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A Streptococcal Collagen-like Protein Interacts with the $\alpha_2\beta_1$ Integrin and Induces Intracellular Signaling*

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The streptococcal collagen-like proteins Scl1 and Scl2 are prokaryotic members of a large protein family with domains containing the repeating amino acid sequence (Gly-Xaa-Yaa)$_n$ that form a collagen-like triple-helical structure. Here, we test the hypothesis that Scl variant might interact with mammalian collagen-binding integrins. We show that the recombinant Scl protein p176 promotes adhesion and spreading of human lung fibroblast cells through an $\alpha_2\beta_1$ integrin-mediated interaction as shown in cell adhesion inhibition assays using anti-$\alpha_2\beta_1$ and anti-$\beta_1$ integrins monoclonal antibodies. Accordingly, C2C12 cells stably expressing $\alpha_2\beta_1$ integrin as the only collagen-binding integrin show productive cell adhesion activities on p176 that can be blocked by an anti-$\alpha_2\beta_1$ integrin antibody. In addition, p176 promotes tyrosine phosphorylation of p125FAK of C2C12 cells expressing $\alpha_2\beta_1$ integrin, whereas parental C2C12 cells do not. Furthermore, adhesion of human lung fibroblast cells to p176 induces phosphorylation of p125FAK, p130CAS, and p68Paxillin proteins. In a domain swapping experiment, we show that integrin binds to the collagenous domain of the Scl protein. Moreover, the recombinant inserted domain of the $\alpha_2$ integrin interacts with p176 with a relatively high affinity ($K_D = 17$ nM). Attempts to identify the integrin sites in p176 suggest that more than one site may be involved. These studies, for the first time, suggest that the collagen-like proteins of prokaryotes retain not only structural but also functional characteristics of their eukaryotic counterparts.

The collagens are a family of extracellular matrix (ECM)$^1$ proteins that provide structural support to all multicellular animals (1). The repeating sequence Gly-Xaa-Yaa (GXY), where $X$ is often proline and $Y$ is often hydroxyproline, is a unique feature of the collagen polypeptides (2–4). Long tracks of repeated GXY sequences fold into left-handed proline polypeptide type II-like chains, and three such chains cooperatively twist around a central axis to form a right-handed rope-like superhelix (2, 5–7), considered a defining feature of collagens. The collagens also contain C- and N-terminal noncollagenous domains, which often are proteolyzed during secretion from the cells (7–9). Mature collagen molecules are deposited in the ECM in the form of fibers, networks, and beaded filaments (8). One group of mammalian proteins that fulfill rudimentary host defense functions such as the complement factor C1q (9) and several mammalian lectins (10) also have collagenous domains, but they are not conventional collagens. These proteins form characteristic lollipop-like structures, where the collagenous domains form the stalks, and globular heads correspond to the noncollagenous regions. Collagen-like molecules also have been found in lower eukaryotes such as mussels, worms, and sponges (11), and collagen-like sequences have been identified from analyses of the genomes of prokaryotes (12–15).

The streptococcal collagen-like proteins Scl1 and Scl2 (also known as ScIA and ScIB) are the best characterized members of the prokaryotic family of collagen-like proteins (16–20). These two related proteins contain long segments of repeated GXY sequences and are located on the cell surface of the human bacterial pathogen Streptococcus pyogenes (group A Streptococcus). The Scl1 and Scl2 proteins have similar organization with four common regions (16). The N-terminal signal sequence and C-terminal cell wall associated regions are conserved between Scl1 and Scl2, whereas the variable (V) and the collagen-like (CL) regions differ significantly in length and primary sequence. In addition, Scl1, but not Scl2, contains a linker region between the CL and the cell wall regions, which is composed of highly conserved tandem repeats. It was previously shown that at least some ScI variants can form collagen-like triple helices, despite the lack of hydroxyprolines (21).

The collagens can act as cell adhesion substrates, induce a reorganization of the cytoskeleton, and promote cellular contractility and motility by their ability to interact with integrins (22–24). Integrins are large glycoproteins expressed on the cell surface as $\alpha\beta$ heterodimers, capable of interacting with ECM proteins in a metal ion-dependent manner (22–28). There are 18 distinct $\alpha$ subunits and 8 $\beta$ subunits in mammals that

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The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; CAS, Crk-associated substrate; JNK, c-Jun N-terminal kinase; FN, Fibronectin; Col I, type I collagen; Col IV, type IV collagen; V, variable; CL, collagen-like; I, inserted; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; SPR, surface plasmon resonance.
combine to form 24 known heterodimers. Recent studies demonstrated that the so-called inserted (I) domains of the α sub-units of α5β1, α6β1, α5β1I, and α1β1I integrins mediate the interactions of these ECM receptors with collagens (29–32) and that the sequence GF/LOGER (O; hydroxyproline) that occurs in a subset of mammalian collagens interacts with I domains of the integrins in the presence of the Ca"2+ and Mg"2+ metal ions (32–35). A crystal structure of the α5 I domain in complex with a collagen peptide has also been reported (36). Integrin-mediated adhesion of cells to ECM substrates triggers intracellular signaling cascades. A general mechanism for integrin signaling has been established; for example, integrin ligand induces phosphorylation of several cytoskeletal and signaling molecules including FAK, PYK2, p72SYK, ILK-1, CAS, paxillin, SRC/FYN, and Shc (37–41). Furthermore, signaling events mediated by these molecules regulate a vast array of cell biological processes including cell migration, proliferation, and differentiation (37–41).

The studies described herein show that a member of the prokaryotic collagen-like proteins can interact with the α2β1 integrin, presumably through the I domain of the α2 subunit. Importantly, this interaction promotes cell adhesion and intracellular signaling, much like eukaryotic collagenases. These studies, for the first time, show that collagen-like structure is functionally conserved in evolutionary distant organisms such as bacteria and humans.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins**—Recombinant proteins were subjected to the Strep-tag II expression and purification system (IBA-GmbH, Goettingen, Germany). The Scl2.28-based pSL163 construct encoding p176 polypeptide has been reported previously (21). To prepare scl1.41-based pSL176 construct encoding recombinant p176 polypeptide, the DNA fragment of the scl1.41 allele (accession number AY452037) was amplified and cloned into an Escherichia coli vector pASK-IBA2 (42). The pSL180 construct encodes p180 polypeptide, containing a glutamate to alanine (E→A) substitution within the GLPGER motif of p176 protein. Single-stranded DNA template of pSL176 harbored in E. coli CJ236 was prepared employing ori F plasmid in the expression vector pASK-IBA2 as described. Oligonucleotide containing new ApaLI restriction site (5′-CCATGGTCTAGCTGCGTGGCGAGG-3′) was phosphorylated, annealed, and extended in vitro using T7 DNA Polymerase (New England Biolabs, Beverly, MA). To identify mutants, the PCR products amplified directly from the E. coli colonies were digested by ApaLI restriction enzyme. Consequently, plasmid pSL163 was used as a core sequence. To prepare pSL181 construct encoding p181 polypeptide, DNA fragment encoding the V region of p176 was PCR-amplified and replaced into the corresponding V region of pSL163. Next, the DNA sequence of the CL region in pSL181 was replaced with a CL region sequence of pSL176. The resulting pSL182 construct contains the V and CL sequence of pSL176 but lacks its linker region. The constructs were subjected to DNA sequencing for correctness. Recombinant proteins were purified by Strep-Tactin Sepharose affinity column, and their identity was confirmed by N-terminal sequencing.

**Rotary Shadowing and Electron Microscopy**—The structural organization of the recombinant proteins was viewed by electron microscopy of the rotary-shadowed rScls, as previously described. Protein samples (100 μg/ml) were dialyzed against 0.1 M ammonium bicarbonate and then mixed with glycerol to a final concentration of 70% (v/v). The samples were atomized onto freshly cleaved mica sheets using an air brush and rotary-shadowed with platinum at an angle of 6 degrees. The replicas were backed with carbon at 90 degrees in a Balzers BAE250 vacuum evaporator. The replicas were floated from the mica in distilled water and placed on copper grids. Photomicrographs were taken with a Philips electron microscope at 80 KV, and a domain binding to p176 was performed as described earlier. Shortly, recombinant proteins were dialyzed overnight at 4 °C against 0.1 M (NH4)2CO3, 1 mM MgCl2 and mixed at the α2-Lp176 (trimer) molar ratio of 4.1:1 in the same buffer. Incubation was carried out at room temperature for 4 h. After that, the sample was mixed with glycerol and rotary-shadowed as described above.

**Circular dichroism (CD) Spectroscopy**—The triple helical conforma-
(MRC-5), parental C2C12, and C2C12-α2+ cells were washed in PBS, detached with in 2 mM EDTA, and washed. The cells were passed through a cell strainer and enumerated. Approximately 0.1 × 10^6 cells were incubated with 1, 5, and 10 μg/ml of anti-α1, anti-α2, anti-α3, anti-α5, anti-β1, and anti-β2 integrin monoclonal antibodies on ice for 30 min. The cells were washed and resuspended in defined medium. The dishes were precoated with 100 nM (considered optimal concentration; no further increase in cell adhesion was observed beyond 100 nM of p176 in prior experiments) p176 and subjected to cell adhesion assays at 37 °C in a CO2 incubator. The use of single dose (10 μl) p176 and subjected to cell adhesion assays at 37 °C in a CO2 incubator. After 30 or 60 min the cells were detached, and maintained in suspension for 45 min at room temperature. The cells were then replated onto dishes coated with indicated substrates at 37 °C in a CO2 incubator. After 30 or 60 min the cells were washed with cold PBS, pH 7.4, and solubilized in cell extraction buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 mM sodium fluoride, 1 mM sodium pyrophosphate, 2 mM sodium orthovanadate, with various protease inhibitors freshly added) for 30 min on ice. Extracts were centrifuged at 21,000 × g for 30 min at 4 °C to remove insoluble material. Protein concentrations of the resulting lysates were determined by the Bio-Rad DC protein assay. For immunoprecipitation analyses, the cell lysates were precleared with mouse IgG-agarose beads at 4 °C for 1 h. Immunoprecipitation, immunoblotting, and de-
variants interact with the collagen-binding integrins of the human cells? To answer these questions, several recombinant Scl proteins were constructed and examined in solid phase integrin binding assays. Recombinant protein p176 derived from the Scl1.41 variant consistently displayed integrin binding activities, whereas the previously characterized (21) recombinant protein p163 was inactive. The p176 variant contains a putative GLPGER cell adhesion motif within its collagenous domain. Analogous sequence motifs GLFGGER (O; hydroxyproline) have been identified in human collagens as binding sites for various collagen-binding integrins (32, 33, 44).

Because triple helix formation presumably is a prerequisite for a collagen-like biological function of the Scl proteins, the structure of each rScl construct was first characterized by CD spectroscopy and electron microscopy. Both rScls were affinity purified to apparent homogeneity, as seen by the presence of single bands on SDS-PAGE (Fig. 1A, right panel). The integrity of p163 and p176 preparations was determined by SDS-PAGE and Western immunoblotting with specific rabbit polyclonal antibodies. p163 was detected with the affinity-purified antibody raised against synthetic peptide corresponding to amino acid residues located in the N-terminal V region of the protein as described previously (17). The p176 protein was detected by an antibody generated in rabbits immunized with the entire recombinant molecule.

The recombinant p176 protein is composed of an 84-amino acid-long V region followed by a CL region that includes 62 GXY triplets. The CD spectra of p176 at 4 °C and 25 °C showed ellipticity maxima at 220 nm, which is consistent with a collagen triple helix-like structure (Fig. 1B). When the p176 sample was heated to 50 °C, the CD spectrum no longer exhibited the characteristics of a collagen triple helix but rather indicated a random coil structure, suggesting that the triple helix had unfolded. Upon cooling to 4 °C again, the signal intensities at 220 nm had increased, indicating that the triple helix could reassemble. The thermal stability was measured with a calculated midpoint temperature of $t_m = 34.9 ^\circ C$. Examination of a rotary-shadowed preparation of p176 (Fig. 1C) revealed a characteristic two domain lollipop-like structure, consistent with what has been previously reported for other Scl variants (21). Recombinant p163, an Scl2.28-derived construct, contains 79 GXY repeats in the collagen-like region and 72 amino acids in

**FIG. 2. Expression of integrins on the surface of fibroblast cells and adhesion assays.** A and B, cells (~$2 \times 10^6$) were incubated in suspension with control mouse IgG, mouse anti-human $\alpha_1$ (TS2/7), $\alpha_2$ (P1E6), $\alpha_3$ (P1B5), $\alpha_4$, $\alpha_5$ (LM609), $\alpha_5$ (P1D6), or $\beta_1$ (4B4) integrin antibodies, as indicated. The cells were then washed and incubated with secondary goat anti-mouse conjugated to FITC (~5.0 $\mu$g/ml), fixed, and subjected to fluorescence-activated cell sorter analysis. C and D, 96-well microtiter plates were coated with increasing concentrations of BSA, FN, and Col I (at 1.25, 2.5, 5, and 10 $\mu$g/ml) and Scl (at 12.5, 25, 50, and 100 nM). Cell adhesion assays were performed at 37 °C in a CO$_2$ incubator as described under “Experimental Procedures.” C, adhesion of MRC-5. D, adhesion of WI-38 fibroblast cells at the end of 45 min. The data are expressed as the means ± S.D. from three replicates and are representative of four independent experiments.
the V region (Fig. 1A, left panel). It adopted a triple-helical conformation and formed lollipop-like structures when viewed by electron microscopy, as reported previously (21).

Collagen-like Protein Promotes Cell Adhesion and Spreading—To examine the ability of Scl proteins to support integrin-dependent cell adhesion and spreading, we used human lung fibroblast (MRC-5 and WI-38) cells. First, the expression levels of various integrin polypeptides on MRC-5 and WI-38 cells were determined using specific antibodies and fluorescence-activated cell sorting. Our data showed that both cell lines expressed collagen-binding \( \alpha_2\beta_1 \) and \( \alpha_5\beta_1 \) integrins at comparable levels as well as \( \alpha_6\beta_1 \), \( \alpha_5\beta_1 \), and \( \alpha_v \), which presumably partners with the \( \beta_3 \) integrin subunit (Fig. 2, A and B).

Next, cell adhesion assays were performed in wells coated with recombinant p163 and p176 proteins or known adhesive proteins (Fig. 3). Adhesion and spreading of fibroblast cells on recombinant Scl proteins. The cells were detached, washed, and resuspended in defined medium, and plated on microtiter plates coated with 100 nM of p176 or 1 \( \mu \)g/ml of either Col I or FN. After the indicated time points, the plates were washed, fixed, and stained. Representative photomicrographs of cells stained with eosin and hematoxylin are shown following 45 and 90 min of adhesion of MRC-5 (A) and WI-38 cells (B) at 200x magnification. Bar, 200 \( \mu \)m. C, cells were grown on coverslips coated with these substrates. After 90 min of permissive adhesion and spreading, the cells were fixed and stained with FITC-phalloidin. Panel a, FN; panel b, Col I; panel c, p176. Magnification, 400x; bar, 10 \( \mu \)m.

FIG. 3. Adhesion and spreading of fibroblast cells on recombinant Scl proteins. The cells were detached, washed, and resuspended in defined medium, and plated on microtiter plates coated with 100 nM of p176 or 1 \( \mu \)g/ml of either Col I or FN. After the indicated time points, the plates were washed, fixed, and stained. Representative photomicrographs of cells stained with eosin and hematoxylin are shown following 45 and 90 min of adhesion of MRC-5 (A) and WI-38 cells (B) at 200x magnification. Bar, 200 \( \mu \)m. C, cells were grown on coverslips coated with these substrates. After 90 min of permissive adhesion and spreading, the cells were fixed and stained with FITC-phalloidin. Panel a, FN; panel b, Col I; panel c, p176. Magnification, 400x; bar, 10 \( \mu \)m.

FIG. 4. Characterization and adhesion of fibroblast cells on recombinant p181 and p182. A and B, effects of domain swapping between p163 and p176. Triple-helix formation and two domain lollipop-like structural organization of the recombinant p181 (A) and p182 (B) were confirmed by CD spectra and electron microscopy analyses, respectively. C and D, adhesion of MRC-5 and WI-38 cells on wells coated with either the p176, p181, or p182 substrates. 96-well microtiter plates were coated with increasing concentrations of either BSA, FN, and Col I (at 1.25, 2.5, 5, and 10 \( \mu \)g/ml) or recombinant Scl proteins (at 12.5, 25, 50, and 100 nM). C, adhesion of MRC-5. D, adhesion of WI-38 cells at the end of 45 min. The data are expressed as the means ± S.D. from three replicates and are representative of four independent experiments.
Scl Protein Interacts with the \( \alpha_2\beta_1 \) Integrin

**The Cell Adhesion Activity of p176 Involves Its Collagen-like Region**—Next, to identify the region of the p176 protein responsible for its ability to support cell adhesion, we generated two chimeric recombinant proteins, p181 and p182, by domain swapping (Fig. 4, A and B). The chimeric rScl protein p181 contains the V region of p176 and the CL region of p163. The p182 construct has both V and CL regions from p176 but lacks its linker region. The CD spectra of the chimeric proteins, p181 (\( t_m = 38.0 ^\circ C \)) and p182 (\( t_m = 36.5 ^\circ C \)), as well as two domain organization revealed by electron microscopy, showed structural features typical for Scl proteins. Cell adhesion assays show that p182 supported attachment of both the MRC-5 and WI-38 cell lines, whereas p181 failed to do so (Fig. 4, C and D). These data show that the collagenous CL region, but not the V region, is responsible for its ability to support cell adhesion, possibly through interacting with one or more of the collagen-binding integrins.

**p176 Interacts Specifically with the \( \alpha_2\beta_1 \) Integrin**—To determine which collagen-binding integrin(s) recognize p176, we tried to inhibit cell attachment using a panel of monoclonal antibodies directed against the extracellular segments of human integrins. Attachment of MRC-5 cells to plates coated with p176 was measured in the presence of increasing concentrations of adhesion-blocking monoclonal antibodies to \( \alpha_1, \alpha_2, \alpha_5, \alpha_6, \alpha_\beta_1, \text{ or } \beta_1 \) integrins or in the presence of EDTA, a metal ion chelating agent (Fig. 5A). Interestingly, only anti-\( \alpha_2\beta_1 \) and -\( \beta_1 \) integrin antibodies inhibited cell adhesion activ-

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ECM proteins. p176 served as a substrate for the attachment of both MRC-5 and WI-38 cells, as did fibronectin (FN), type IV collagen (Col IV), and type I collagen (Col I). Cell adhesion on p176 substrate was maximal at 100 nM (Fig. 2, C and D); no further increase in cell adhesion activity was evident beyond 100 nM concentration (data not shown). In contrast to p176 that supported cell adhesion, p163 and BSA failed to do so under the same experimental conditions.

The cell attachment results were complemented with cell spreading assays. Detached WI-38 and MRC-5 cells were allowed to reattach onto dishes coated with FN, Col I, and p176 substrates for 45 or 90 min. p176 induced considerable cell spreading, as did fibronectin (FN), type IV collagen (Col IV), and type I collagen (Col I). Cell adhesion assays were performed after 45 min. C, indicated cells (0.5 \( \times \)10^6) were plated onto dishes coated with BSA (control), p163 (100 nM), and p176 (100 nM) substrates. C2Cl2-\( \alpha_5 \) cells were preincubated with optimal concentration (10 \( \mu \)g/ml) of adhesion blocking anti-human integrin monoclonal antibodies for 30 min. The cells were washed and resuspended in defined medium and then replated onto dishes coated with Scl p176 (100 nM) and subjected to cell adhesion assay after 45 min. The data are expressed as the means \( \pm \) S.D. from four replicates and are representative of two to four independent assays. The values are adjusted against background readings obtained from plates incubated in absence of cells. *, \( p < 0.0001 \); \#, \( p < 0.001 \). D, indicated cells were serum-starved overnight, detached using 3 mM EDTA, pH 7.5, washed, and replated on dishes coated either with Fn (10 \( \mu \)g/ml) or p176 (100 nM) for 45 min. The cells were solubilized in radioimmune precipitation assay buffer and subjected to Western blot (WB) analyses with rabbit anti-p-397Y-FAK polyclonal (\( \alpha \)Ab). E, equal loading of total lysate was analyzed by immunoblotting with monoclonal (\( \alpha \)Ab) anti-FAK (2A7) antibodies. All of the blots shown are representative of those obtained in at least three separate experiments, with similar results.

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**Fig. 5. Cell adhesion onto immobilized p176 is mediated by \( \alpha_2\beta_1 \) integrin.** A, MRC-5 (1 \( \times \)10^5) cells were preincubated with 1, 5, or 10 \( \mu \)g/ml anti-human IgG (c), \( \alpha_1 \) (TS2/7), \( \alpha_5 \) (P1E6), \( \alpha_2 \) (P1B5), \( \alpha_5\beta_1 \) (LM609), \( \alpha_6 \) (P1D6), and \( \beta_1 \) (4B4)\# anti-integrin antibodies in PBS containing Ca^2+/Mg^2+ on ice for 30 min. After washing, the cells were suspended in defined medium and replated onto microplates precoated with 100 nM of p176. Untreated control cells (bar C) or cells treated with 1, 2, and 5 mM of EDTA were also analyzed. The cells were allowed to attach at 37 °C in a CO2 incubator. After 45 min, cell adhesion was determined. B, indicated cells were plated onto dishes coated with 0.5 \( \mu \)g/ml of Col I, Col IV, vitronectin (Vn), and FN. Cell adhesion assays were performed after 45 min. C, indicated cells (0.5 \( \times \)10^6) were plated onto dishes coated with BSA (control), p163 (100 nM), and p176 (100 nM) substrates. C2Cl2-\( \alpha_5 \) cells were preincubated with optimal concentration (10 \( \mu \)g/ml) of adhesion blocking anti-human integrin monoclonal antibodies for 30 min. The cells were washed and resuspended in defined medium and then replated onto dishes coated with Scl p176 (100 nM) and subjected to cell adhesion assay after 45 min. The data are expressed as the means \( \pm \) S.D. from four replicates and are representative of two to four independent assays. The values are adjusted against background readings obtained from plates incubated in absence of cells. *, \( p < 0.0001 \); \#, \( p < 0.001 \). D, indicated cells were serum-starved overnight, detached using 3 mM EDTA, pH 7.5, washed, and replated on dishes coated either with Fn (10 \( \mu \)g/ml) or p176 (100 nM) for 45 min. The cells were solubilized in radioimmune precipitation assay buffer and subjected to Western blot (WB) analyses with rabbit anti-p-397Y-FAK polyclonal (\( \alpha \)Ab). E, equal loading of total lysate was analyzed by immunoblotting with monoclonal (\( \alpha \)Ab) anti-FAK (2A7) antibodies. All of the blots shown are representative of those obtained in at least three separate experiments, with similar results.
ities in a dose-dependent manner. In contrast, antibodies against other integrins did not block cell attachment. As expected, EDTA also inhibited cell attachment in a dose-dependent manner. These data show that the \( \alpha_2^\beta_1 \) integrin is a cellular receptor for the p176 protein.

To further examine the role of the \( \alpha_2^\beta_1 \) integrin in cell adhesion to p176, collagen receptor-deficient C2C12 myoblast cells and C2C12 cells stably expressing the human wild-type \( \alpha_2^\beta_1 \) integrin subunit (designated C2C12-\( \alpha_2^\beta_1 \)) were used. Upon expression in C2C12 cells, wild-type \( \alpha_2^\beta_1 \) integrin polypeptide combines with the endogenous \( \beta_1 \) subunit to form a functional \( \alpha_2^\beta_1 \) integrin (49). Col I, Col IV, FN, and vitronectin were included as positive controls in cell adhesion assays. As expected, Col I and Col IV supported adhesion of C2C12-\( \alpha_2^\beta_1 \) cells but not of parental C2C12 cells (Fig. 5B). Similarly, whereas C2C12-\( \alpha_2^\beta_1 \) cells attached to p176, adhesion of parental C2C12 cells to p176 was negligible (Fig. 5C). Attachment of both parental C2C12 and C2C12-\( \alpha_2^\beta_1 \) cells to BSA (negative control) and p163 were insignificant (Fig. 5C). Furthermore, adhesion of C2C12-\( \alpha_2^\beta_1 \) cells to p176 was blocked effectively by anti-\( \alpha_2 \) and anti-\( \beta_1 \) integrin monoclonal antibodies (Fig. 5B).

**Fig. 6.** SPR analyses of \( \alpha_2^\beta_1 \) integrin subunit I domain (\( \alpha_2^\beta_1 \)I) binding to recombinant Scl proteins. Different concentrations of \( \alpha_2^\beta_1 \)I were injected at a flow rate of 20 \( \mu l/min \) for 6 min in the presence of 1 mM MgCl\(_2\). A, representative sensorgram of the relative SPR responses of \( \alpha_2^\beta_1 \)I over immobilized p176. B, the responses of \( \alpha_2^\beta_1 \)I over a blank cell were subtracted. In the concentration range of 3 to 3000 nM, the dissociation constant \( (K_d) \) for \( \alpha_2^\beta_1 \)I over p176 is ~17 nM. C, no significant binding of \( \alpha_2^\beta_1 \)I with immobilized p181. Similar results were obtained from SPR measurements when p176 was passed over immobilized \( \alpha_2 \) domain protein (data not shown). The data are representative of those obtained from at least two to three separate experiments, with similar results. D, cell adhesion onto immobilized p180. MRC-5 \( (1 \times 10^5) \) cells were preincubated with 1, 5, or 10 \( \mu g/ml \) anti-human (IgG (c), \( \alpha_2^\beta_1 \)TS2/7), \( \alpha_2 \) (P1E6), \( \alpha_3 \) (P1B5), \( \alpha_5^\beta_1 \)LM609, \( \alpha_5 \) (P1D6), and \( \beta_1^\beta_1 \) (4B4) integrin monoclonal antibodies in PBS containing Ca\(^{2+} \)/Mg\(^{2+} \) on ice for 30 min. After washing, the cells were suspended in defined medium and replated onto dishes coated with 100 nM of p180. In addition, cell adhesion assays were also performed in the presence of 1, 2, and 5 mM of EDTA. Cell adhesion assays were carried out for 45 min at 37 °C in a CO\(_2\) incubator, as described in the legend to Fig. 5A. The data are expressed as the means ± S.D. from four replicates, and the experiments were performed three times. The values are adjusted against background readings obtained from plates incubated in absence of cells. *

\( p < 0.01. \)
The I Domain of α₂ Integrin (α₂-I) Interacts with p176 but Not with p181—The so-called I domain of the α components of collagen-binding integrins has been demonstrated to bind to specific sites in triple helix collagens (29, 32, 33, 47). To study whether the I domain of the α₂ integrin subunit interacts with p176, we expressed α₂-I as a recombinant protein and examined its binding to p176 and p181 by SPR analysis. Different concentrations of α₂-I were passed over Biacore chips to which the recombinant Scl proteins had been coupled. A concentration-dependent binding of α₂-I to p176 was noted with rapid on and off rates and defined equilibrium (Fig. 6A). A $K_D$ of 17 nM was calculated from a Scatchard plot of the equilibrium data (Fig. 6B). No significant binding was observed when increasing concentrations (up to 300 nM) of α₂-I were run over p181 (Fig. 6C). These results demonstrate that p176 contains a site to which α₂-I domain binds with high affinity.

Mapping of the Integrin α₂β₁-binding Site on p176—The p176 protein contains a GLPGER sequence that is similar to the sequence motifs GL/FOGER (O; hydroxyproline) that have been identified in human collagens as binding sites for various collagen-binding integrins (32, 33, 44). Furthermore, the glutamyl residue plays a crucial role in those interactions (31, 36). To test the involvement of the GLPGER motif of p176 in α₂β₁ binding, we generated a mutant of p176 called p180 in which the GLPGER sequence has been changed to GLPGAR. Importantly, we expected that the resulting GAR triplet will not destabilize the triple helix (50). As predicted, the recombinant p180 variant containing the GLPGAR sequence formed a stable triple helix ($T_m = 36.0\, ^\circ C$) and displayed a typical lollipop-like structure (data not shown).

In a cell adhesion assay, p180 mutant (GER to GAR) protein supported the attachment of the MRC-5 cells at the level comparable with that obtained with the parental protein p176 (Fig. 6D). Furthermore in surface plasmon resonance analyses p180 immobilized on a chip supported the concentration-dependent binding of the α₂-I domain. A $K_D$ of 32 nM was calculated for this interaction compared with a $K_D$ of 17 nM determined for the binding of the α₂-I domain to immobilized p176. These unexpected results suggest that the α₂β₁ integrin recognize the GLPGER sequence present in p176 and absent in p180 (because of the differences in $K_D$ values) but also additional binding site(s) present in both p176 and p180.

To locate the α₂β₁-binding sites within the collagenous domain of p176, we incubated p176 with the α₂-I domain, subjected the complex to rotary shadowing, and examined by electron microscopy (Fig. 7). Spherically shaped α₂-I domains (Fig. 7B) were seen bound to p176 (Fig. 7, C–E, black arrows). The GLPGER motif stretches over the 8th and 9th repeats in a 62-GXY repeat-long CL region and is spaced from its N terminus at a calculated distance corresponding to $\sim{1/6}$ of the total length. In addition, a second
binding site was located at a distance of $\sim 1/5$ of the total length as measured from the globular V domain (Fig. 7, C and E–G, white arrows). We could not identify any sequence in this region that would resemble a previously reported integrin-binding motif (31, 36, 52), and we have not yet identified this second binding site.

p176 Induces Phosphorylation of p125FAK, p130CAS, Paxillin, and JNK—The results presented above demonstrate that p176 can ligate $\alpha_\beta_1$ integrin. Cell adhesion-induced phosphorylation of p125FAK, p130CAS, paxillin, and JNK proteins are considered to be integrin-mediated signaling events (37–41). To investigate whether p176 induces integrin signaling, we evaluated the phosphorylation states of these proteins. The cells kept in suspension and cells adhering to Col I and FN substrates served as negative and positive controls, respectively. Cell attachment was allowed to proceed for 30 min, a time required for FAK activation (Fig. 8). We found that the phosphorylation of p125FAK at tyrosine 397 is markedly increased in response to adhesion onto p176, FN, and Col I (Fig. 8A). Because paxillin and p130CAS proteins are key focal adhesion-related molecules that undergo phosphorylation in response to activation of p125FAK, immune complexes were analyzed by immunoblotting with an anti-phosphotyrosine antibody. Like p125FAK, both p130CAS and paxillin were phosphorylated in response to adhesion onto p176, FN, and Col I (Fig. 8, C and E).

In contrast, these proteins did not exhibit any change in phosphorylation state in the cells that remained in suspension, mimicking the unattached cells on p163. Similarly, phosphorylation of JNK was phosphorylated in response to adhesion onto p176, FN, and Col I (Fig. 8G) but not under control conditions (cells in suspension). All of the blots were stripped and reprobed with indicated antibodies to determine the amounts of protein loading (Fig. 8, B, D, F, and H). Our data indicate that $\alpha_\beta_1$ integrin-dependent adhesion to p176 induces phosphorylation of FAK, CAS, paxillin, and JNK proteins.

**DISCUSSION**

We have previously shown that group A streptococci express two related families of cell surface proteins with domains containing repeated GXy sequence. This sequence is reminiscent of the characteristic collagen sequence required for triple helix formation. In fact, the collagen-like domains of at least some Scl proteins can form triple helix structures resembling those formed by mammalian collagens (21).

In higher organisms collagens are traditionally known for providing the structural integrity to the tissues (1). However, the collagens can also directly or indirectly affect cellular behavior by interacting with specific cellular receptors or with other biologically active extracellular molecules (27, 30–33). Because of the structural similarities between the collagen-like domains of Scl proteins and collagen, we have explored the possibility that these streptococcal proteins affect cellular behavior in the host by mimicking the mammalian collagen. In this paper, we report that a recombinant protein, p176, based on Scl1 from M type 41 supports $\alpha_\beta_1$ integrin-dependent cell adhesion of human lung fibroblasts. The p176 substrate not only supports the attachment of lung fibroblast cells but also induces integrin signaling and cell spreading. In contrast to the p176 polypeptide that supports cell binding and integrin signaling, p163, a structurally similar Scl protein does not show such activity. These data suggest that integrin ligation is not a general property of Scl proteins, and several other recombinant proteins based on different Scl sequences did not support cell adhesion.

These observations suggest that specific sequences present in some but not all Scl variants are recognized by the integrins. In fact, previous studies by us and others have shown that the $\alpha_\beta_1$ and $\alpha_\beta_2$ integrins interact with specific sequences in mammalian collagens (31–33, 44). The substrate specificity of the $\alpha_\beta_1$ and $\alpha_\beta_2$ integrins appears to be similar but not identical. Thus, $\alpha_\beta_1$ but not $\alpha_\beta_2$ integrin requires a hydroxyproline residue in the binding site for full activity (51). This requirement could explain our results why $\alpha_\beta_2$ but not $\alpha_\beta_1$ mediates cell adhesion to the p176 protein, which lacks hydroxyprolines.

Recent studies demonstrated that several GXXGER motifs that occur in human fibillar collagens interact with integrin with varied affinities that also depended on the activation state of the integrin (52). Five different GXXGER motifs (GRNGER, GLQGER, GPAGER, GPRGER, and GKDGER) are present within the collagen-like domain of p163 protein, which did not bind integrins. To the contrary, an integrin-binding variant p176 harbors a single GLPGER motif that resembles the high affinity integrin-binding site of mammalian collagens, GLOGER. However, our initial studies indicated that the GLPGER sequence might not be the only integrin-binding site in the p176 protein. The p176 protein does not contain any other known integrin-binding sites, and it appears that this domain contain previously unidentified active sites. Studies are currently underway to define the novel $\alpha_\beta_1$-binding site(s) in p176.

It is presently unclear whether an integrin interaction through an Scl protein plays a role in the infectious process of *S. pyogenes*. Integrins are involved in streptococcal host cell invasion (53). The fibronectin binding MSCRAMM on Gram-positive bacteria can recruit soluble fibronectin, and the bacteria bound adhesive protein is then recognized by the $\alpha_\beta_1$ integrin in a process that leads to host cell invasion (54). It is tempting to speculate that a direct binding of the Scl protein to an integrin could have similar consequences, but experimental data supporting an Scl integrin-mediated cellular invasion are not yet available. The $\alpha_\beta_1$ integrin is also a prominent collagen receptor on platelets (55), and it is possible that Scl proteins may induce a platelet signaling through interacting with the platelet integrin.

The Scl protein-integrin interaction reported in this work suggest that these bacterial proteins are mimicking collagen ligands when it comes to $\alpha_\beta_1$ integrin. It is likely that Scl proteins could also behave as collagens in other systems and that this molecular mimicry allows the bacteria to manipulate host biology at a number of different levels. In addition to the known mechanisms (56, 57), we propose that a structurally conserved prokaryotic collagen-like protein is capable of interacting with mammalian integrin receptors, raising the possibility of *S. pyogenes* expressing p176 to affect host cells via the integrin-signaling pathway.

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**REFERENCES**