A Streptococcal Collagen-like Protein Interacts with the α₂β₁ Integrin and Induces Intracellular Signaling*

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The streptococcal collagen-like proteins Sc1 and Sc2 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 Sc1 variant might interact with mammalian collagen-binding integrins. We show that the recombinant Sc1 protein p176 promotes adhesion and spreading of human lung fibroblast cells through an α₂β₁ integrin-mediated interaction as shown in cell adhesion inhibition assays using anti-α₂β₁ and anti-β₁ integrins monoclonal antibodies. Accordingly, C2C12 cells stably expressing α₂β₁ integrin as the only collagen-binding integrin show productive cell adhesion activities on p176 that can be blocked by an anti-α₂β₁ integrin antibody. In addition, p176 promotes tyrosine phosphorylation of p125FAR of C2C12 cells expressing α₂β₁ integrin, whereas parental C2C12 cells do not. Furthermore, adhesion of human lung fibroblast cells to p176 induces phosphorylation of p125FAR, p130CAS, and p6βaxitin proteins. In a domain swapping experiment, we show that integrin binds to the collagenous domain of the Sc1 protein. Moreover, the recombinant inserted domain of the α₂ integrin interacts with p176 with a relatively high affinity (Kₐ = 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 retained not only structural but also functional characteristics of their eukaryotic counterparts.

The collagen family of extracellular matrix (ECM) 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 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 are often 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 Sc1 and Sc2 (also known as Sc1A and Sc1B) 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 Sc1 and Sc2 proteins have similar organization with four common regions (16). The N-terminal signal sequence and C-terminal cell wall associated regions are conserved between Sc1 and Sc2, whereas the variable (V) and the collagen-like (CL) regions differ significantly in length and primary sequence. In addition, Sc1, but not Sc2, 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 Sc1 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 αβ heterodimers, capable of interacting with ECM proteins in a metal ion-dependent manner (22–28). There are 18 distinct α subunits and 8 β subunits in mammals that...

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1 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 α₁β₁, α₂β₁, α₁δβ₁, and α₁β₂ 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 α 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 ligation 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 α₂β₂ integrin, presumably through the I domain of the α₂ subunit. Importantly, this interaction promotes cell adhesion and intracellular signaling, much like eukaryotic collagens. 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, Gottingen, Germany). The Scl2.28-based pSL163 construct encode p163 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 AF542037) 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 F1 present in the expression vector pASK-IBA2 as described. Oligonucleotide containing new ApaLI restriction site (5′-CTTATTAGCTCCTAGTTAGCTGGACCCAGG-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 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-termini sequencing.

**Rotary Shadowing and Electron Microscopy**—The structural organization of the recombinant proteins was viewed by electron microscopy of the rotary-shadowed rScs, 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 Phillips 410 Transmission electron microscope operated at 80 KV. A 0.1-mm 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 α₁β₂-p176 (trimer) molar ratio of 4:1 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 conformation of recombinant rScs was analyzed by CD, as described previously (44). The spectra were recorded on a Jasco J720 spectroptometer equipped with a variable temperature unit. The samples were dissolved in 0.001 M PBS, pH 7.4. The Measurements were taken with a 0.5-cm path length, and the data were integrated for 1 s at 0.2-nm intervals with a bandwidth of 1 nm. A wavelength scan was performed for each protein before unfolding (45 and 25 °C) and after unfolding (50 °C) or after subsequent refolding (4 °C, renatured).

**Reagents and Antibodies**—Human fibronectin, type I and type IV collagen, control IgG, anti-α₁ (TS2/7), anti-α₂ (P1E6), anti-α₃ (P1B5), α₁β₁ (P16D1), α₁β₂ (P16D6) integrin antibodies, and cell culture materials were purchased from Invitrogen. Anti-FAK (2A7), anti-p130 CAS, anti-paxillin, and anti-phosphotyrosine (4G10) mouse monoclonal antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-phospho-JNK and anti-phospho-FAK (Y397) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-α₁β₁ (LM609) was obtained from Chemicon (Temecula, CA). Anti-β₁ (4B4) integrin monoclonal antibody was procured from BD Biosciences (San Jose, CA).

**Cells and Cell Culture**—Human lung fibroblast cell lines (MRC-5 and WI-38) were obtained from ATCC (CCL-171; CCL-75) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics. Parental mouse myoblast C2C12 (control cells) and C2C12 cells stably transfected with the Scl integrin subunit (Scl1.41-α₁β₁) were kindly provided by Dr. Donald Gulberg (Uppsala University, Uppsala, Sweden) and maintained in 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium in the absence or presence of 10 μg/ml puromycin, respectively. Methods for cell culture have been previously described (45, 46).

**Fluorescent Activated Cell Sorting**—The cells were washed in PBS, pH 7.4, and then dissociated with 2 mM EDTA in PBS, pH 7.4, and washed twice in PBS. The cells were resuspended through a cell strainer and enumerated. The cells (2 × 10⁶) were then incubated with saturating amounts of control mouse IgG or anti-human integrin adhesion-blocking monoclonal antibodies: anti-α₁ (TS2/7), anti-α₂ (P1E6), anti-α₃ (P1B5), anti-α₁β₁ (LM609), anti-α₁β₂ (P16D6), and anti-β₁ (4B4) for 30 min at 4 °C. In all cases, secondary labeling was performed for 1 h at 4 °C with a goat anti-mouse IgG conjugated to FITC (PharMingen). The cellular DNA was stained with propidium iodide (Molecular Probes). Cells were washed twice with PBS, fixed with 0.5% p-formaldehyde, and analyzed with the FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).

**Cell Adhesion and Spreading Assays**—Most cell adhesion assays were carried out in a 24-well tissue culture dish coated with specified substrates as has been described previously (46). Briefly, the test substrates (Scl recombinant proteins) were prepared at a concentration of 12.5, 25, 50, and 100 μg/ml in Tris-buffered saline (4°C, pH 7.4, 150 mM NaCl, 0.02% sodium azide) and coated onto sterile silicon microplates overnight at 4 °C. Human fibroblast, type IV collagen, type I collagen, and bovine serum albumin (BSA) (1.25, 2.5, 5, and 10 μg/ml) were included as controls. BSA was filtered through a 0.22-μm membrane and heat-inactivated prior to use. After washing with PBS, cells were preincubated with 0.5% BSA in Tris-buffered saline, pH 7.4, for 5 min at 37 °C. The plate was washed twice with cold 1% formaldehyde-fixed cells were stained with eosin and hematoxylin and absorbance was measured at 590 nm. To visualize cell spreading, 37 °C incubator. Human lung fibroblast MRC-5 and WI-38 or C2C12 cells starved overnight in serum-free medium were detached in PBS containing Ca²⁺ and Mg²⁺, and washed twice in PBS containing Ca²⁺ and Mg²⁺, then washed with 1% formaldehyde, and analyzed with the FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).

**Cell Adhesion Blocking Assay**—For blocking assay, antibodies were dialyzed overnight in sterile 1× Tris-buffered saline, pH 7.4, to remove any traces of sodium azide and minor impurities. The integrity and concentration of antibodies were determined by 10% SDS-PAGE prior to experiment. The cell adhesion blocking assay has been previously described (46). In brief, 70% confluent human lung fibroblast...
protein surface was achieved by running 10
description (46).
integrin monoclonal antibody for C2C12-
assay was considered optimal. Cold (4 °C) PBS, pH 7.4, containing 1 mM
37 °C in a CO2 incubator. The use of single dose (10
p176 in prior experiments) p176 and subjected to cell adhesion assays at
proteins in 25 mM HEPES, 150 mM NaCl, pH 7.4, buffer containing 5 mM
L
to scale). Integrin subunit was amplified by PCR from a human hepatoma cDNA
sequences was verified by dideoxy DNA sequencing
affinity chromatography as previously described (44).
(4 °C, renatured). C, electron microscopy of the rotary-shadowed p176, demonstrating the formation of lollipop-like structures.
(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 × 10⁶ cells were incubated with 1, 5, and 10 μg/ml of anti-α1, anti-α2, anti-α3, anti-αv, anti-β3, 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 μg/ml) of anti-integrin monoclonal antibody for C2C12-α2+ cell adhesion blocking assay was considered optimal. Cold (4 °C) PBS, pH 7.4, containing 1 mM of Ca²⁺ and Mg²⁺ was used for washing cells. Statistical analyses were
performed as previously described (46).
Recombinant I Domain of α₂ Integrin Subunit—Cloning, expression, and purification of the I domain of α₂ integrin have been previously described (47). In brief, cDNA fragment encoding the I domain of α₂ integrin subunit was amplified by PCR from a human hepatoma cDNA library and subcloned into the expression vector pQE30 (Qiagen). The accuracy of the DNA sequence was verified by dyeodeoxy DNA sequencing (Lonestar Laboratory, Houston, TX). Large scale expression and purification of the recombinant α₂-I were carried out using Ni²⁺-chelating affinity chromatography as previously described (44).
Surface Plasmon Resonance (SPR) Analysis—Measurements were performed at room temperature in a BIACORE 3000 instrument (BIACore, Uppsala, Sweden) as described previously (47). Briefly, p176ScI (990 response units) and p18IScI (717 response units) proteins were immobilized onto the flow cells on a CM5 chip. Different concentrations of α₂-I proteins in 25 mM HEPES, 150 mM NaCl, pH 7.4, buffer containing 5 mM β-mercaptoethanol, 1 mM MgCl₂, and 0.05% octyl-D-glucopyranoside were passed over these surfaces at 20 μl/min for 6 min. Regeneration of the ScI protein surface was achieved by running 10 μl of a solution of 0.01% SDS. Binding of α₂-I to a reference flow cell, which had been activated and deactivated without the coupling of proteins, was also measured and subtracted from the binding to ScI protein-coated chips. SPR sensorsgrams from different injections were overlaid using the BIAevaluation software (BIACore AB). Data from the equilibrium portion of these sensorsgrams were used for Scatchard analysis. Based on the correlation between the
SPR response and change in soluble I domain protein binding to the immobilized ScI proteins, values for the binding ratio, rbound and the concentration of free protein, [P]freet were calculated using the equations described previously (47). Scatchard analysis was performed by plotting rbound/[P]freet against rbound in which the negative reciprocal of the slope is the dissociation constant, Kd.
Biochemical Methods—The cells were serum-starved overnight, 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 detection protocols were performed as described previously (45, 46, 48). For immunoprecipitation, 2–3 μg of antibodies were used for each sample. For immunoblotting analyses, the antibodies were prepared at a concentration of 0.5–2 μg/ml in 1× Tris-buffered saline, pH 7.4, with 3% BSA.

RESULTS
Characterization of Recombinant ScI (rScI) Proteins—We recently demonstrated that two different rScI proteins, p144 derived from the ScI1.1 variant produced by the M1-type group A Streptococcus and p163 (ScI2.28) derived from the ScI2 variant of the M28-type group A Streptococcus, contain domains that form collagen-like triple helices, despite the lack of hydroxyproline commonly found in mammalian collagens and a significant variation in primary sequence (21). This observation raised the question: can mammalian cells recognize the ScI proteins as collagens? For example, do collagen-like domains of some ScI
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 GL/F/PGER (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.

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 $t_m = 34.9$ °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 ($\sim 2 \times 10^6$) were incubated in suspension with control mouse IgG, mouse anti-human $\alpha_1$ (TS2/7), $\alpha_2$ (P1E6), $\alpha_5$ (P1B5), $\alpha_6$, (LM609), $\alpha_9$ (P1D6), or $\beta_1$ (4B4) integrin antibodies, as indicated. The cells were then washed and incubated with secondary goat anti-mouse conjugated to FITC ($\sim 5.0$ μg/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 μg/ml) and Scl (at 12.5, 25, 50, and 100 nM). Cell adhesion assays were performed at 37 °C in a CO2 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 $\alpha_3\beta_1$, $\alpha_6\beta_1$, 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
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 (at \( t_m = 38.0 \, ^\circ\text{C} \)) and p182 (at \( t_m = 36.5 \, ^\circ\text{C} \)), 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 globular V region or the linker region, of p176 supports cell adhesion, possibly through interacting with one or more of the collagen-binding integrins.

**p176 Interacts Specifically with the \( \alpha_2\beta_1 \) Collagen-binding Integrin**—To determine which collagen-binding integrin(s) recognizes 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 \( \alpha_1 \), \( \alpha_2 \), \( \alpha_5 \), \( \alpha_6 \), \( \alpha_\beta_1 \) 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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**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 \( \mu \text{g/ml} \) anti-human IgG (c), \( \alpha_1 \) (TS2/16), \( \alpha_2 \) (P1E6), \( \alpha_5 \) (P1B5), \( \alpha_\beta_1 \) (LM609), \( \alpha_6 \) (P1D6), and \( \beta_1 \) (4B4) \( \alpha_2\beta_1 \) 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 on microplates precoated with 100 \( \mu \text{M} \) of p176. Untreated control cells (bar C) or cells treated with 1, 2, and 5 \( \mu \text{M} \) of EDTA were also analyzed. The cells were allowed to attach at 37 °C in a CO\(_2\) incubator. After 45 min, cell adhesion was determined. B, indicated cells were plated onto dishes coated with 0.5 \( \mu \text{g/ml} \) of Col I, Col IV, vitronectin (Vn), and FN. Cell adhesion assays were performed after 45 min. C, indicated cells (0.5 \times 10^5) were plated onto dishes coated with BSA (control), p183 (100 \( \mu \text{M} \)), and p176 (100 \( \mu \text{M} \)) substrates. C2C12-\( \alpha_2 \) cells were preincubated with optimal concentration (10 \( \mu \text{g/ml} \)) 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 \( \mu \text{M} \)) and subjected to cell adhesion assay after 45 min. The data are expressed as the means ± 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 \( \mu \text{M} \) EDTA, pH 7.5, washed, and replated on dishes coated either with Fn (10 \( \mu \text{g/ml} \)) or p176 (100 \( \mu \text{M} \)) 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 (pAb). E, equal loading of total lysate was analyzed by immunoblotting with monoclonal (mAb) anti-FAK (2A7) antibodies. All of the blots shown are representative of those obtained in at least three separate experiments, with similar results.

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 \( \mu \text{M} \) (Fig. 2, C and D); no further increase in cell adhesion activity was evident beyond 100 \( \mu \text{M} \) concentration (data not shown). In contrast to p176 that 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 (at \( t_m = 38.0 \, ^\circ\text{C} \)) and p182 (at \( t_m = 36.5 \, ^\circ\text{C} \)), 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 globular V region or the linker region, of p176 supports cell adhesion, possibly through interacting with one or more of the collagen-binding integrins(s).
Scl Protein Interacts with the \( \alpha_2\beta_1 \) Integrin

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 \( \alpha_2\beta_1 \)/H9251/H9252 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 \( \alpha_2 \) integrin subunit (designated C2C12-\( \alpha_2 \)+) were used. Upon expression in C2C12 cells, wild-type \( \alpha_2 \) 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-\( \alpha_2 \)+ cells but not of parental C2C12 cells (Fig. 5B). Similarly, whereas C2C12-\( \alpha_2 \)+ cells attached to p176, adhesion of parental C2C12 cells to p176 was negligible (Fig. 5C). Attachment of both parental C2C12 and C2C12-\( \alpha_2 \)+ cells to BSA (negative control) and p163 were insignificant (Fig. 5C). Furthermore, adhesion of C2C12-\( \alpha_2 \)+ cells to p176 was blocked effectively by anti-\( \alpha_2 \) and anti-\( \beta_1 \) integrin monoclonal antibodies (Fig. 5C). In addition, p176 substrate induced tyrosine phosphorylation of FAK of C2C12-\( \alpha_2 \)+ cells; in contrast, no significant phosphorylation was observed in parental C2C12 cells (Fig. 5, D and E). Clearly, these data indicate that adhesion of cells to p176 is mediated specifically by the \( \alpha_2\beta_1 \) integrin.
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 collagen-binding integrins has been demonstrated to bind to these sites (29, 32, 33, 44). Furthermore, the glutamyl residue plays a crucial role in those interactions (31, 36).

To locate the αβ1-binding sites within the collagenous domain of p176, we incubated p176 with the αβ1-I domain, subjected the complex to rotary shadowing, and examined by electron microscopy (Fig. 7). Spherically shaped αβ1-I domains (Fig. 7B) were seen bound to (Fig. 7, C–G) the linear collagenous tails of p176. In the complex, at least two different recognition sites were seen. One binding site was located close to the globular domain and could well correspond to the GLPGER site (marked with a black arrowhead) and newly observed Site 2 (white arrowheads). Bar, 50 nm.

Mapping of the Integrin αβ1-binding Site on p176—The p176 protein contains a GLPGER sequence that is similar to the GLPGER motif (29, 32, 33, 47). The GLPGER motif (marked with a solid diamond) and the observed second binding site (marked with an open diamond). B. rotary-shadowed preparation of the αβ1-I domain. C–G, micrographs of the αβ1-I/p176 complexes at presumed GLPGER site (black arrowheads) and newly observed Site 2 (white arrowheads). Bar, 50 nm.

Fig. 7. Visualization of the αβ1-I domain-binding sites on p176 by electron microscopy. A, schematic representation of p176 with GLPGER motif (marked with a solid diamond) and the observed second binding site (marked with an open diamond). B, rotary-shadowed preparation of the αβ1-I domain. C–G, micrographs of the αβ1-I/p176 complexes at presumed GLPGER site (black arrowheads) and newly observed Site 2 (white arrowheads). Bar, 50 nm.

Further in surface plasmon resonance analyses p180 immobilized on a chip supported the concentration-dependent binding of the α2-I domain. A KD of 32 nM was calculated for this interaction compared with a KD of 17 nM determined for the binding of the α2-I domain to immobilized p176. These unexpected results suggest that the αβ1 integrin recognize the GLPGER sequence present in p176 and absent in p180 (because of the differences in KD values) but also additional binding site(s) present in both p176 and p180.

To locate the αβ1-binding sites within the collagenous domain of p176, we incubated p176 with the αβ1-I domain, subjected the complex to rotary shadowing, and examined by electron microscopy (Fig. 7). Spherically shaped αβ1-I domains (Fig. 7B) were seen bound to (Fig. 7, C–G) the linear collagenous tails of p176. In the complex, at least two different recognition sites were seen. One binding site was located close to the globular domain and could well correspond to the GLPGER site (Fig. 7, C–G, black arrow). 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 ~1/3 of the total length. In addition, a second

Fig. 8. p176 induced phosphorylation of FAK, paxillin, CAS, and JNK in MRC-5 cells. Serum-starved MRC-5 cells were detached using 3 mM EDTA, pH 7.5, washed, and replated on dishes coated either with FN, Col I, or p176. A and G, total lysates were analyzed by immunoblotting with indicated anti-phospho-specific antibodies. B, D, and F, the resulting immune complexes were analyzed by immunoblotting, with anti-phosphotyrosine antibodies. B, D, F, and H, equal amounts of protein loading were determined by immunoblotting with indicated non-phospho-specific antibodies. All of the blots shown are representative of those obtained in at least three separate experiments with similar results.
binding site was located at a distance of ~1/3 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 αβ1 integrin. Cell adhesion-induced phosphoryla-
tion of p125FAK, p130CAS, paxillin, and JNK proteins are con-
sidered to be integrin-mediated signaling events (37–41). To
investigate whether p176 induces integrin signaling, we eval-
uated the phosphorylation states of these proteins. The cells
kept in suspension and cells adhering to Col I and FN sub-
strates 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 phos-
phorylation 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 phos-
phorylation state in the cells that remained in suspension,
mimicking the unattached cells on p163. Similarly, phospho-
JNK immunoblotting analysis of total lysates showed that JNK
was phosphorylated in response to adhesion onto p176, FN, and
Col I (Fig. 8G) but not under control conditions (cells in sus-
ension). All of the blots were striped and reprobed with
indicated antibodies to determine the amounts of protein load-
ing (Fig. 8, B, D, F, and H). Our data indicate that αβ1
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 con-
taining 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 αβ1 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 sig-
naling, 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 αβ1 and αβ1 integrins interact with specific se-
quences in mammalian collagens (31–33, 44). The substrate
specificity of the αβ1 and αβ1 integrins appears to be similar
but not identical. Thus, αβ1 but not αβ1 integrin requires a
hydroxyproline residue in the binding site for full activity (51).
This requirement could explain our results why αβ1 but not
αβ1 mediates cell adhesion to the p176 protein, which lacks
hydroxyprolines.

Recent studies demonstrated that several GXGGER motifs
that occur in human fibrillar collagens interact with integrin
with varied affinities that also depended on the activation state
of the integrin (52). Five different GXGGER motifs (GRNGER,
GLGGER, PGAGER, GPGRER, 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 GLGGER motif that resembles the highly affin-
integrin-binding site of mammalian collagens, GLOGER. How-
ever, our initial studies indicated that the GLGGER 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 αβ1-integrin 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 bacte-
ria bound adhesive protein is then recognized by the αβ1
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 αβ1 integrin is also a prominent cola-
gen receptor on platelets (55), and it is possible that Scl pro-
teins 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 αβ1 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 inter-
acting with mammalian integrin receptors, raising the possi-
bility of S. pyogenes expressing p176 to affect host cells via the
integrin-signaling pathway.

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