SPBA. The I domain was applied to the Collagen II and III Toolkit peptides in the presence or absence of magnesium and detected using anti-GST antibody, and data are shown in Fig. 1. The background response is indicated by wells coated with GPP10 or BSA. A peptide containing the GFOGER motif was used as a positive control in these and all other experiments. The $\alpha 1$ I domain bound in a Mg^{2+} -dependent manner to the Toolkit peptides in the rank order II-28, II-7, II-8 = II-18 = II-22, II-55 = II-23 (Fig. 1a) and bound to III-7 and III-4 and weakly to III-31, III-18, and III-8 (Fig. 1b). Most of the reactive peptides contain a previously identified integrin-specific motif: GFOGER in II-28, GLOGER in II-7 and II-8, GAOGER in II-18, and GROGER in III-4. GLKGEN and GLOGEN are the two motifs found in peptide III-7, the best binder in Toolkit III, which supports better adhesion to the $\alpha 1$ I domain compared with GFOGER (Fig. 1b).

Binding of $\alpha 1$ I Domain to Short Triple-helical Motifs—To determine the potential adhesiveness of each of the GLKGEN and GLOGEN motifs, two short triple-helical peptides were tested alongside other known $Gxx'GEx''$ -containing peptides and III-7. Fig. 1c shows the results, which indicate that binding of the $\alpha 1$ I domain to III-7 is mediated by its GLOGEN motif only. This peptide supported more adhesion than any other motif tested, including GFOGER and GLOGER. In addition, the $\alpha 1$ I domain bound weakly to GLOGEA, GFPGER, GMOGER, and GROGER, all high or moderate affinity motifs for the $\alpha 2$ I domain (39, 46), but not to GAOGER, GLKGEN, or GFOGDR. For comparison, collagens were used in these experiments, and collagen IV proved to be the best substrate, with collagens I, II, and III supporting good adhesion, and collagen V, which lacks GLOGEN or other high affinity motifs, being only slightly more adhesive than negative controls.

Binding of PC12 Cells to Toolkits II and III—The PC12 cell line expresses $\alpha 1\beta 1$ as its sole collagen-binding integrin (47, 48) and was therefore used to investigate the binding of $\alpha 1\beta 1$ to collagen in a cellular context. Adhesion to Toolkit II and III peptides was performed first using SPBA, with lactate dehydro-

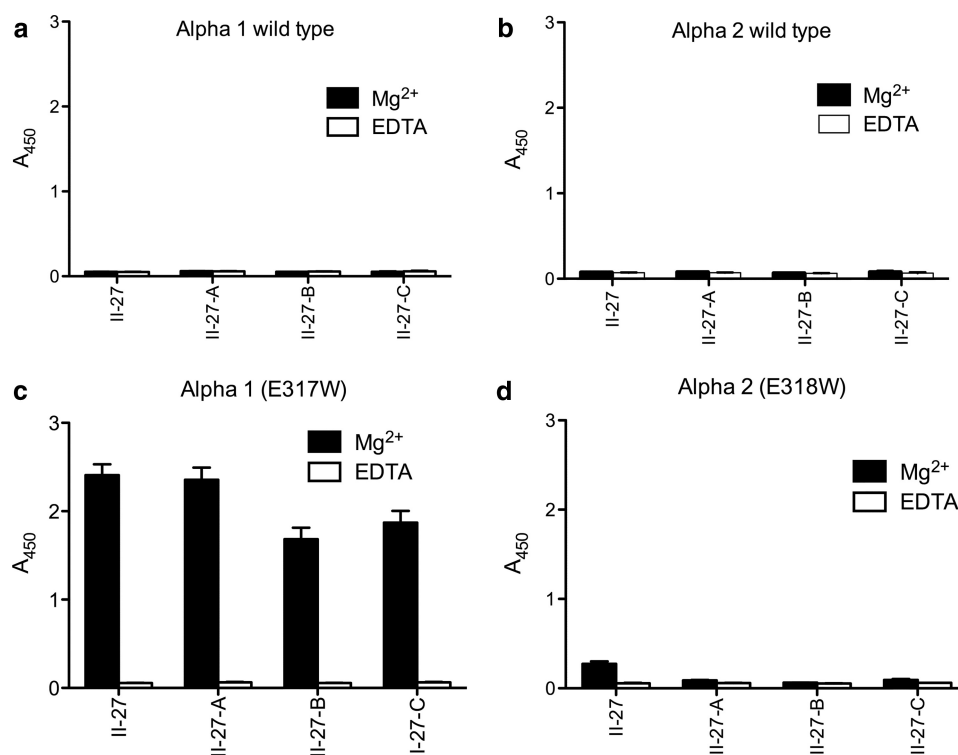


FIGURE 5. Binding of wild-type and active $\alpha 1$ and $\alpha 2$ I domains to peptide II-27. Experiments were performed as described in the legend to Fig. 1 using 0.1 μ g of protein/well. Solid-phase adhesion of I domains to peptide II-27 or its shorter derivatives (II-27-A, II-27-B, and II-27-C) is shown. *a*, adhesion of the wild-type $\alpha 1$ I domain. *b*, adhesion of the wild-type $\alpha 2$ I domain. *c*, adhesion of the constitutively active E317W $\alpha 1$ I domain. *d*, adhesion of the constitutively active E318W $\alpha 2$ I domain. Data are the mean $\pm$ S.E. of four independent experiments, each performed in triplicate.

genase activity reporting the presence of adherent cells as described (45). PC12 cells bound in a Mg^{2+} -dependent manner to peptide II-28 and less well to peptides II-7 and II-27 (Fig. 2*a*). Tested in matched experiments with Toolkit III, PC12 cells showed very strong binding to peptide III-7 (Fig. 2*b*).

Second, we used the impedance-based xCELLigence system, which allows sensitive label-free real-time monitoring of cell adhesion (49, 50), to investigate selected established and potential integrin-binding Toolkit peptides. In rank order, III-7 (containing GLOGEN) showed the highest level of binding to PC12 cells, followed by II-28 (containing GFOGER), III-31, II-8, II-31, II-27, III-4, and II-7 (Fig. 2*c*). Using the short peptide set together with III-7 and II-28 as controls, III-7 supported the highest binding, followed by GLOGEN, GLOGER, II-28, and GLOGEA. Moderate binding to GFOGER, GMOGER, and GAOGER was observed, whereas GLKGEN (also present in III-7 alongside GLOGEN) and GROGER showed only slight activity. GFPGER, presented as a short peptide within (GPP)₅ hosts, supported weak binding (lower compared with GFOGER), whereas in the context of more primary sequence (long GFPGER), it was almost as active as its hydroxylated equivalent, II-28 (Fig. 2*d*). It should be noted that this real-time technique includes no physical perturbation such as wash steps and reports cell adhesion with great sensitivity. In these experiments, particular motifs tended to show greater adhesion when presented in the context of longer stretches of native sequence, as in Toolkit peptides, and the recombinant I domain bound to a wider range of peptides than the PC12 cells. This applied to GFPGER (supplemental Fig. 1), as well as to GFOGER.

Inhibition of PC12 Cell Binding to III-7 Using Anti- $\alpha 1$ Antibody—Adhesion of PC12 cells to peptide III-7, detected using the xCELLigence platform, was inhibited to a similar level upon EDTA treatment or preincubation of cells with anti- $\alpha 1$ inhibitory antibody, but not with inhibitory anti- $\alpha 2$ antibody or control IgG, each used at 10 μ g/ml (Fig. 3). Fig. 3*a* shows that preincubation with anti- $\alpha 2$ antibody or mouse IgG did not affect the time dependence of cell binding to peptide III-7 and Fig. 3*b* that anti- $\alpha 1$ inhibitory antibody showed specific dose-dependent inhibition of PC12 cell binding to peptide III-7, with $IC_{50} \sim 40$ ng/ml and near-complete blockade at 180 ng/ml.

Peptide II-27 and GVOGEA-binding Motif—Peptide II-27 bound PC12 cells moderately well using real-time assay (Fig. 2*c*) but contains no known integrin-binding site. However, a candidate sequence is GVOGEA, conforming to the general GxOGEx" motif. To assess the role of GVOGEA, three peptides containing GVOGEA within sequence derived from II-27 (to provide a larger local context) were synthesized within the host peptide backbone, (GPP)₅, and named II-27-A, II-27-B, and II-27-C (Fig. 4). PC12 cells bound to peptides II-27-A, II-27-B, and II-27-C to $\sim 40\%$ of the level attained with II-27, whereas no adhesion was observed in the presence of EDTA. In contrast, the wild-type $\alpha 1$ and $\alpha 2$ I domains did not bind to peptide II-27 or its short derivatives, II-27-A, II-27-B, and II-27-C (Fig. 5, *a* and *b*).

Active Forms of Integrin $\alpha 1$ Bind to GVOGEA—To investigate the sequence GVOGEA further, we used constitutively active constructs of the $\alpha 1$ I domain (E317W) (51) and of the $\alpha 2$ I domain (E318W) (52). E317W but not E318W bound strongly

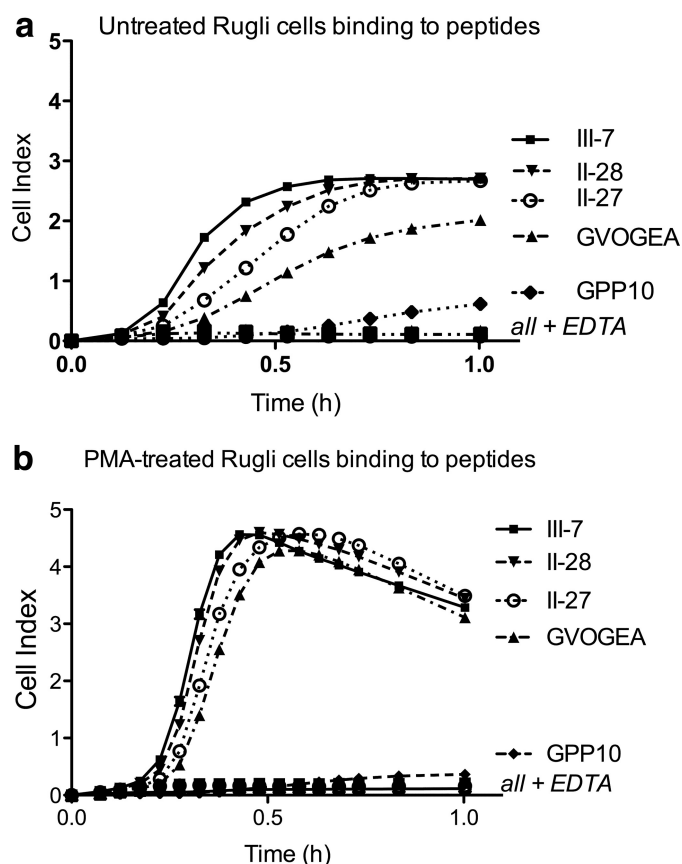


FIGURE 6. Binding of Rugli cells to selected Toolkit peptides before and after activation by PMA. Rugli cells were applied to peptide-coated E-Plates™ prepared as described under “Experimental Procedures,” and the cell index was recorded at 1-min intervals. For clarity, fewer time points are shown in each graph. The mean cell index $\pm$ S.D. is shown from one representative experiment, performed in triplicate, from three similar data sets. The peptides used are indicated. Traces close to the base line include adhesion to the GPP10 control and to the indicated peptides in the presence of EDTA. *a*, adhesion of control Rugli cells. *b*, adhesion of Rugli cells activated, in parallel with controls, for 30 min with 1 μ M PMA.

to II-27 and to each of its short derivatives (Fig. 5, *c* and *d*). Although substitution of Mg^{2+} with Mn^{2+} , widely used to enhance integrin-binding activity, increased PC12 cell binding to II-27 (65%), GFOGER (40%), and III-7 (26%), measured with real-time binding assay, it had little effect (13%) on PC12 cell adhesion to GVOGEA (data not shown). However, preincubation of another $\alpha 1 \beta 1$ -expressing cell line, the rat glioma Rugli cell, with 1 μ M phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, raised its adhesion to GVOGEA to the same level as to the parent peptide, II-27 (Fig. 6, *a* and *b*). To highlight the selectivity of particular motifs for different integrins, we used the mouse myoblast C2C12 cell line in its parental form, lacking collagen-binding integrins (44), and its derivatives, C2C12- $\alpha 2^+$ (expressing human $\alpha 2 \beta 1$) and C2C12- $\alpha 11^+$ (expressing human $\alpha 11 \beta 1$). None of these cells showed any specific binding to II-27 or GVOGEA (or to II-27-B and II-27-C; data not shown) but showed better binding to II-28 than to III-7. In contrast, PC12 cells bound most strongly to III-7 and then to II-27 and II-28 (Fig. 7). Note that the parental C2C12 cells and transfected counterparts bound more to the control peptide (GPP10) than to II-27 or GVOGEA.

Inhibition of $\alpha 1$ I Domain Binding to Collagen IV by GLOGEN— $\alpha 1 \beta 1$ is known to have high affinity for collagen IV (30). To evaluate the relative affinity of $\alpha 1 \beta 1$ for GLOGEN and GFOGER, we used peptide to compete for the $\alpha 1$ I domain from collagen IV, measured using SPBA. The $\alpha 1$ I domain was preincubated with increasing doses of either GLOGEN or the control peptide (GPP10) before addition to collagen IV-coated plates. Fig. 8*a* shows the specific inhibition of $\alpha 1$ binding to collagen IV by the peptide, with $IC_{50} = 2.8 \mu M$ (30 $\mu g/ml$). For comparison, GFOGER showed a lesser ability ($IC_{50} \sim 90 \mu M$, $\sim 1000 \mu g/ml$) to compete with collagen IV for the $\alpha 1$ I domain.

Using the SRU Biosystems Explorer platform, we measured the affinity of the wild-type $\alpha 1$ I domain for immobilized peptides (Fig. 8*b*). Saturation was observed for GLOGEN ($K_d = 13 \pm 1.9 \mu M$), but adsorption to GFOGER and GVOGEA was not saturated at a protein concentration up to 60 μM . Estimates of K_d of 53 ± 6 and $140 \pm 37 \mu M$ were obtained for GFOGER and GVOGEA, respectively. All values differed ($p < 0.0001$). In a competitive binding experiment, GLOGEN proved readily able to displace the $\alpha 1$ I domain from coatings of GFOGER or of itself (supplemental Fig. 1, *a* and *b*), whereas GFOGER was inactive at up to 40 $\mu g/ml$. Conversely, GFOGER in solution was able to displace the $\alpha 2$ I domain from GFOGER coatings and was a potent competitor of $\alpha 2$ I domain binding to GLOGEN. GLOGEN had little effect on $\alpha 2$ I domain binding to GFOGER while being able to displace the $\alpha 2$ I domain from itself (supplemental Fig. 1, *c* and *d*).

DISCUSSION

We set out to identify sites within the fibrillar collagens that might bind the resting forms of $\alpha 1 \beta 1$ on the cell surface and be selective for integrin $\alpha 1 \beta 1$ over $\alpha 2 \beta 1$. To this end, we applied recombinant I domains and suitable cells to the Collagen Toolkits and to selected shorter peptides. The use of the recombinant $\alpha 1$ I domain confirmed its ability to bind the expected Toolkit peptides II-28, II-7, II-8, and II-18, which contain known integrin-binding motifs GFOGER, GLOGER, and GAOGER. The $\alpha 1$ I domain also showed moderate reactivity with II-22, II-23, and II-55, all of which contain at least one candidate glutamate residue, but we have not pursued these further. Toolkit III contains just two peptides that bound the $\alpha 1$ I domain strongly, III-4 and III-7, containing GROGER and GLOGEN, respectively, already known as integrin-recognizing motifs (39, 46). We have shown here that GLKGEN, also present in III-7, has only slight integrin-binding activity. Comparing all of these binding motifs suggested that GLOGEN, in the setting of Toolkit peptide III-7 or alone within the (GPP)₅ hosts, has the greatest ability to bind the $\alpha 1$ I domain, with GLOGER being of similar affinity and GLOGEA binding weakly.

The recently introduced xCELLigence label-free detection system measures cell adhesion with no washing step, so weaker interactions can be revealed. The method yielded data confirming the ability of PC12 cells to bind the higher affinity Toolkit peptides, III-7, II-7, and II-28 but revealed substantial activity in II-8, II-27, II-31, III-4, and III-31 (all GxOGEx"-containing peptides), which was less obvious from SPBA.

Peptide II-27 proved to be a moderate ligand for $\alpha 1 \beta 1$ in PC12 and Rugli cells as determined using either SPBA or xCEL-

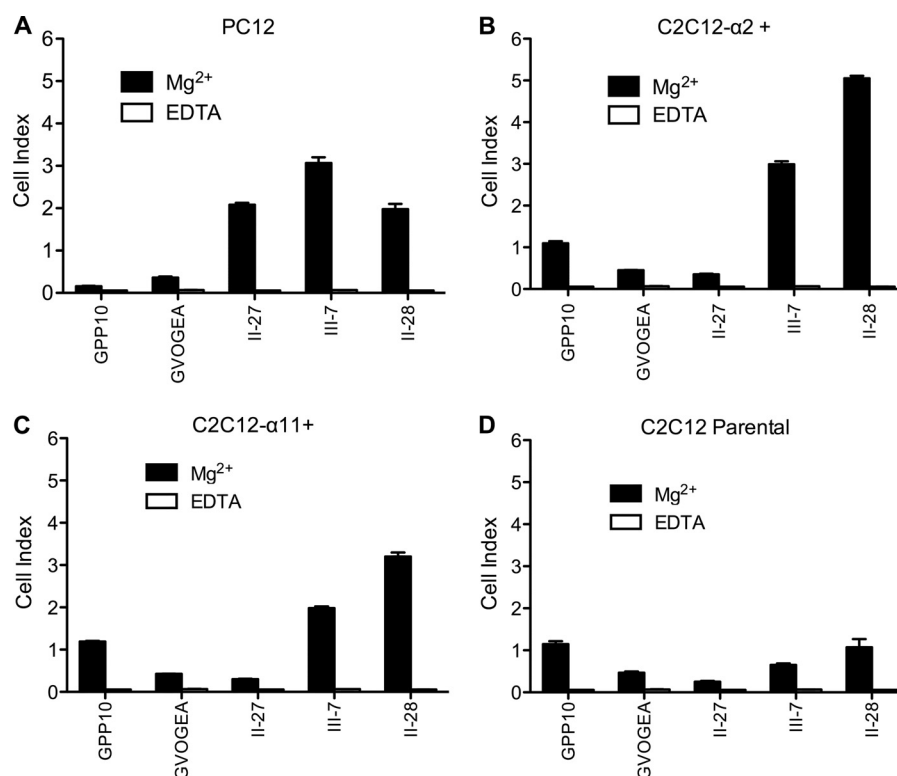


FIGURE 7. Real-time adhesion of PC12, C2C12- $\alpha 2^+$, and C2C12- $\alpha 11^+$ cells to selected peptides. The binding of integrin-expressing cells (25,000 cells/well) was measured using real-time adhesion assay as described under "Experimental Procedures" and in the legend to Fig. 2. Peptides III-7, II-27, II-28, and GVOGEA were used as adhesive coatings, with GPP10 as the control. The cell line and integrin expressed are indicated above each panel, which shows the mean cell index after $2 \pm$ S.E. from triplicate determinations from each of three experiments.

Ligence. It contains two candidate glutamate-containing motifs, GPOGEG and GVOGEA, in its sequence (GPOGPOGEGGKOGDQGVOGEAGAOGV). GPOGEG was discounted because it also occurs in the inactive II-26 peptide. We synthesized the shorter peptides GVOGEA, GDQGVGEAGAO, and GKOGDQGVOGEAGAO, all within GPP hosts. Each supported PC12 cell adhesion (measured using label-free detection) but at about one-third of the level supported by II-27 itself.

$\alpha 1\beta 1$ has been shown to bind a sequence in collagen IV (31) containing an aspartate residue (Asp-441 in the $\alpha 1$ chain) considered to coordinate its MIDAS metal ion. This is consistent with the flatter surface of the $\alpha 1$ I domain compared with that of $\alpha 2$, which requires the longer glutamate side chain to reach the bound metal ion (7). However, because the binding of PC12 cells to GKOGDQGVOGEAGAO was no greater than to GVOGEA, we find no evidence that GKOGDQ can serve as an integrin recognition site, despite its similarity to GPOGDQ, which forms part of the $\alpha 1\beta 1$ -binding locus in collagen $\alpha 1(\text{IV})$. Docking studies suggest that Lys-456 in the corresponding GAKGRA sequence of collagen $\alpha 2(\text{IV})$ is essential, forming a salt bridge with the I domain surface (32). Consistent with this role for the residues surrounding the carboxylate anion of the ligand, homotrimeric GFOGDR proved inactive, suggesting that aspartate, even within an integrin-reactive motif, cannot in isolation substitute for glutamate. Investigating GVOGEA further, we found that the recombinant wild-type $\alpha 1$ I domain was unable to bind any GVOGEA-containing triple-helical peptide as measured using SPBA. Accordingly, we treated Rugli cells with PMA and found that their moderate affinity for the shorter

GVOGEA-containing peptides increased to that for the parent peptide, II-27, confirming GVOGEA as a good ligand for $\alpha 1\beta 1$ after activation of the integrin. Of note, treatment with PMA increased both the rate of onset of adhesion and the peak cell index (Fig. 6b). C2C12 cells stably expressing either $\alpha 2\beta 1$ or $\alpha 11\beta 1$ did not bind GVOGEA or its parent peptide (II-27), providing further evidence of $\alpha 1\beta 1$ specificity.

GVOGEA in collagen II is unique among the human collagens, but two other related motifs, GVOGER (found in collagens $\alpha 2(\text{VIII})$ and $\alpha 1(\text{XXVIII})$) and GVOGEK (in collagen $\alpha 2(\text{XI})$) are promising ligands for $\alpha 1\beta 1$ and, given the presence of the positively charged Arg or Lys residues, should be investigated in the context of other collagen-binding integrins. GVOGDL, occupying in collagen $\alpha 1(\text{I})$ the same position as GVOGER in collagen $\alpha 1(\text{II})$, is also worthy of investigation.

Taking all of our data together, we conclude that GLOGEN, the first Gxx'GEN motif identified as an integrin ligand (39), is the most potent ligand for $\alpha 1\beta 1$ discovered among the fibrillar collagen sequences to date. The exact motif occurs only in collagens III and XXII. Collagen III may form heterotypic fibrils with collagen I in skin and other tissues, and collagen XXII is a FACIT collagen (53). Both may therefore enhance the $\alpha 1\beta 1$ reactivity of collagen fibers. Of other such motifs, GSOGEN occurs in collagen II (Toolkit peptides II-6 and II-7) but displays little activity. GSOGEN is conserved at the same locus in collagen $\alpha 1(\text{I})$, aligning with GAOGEN in the $\alpha 2(\text{I})$ chain. In general, where $x = \text{A}$, Gxx'GEx'' motifs are poor integrin ligands (37, 38), consistent with the preference for long hydrophobic residues in this position that support extended contact with the I domain

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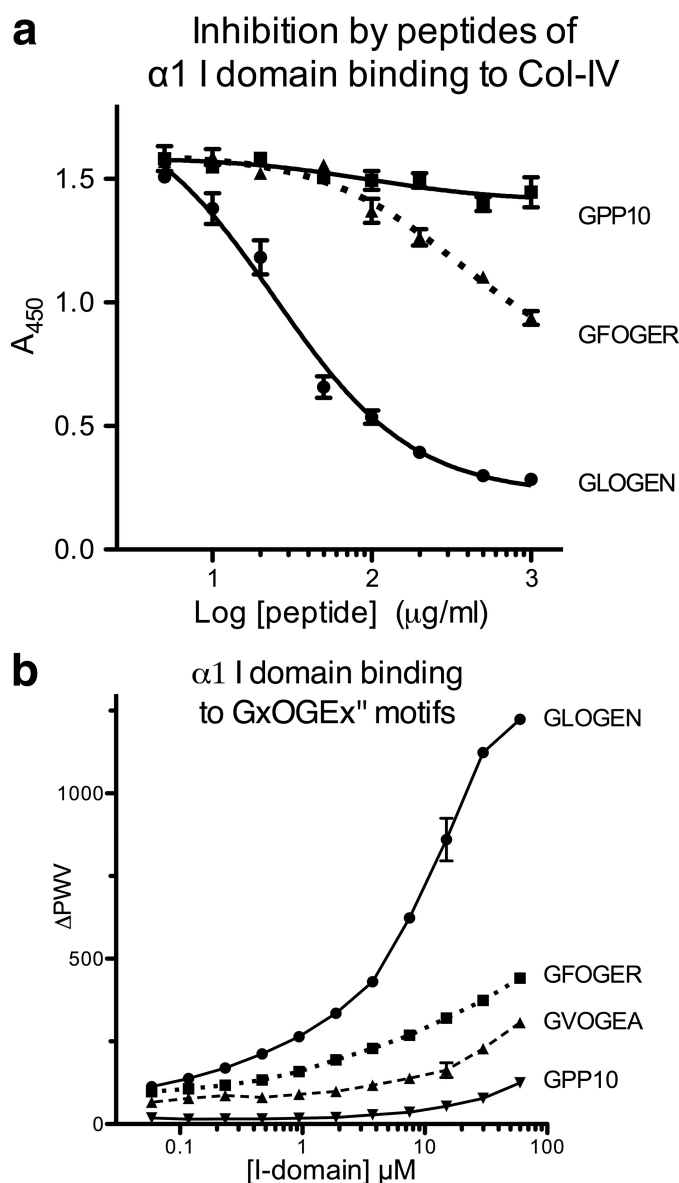


FIGURE 8. Equilibrium binding of $\alpha 1$ I domain to collagenous substrates. *a*, inhibition of binding of the wild-type $\alpha 1$ I domain to collagen IV (Col-IV) was determined as described in the legend to Fig. 1 in the presence of peptides. The I domain was preincubated with the indicated amount of GLOGEN, GFOGER, and GPP10. Data show the mean $\pm$ S.E. of three experiments. *b*, adhesion of the wild-type $\alpha 1$ I domain to GLOGEN, GFOGER, GVOGEA, and GPP10 was measured using the SRU Biosystems BIND Explorer as described under "Experimental Procedures", allowing K_D to be estimated. Δ PPWV, shift in peak wavelength value.

surface. Recently, a related but unnatural motif (GFPGEN) was expressed within *Streptococcus pyogenes* collagen-like molecules and found to be a selective ligand for $\alpha 1\beta 1$ (40). Like GLOGEN, GFPGEN showed higher affinity for the $\alpha 1$ I domain compared with GFPGER, which we found previously to be a poor ligand for $\alpha 1\beta 1$ -expressing Rugli cells (54).

Finally, using competition assays, we have shown that the triple-helical peptide GLOGEN in solution is able to displace the $\alpha 1$ I domain from immobilized collagen IV with much higher affinity ($IC_{50} \sim 2.8 \mu M$) compared with GFOGER ($IC_{50} \sim 90 \mu M$). These data support the selective nature of the two integrins for our peptide ligands, as well as for the native collagens.

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**Glycobiology and Extracellular Matrices:
Mapping of Potent and Specific Binding
Motifs, GLOGEN and GVOGEA, for
Integrin $\alpha 1\beta 1$ Using Collagen Toolkits II
and III**



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