**β₁ Integrin-dependent Cell Adhesion to EMILIN-1 Is Mediated by the gC1q Domain**

Paola Spessotto‡, Marta Cervi‡‡, Maria Teresa Mucignat‡, Gabriella Mungiguerra‡, Ida Sartoretto‡, Roberto Doliana‡, and Alfonso Colombatti‡¶**

From the ‡Divisione di Oncologia Sperimentale 2, Centro di Riferimento Oncologico, Aviano, the ¶Dipartimento di Scienze e Tecnologie Biomediche and the §Microgravity, Ageing, Training, and Immobility Center of Excellence, Università di Udine, Udine 33100, Italy

EMILIN-1 (Elastin Microfibril Interface Located Protein), the prototype of the EMILIN family, consists of a cysteine-rich domain (EMI domain) at the N terminus, an extended region with a high potential coiled-coil structure, a short collagenous stalk, and a self-interacting globular gC1q-l domain. EMILIN-1 is an adhesive extracellular matrix constituent associated with elastic fibers, detected also in the proximity of cell surfaces. To localize the cell attachment site(s), monoclonal antibodies (mAbs) against EMILIN-1 or the gC1q-1 domain were used to inhibit cell attachment to EMILIN-1. Thus, one mAb mapping to the gC1q-1 domain caused complete inhibition of cell attachment. EMILIN-1 and gC1q-1 displayed a comparable dose-dependent ability to promote cell adhesion. Adhesion kinetics was similar to that of fibronectin (FN), reaching the maximum level of attachment at 20 min, but in the absence of cations adhesion was negligible. The relative adhesion strength to detach on the substrate with prominent stress fibers and plated on FN or vitronectin, which appeared well spread was 50% of the cells was similar for EMILIN-1 and gC1q-1 was completely blocked was negligible. The relative adhesion strength to detach (250–270 × g) but lower than that for FN (>>500). Cell adhesion to EMILIN-1 or gC1q-1 was completely blocked by a function-blocking β₁ integrin subunit mAb. In contrast, adhesion to the complement C1q component was totally unaffected. Among the various function-blocking mAbs against the α integrin subunits only the anti-α₁ fully abrogated cell adhesion to gC1q-1 and up to 70% to EMILIN-1. Furthermore, only K562 cells transfected with the α₁ integrin chain, but not wild type K562, were able to adhere to EMILIN-1 and were specifically inhibited by anti-α₁ function-blocking mAb. Finally, cells attached to EMILIN-1 or gC1q-1, compared with cells plated on FN or vitronectin, which appeared well spread out on the substrate with prominent stress fibers and focal contacts, were much smaller with wide ruffles and a different organization status of the actin cytoskeleton along the cell periphery. This pattern was in accord with the ability of EMILIN-1 to promote cell movement.

EMILIN¹-1 (1) is the prototype of a new family of glycoproteins, EMILINs (2–5), which are expressed in a tissue-specific and developmentally regulated manner and whose biological activities are still to be defined properly. EMILIN-1 is composed of a cysteine-rich domain (EMI domain) at the N terminus (6), a long segment of about 650 residues with a high potential for forming coiled-coil helices, a short uninterrupted collagenous stalk, and a C1q-like globular domain at the C terminus (gC1q-l), representing a structurally unique component. The gC1q-l domain is necessary for the noncovalent formation of homotrimers that are then linked by disulfide bonds giving rise to very large extracellular aggregates (7). EMILINs are members of the large C1q/tumor necrosis superfamily of proteins that are characterized by the presence of a gC1q domain; the superfamily (1) includes several collagens among which types VIII and X, the recognition component of the classical complement pathway C1q-C₃, and AdipoQ.

Elastic fibers and associated fibrillin-containing microfibrils (8) are important structural components of the extracellular matrix (ECM) of most connective tissues. EMILIN-1 forms a fibrillar network in vitro and in the ECM of several tissues including blood vessels, skin, heart, lung, kidney, and cornea (9–13). This glycoprotein codistributes with elastin in most sites and likely constitutes an associated component of elastic fibers. EMILIN-1 is localized mainly at the interface between amorphous elastin and the surrounding microfibrils, and it has been implicated in the correct deposition of elastin in vitro (14). In addition, EMILIN-1-reacting structures were often observed in vivo closely adjacent to the surface of cells (15, 16), and thus it seemed likely that EMILIN-1 could also interact directly with cell membrane receptors. The constituents of elastic fibers and microfibrils not only display a repertoire of multiple interactions but are recognized by integrin cell surface receptors. Integrons are widely distributed heterodimeric glycoproteins that play a fundamental role in cell adhesion and migration, in cell differentiation, and in many different biological processes. In particular the integrins α,β₅, a promiscuous RGD-dependent integrin (17), was shown to mediate adhesion and spreading of many cell types on elastic fiber-associated constituents such as fibrillin-1 and -2, MAGP-2, and fibulin-5 (18–22). EMILIN-1 does not contain RGD motifs (1), but in preliminary studies it was shown to behave as a ligand for some tumor cells (1, 2), although the receptors involved had not been investigated.

In this study with the use of purified EMILIN-1 and its fragments we addressed the adhesive function of EMILIN-1 located protein; BSA, bovine serum albumin; C1q-C₃, C1q complement component; CAFC, centrifugal assay for fluorescence-based cell adhesion; DABCO, 1,4-diazabicyclo(2.2.2)octane; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FN, fibronectin; gC1q-l, globular C1q-like domain of EMILIN-1; mAbs, monoclonal antibody(ies); PBS, phosphate-buffered saline; VCAM, vascular cell adhesion molecule; VN, vitronectin; LN, laminin.

6160 This paper is available on line at http://www.jbc.org
and pinpointed the gC1q-1 domain as the major and sufficient domain responsible for cell adhesion. Our results suggest that the gC1q-1 domain of EMILIN-1, in addition to its proven function in the initial steps of the assembly of EMILIN-1 homotrimers and multimers (7,12), has a novel cell adhesive role and highlight that gC1q-1 is the first globular C1q domain that is a ligand for a β1 integrin, namely αβ1.

EXPERIMENTAL PROCEDURES

Materials—Function-blocking anti-integrin monoclonal antibodies (mAbs) were obtained as follows. Anti-α6 (clone 12171) was from Virgil Teixido (Centro de Investigaciones Biológicas, Department of Immunology, Madrid, Spain) and maintained in RPMI medium containing 10% fetal calf serum. K562 cells stably transfected with the constructs for EMILIN-1 or the LEU-COL-gC1q-1 as described previously (12). The cells expanded to 293 cells, constitutively expressing the EBNA-1 protein (293-EBNA), were transfected with the constructs for EMILIN-1 or the polypeptide produced in 293-EBNA cells as well as the gC1q-1 polypeptide. The recombinant protein was eluted from the affinity column in 0.6 M NaCl, 20 mM Tris-HCl, pH 8.0) at 5 volumes/g, wet weight. The culture was then centrifuged at 4,000 × g for 20 min, and the cell pellet was resuspended in sonication buffer (50 mM sodium phosphate, 0.3 M NaCl, pH 8.0) at 5 volumes/g, wet weight. The sample was frozen in a dry ice/ethanol bath, thawed in cold water, and sonicated on ice (1-min burst/1-min cooling; 2–300 watts), and cell breakage was monitored by measuring the release of nucleic acids at A260 nm. The cell lysate was centrifuged at 10,000 × g for 20 min, the supernatant was collected, and purification of the His6- tagged recombinant fragment was performed by affinity chromatography using nickel-nitrilotriacetic acid resin (Qiagen GmbH) under native conditions. The recombinant protein was eluted from the affinity column in sonication buffer, pH 6.0, containing 10% glycerol and 0.2 M imidazole, and the native status of gC1q-1 was checked by circular dichroism. The EMILIN-1 proteins that were used in the present study, including the recombinant human full-length EMILIN-1 and the LEU-COL-gC1q-1 polypeptide produced in 293-EBNA cells as well as the gC1q-1 polypeptide produced in Eiucheria coli cells (Fig. 1A), were analyzed by SDS-PAGE under reducing conditions using 8% gels and Coomassie Brilliant Blue staining and are shown in Fig. 1B.

Production of mAbs—BALB/c mice were immunized with EMILIN-1 or with the recombinant gC1q-1 polypeptide. 0.1 mg of recombinant protein for each mouse was emulsified with complete Freund’s adjuvant and injected intraperitoneally. Four repeated injections every 10–14 days with the same amount of protein emulsified with incomplete Freund’s adjuvant were administered. Three days after the last booster injection, the spleens were removed and the splenocytes fused with the cell line P3X63Ag8-653 (26). Culture fluids of the resulting hybridomas were screened for anti-EMILIN-1 activity in ELISA and Western blotting. Several hybridomas that recognized EMILIN-1 and reacted with the antibodies in ELISAs were selected and subcloned twice before using. Rabbit antiserum against human EMILIN-1 obtained by immunization with purified recombinant protein was absorbed onto a CNBr-activated Sepharose resin saturated with mock-transfected cell proteins as described before (1).
Competitive ELISA—Poly styrene wells coated with gC1q-1 and saturated with 2% (w/v) BSA were first incubated for 1 h with 3-fold dilutions of unlabeled mAbs against EMILIN-1 purified by protein A-Sepharose (Amersham Biosciences) followed by a 1-h incubation with 15–20 ng of different biotin-labeled mAbs. Purified mAbs were labeled by the biotin procedure as recommended by the manufacturer (Pierce). 100% binding was calculated as the binding obtained in the presence of an unrelated mAb as inhibitor.

Cell Adhesion Assay—The quantitative cell adhesion assay used in this study is based on centrifugation and has been described previously (27, 28). Briefly, six-well strips of flexible polyvinyl chloride denoted CAFCA (centrifugal assay for fluorescence-based cell adhesion) miniplates, covered with double-sided tape (bottom units), were coated with the appropriate secondary antibodies and with 2% (w/v) poly (l-lysine) for 2 min. The cells were then incubated with 4% (w/v) formaldehyde for 10 min and permeabilized in PBS containing 0.1% Triton X-100 for 2 min. Cells were then plated onto the wells of the bottom miniplates) and cells that fall to bind to the substrate. The miniplates were then incubated for 20 min at 37 °C and subsequently mounted together with a similar CAFCA miniplate to create communicating chambers for subsequent reverse centrifugation. In some instances, cells were preincubated with blocking antibodies or nonblocking control antibodies for 30 min and then added to the miniplates coated with the various substrates. Another series of experiments, in which the CAFCA miniplate assemblies were centrifuged at 43, 170, and 380 × g, were also performed with the aim of determining the relative strength of cell adhesion to the various substrates. The relative number of cells bound to the substrate (i.e. remaining in the wells of the bottom miniplates) and cells that fall to bind to the substrate (i.e. remaining in the wells of the top miniplates) was estimated by top/bottom fluorescence detection in a computer-interfaced SPEC-TRAFluor Plus microplate fluorometer (TECAN). Fluorescence values were elaborated by the CAFCA software (TECAN) to determine the percentage adherent cells, of the total cell population analyzed, according to a previously published formula (28, 29). Statistical significance determined by Student’s t test was set at p < 0.001. In experiments aimed at examining the effects of blocking antibodies, the various antibodies were added directly to the wells, just before plating the cells. In other experiments aimed at examining the effect of inhibition of cell adhesion by purified integrins, soluble α5β1 or α5β2 was added (5 μg/ml directly to the wells, just before plating the cells).

Immunofluorescence—Multwll plates or acid-washed coverslips were coated with 10 μg/ml FN, 10 μg/ml EMILIN-1, 20 μg/ml gC1q-1, 10 μg/ml VN, 20 μg/ml C1q-C, and the 20 μg/ml synthetic peptide CS-1 for 16 h at 4 °C. Non-specific binding was blocked with 1.0% radioimmune assay grade BSA (Sigma). Cells were then plated onto the various substrates for 30–40 min as indicated, then they were fixed with 4% (w/v) formaldehyde for 10 min and permeabilized in PBS containing 0.1% Triton X-100 for 2 min. The cells were then incubated for 1 h with primary antibodies in PBS, washed, and incubated further with the appropriate secondary antibodies and with 2 μg/ml Texas Red-conjugated phalloidin (Molecular Probes) for 1 h. After extensive washes, coverslips were mounted in Mowiol 4–88 (Calbiochem-Novabiochem) containing 2.5% (w/v) DABCO (Sigma). Images were acquired with a Bio-Rad MRC-1024 confocal system using Bio-Rad Lasersharp software and a 60 × phase/fluorescence objective on a Diaphot 200 Nikon.

Motility Assay—Migration experiments involving haptotactic movement of the cells through a porous membrane were performed using Transwells (Corning Costar Corporation, Cambridge, MA). The under side of the insert membrane was coated with the various molecules in bicarbonate buffer at 4 °C overnight and blocked with 1% BSA for 1 h at room temperature. Cells were aliquoted into the upper side of each insert unit (1 × 104 cells/insert), and after 4 h the number of migrated cells/field was evaluated under the microscope.

RESULTS

Cell Adhesion—The potential cell adhesion capacity of recombimant EMILIN-1 had been already assessed using several cell lines and found to be of comparable potency but of distinct pattern with that of FN, a prototype adhesive molecule (2). To investigate further the site(s) of cell attachment, mAbs against the full sized recombinant EMILIN-1 or the gC1q-1 domain were generated and used in conjunction with recombinant proteins to localize the reactive epitopes by solid phase ELISA. The localization of the antibody epitopes is shown schematically in Fig. 2A. MAbs 837C and 1H2 were the only one that bound to the full sized EMILIN-1 and not to the other polypeptides, indicating that its epitope maps to the coiled-coil or the EM1 domain region. Instead, several mAbs bound to both EMILIN-1 and the LEU-COL-gC1q-1 polypeptide, indicating that they map in the COL domain or in the short upstream region. Two mAbs (2F1 and 2C8) bound only to the LEU-COL-gC1q-1 polypeptide, suggesting that their epitopes are masked in full sized EMILIN-1 during its assembly process. Finally, seven mAbs recognized all three polypeptides and thus map to the gC1q-1 domain.

Next, to determine which domain(s) would be necessary and sufficient to promote cell adhesion, cells were allowed to adhere to EMILIN-1 in the presence of mAbs directed against different epitopes of the EMILIN-1 molecule. Although the presence of antibody 1H2 (mapping to the gC1q-1 domain) caused complete inhibition of SW982 cell attachment (Fig. 2B), mAbs mapping to other domains were totally ineffective. Also, several mAbs mapping to the gC1q-1 domain were unable to block cell adhesion. Because by increasing the amounts of nonblocking antibodies added we could not obtain lower attachment levels (data not shown), it seems reasonable to conclude that blocking by 1H2 was highly specific. To investigate further the binding site specificity of mAb 1H2 on the gC1q-1 domain a competitive inhibition solid phase ELISA binding assay was performed using EMILIN-1 as a substrate and a number of mAbs as competitors. Binding of biotin-labeled 1H2 to EMILIN-1 was unaffected by several mAbs mapping on the gC1q-1 domain except 1H2 itself (Fig. 2C), suggesting that this mAb detects a unique epitope involved in cell recognition and adhesion.
Characterization of Cell Adhesion to EMILIN-1 and gC1q-1—Although EMILIN-1 promoted an effective cell adhesion, the very large disulfide-bonded aggregates of this protein produced by transfected 293-EBNA cells make it less suitable than gC1q-1 to investigate its role in cell adhesion phenomena further. As a preliminary analysis we sought then to compare the adhesive function of full sized EMILIN-1 with that of the gC1q-1 polypeptide on a larger spectrum of cells (Table I). The two ligands displayed a comparable ability to promote cell adhesion, with EMILIN-1 displaying a somewhat higher percentage of cell attachment. Only HT-1080 cells were found not to adhere to both ligands to the same extent, but clearly preferred EMILIN-1 (97% adhesion on EMILIN-1 versus 45% on gC1q-1).

Next, the kinetics of cell adhesion was investigated. Cell attachment on both EMILIN-1 and gC1q-1 showed a kinetics similar to that on FN, as 50% cell binding was observed within 10 min of plating, and the maximum level of attachment was reached with all ligands at 20 min (Fig. 3A). Furthermore, SW982 cells attached to both EMILIN-1 and gC1q-1 in a dose-dependent manner (Fig. 3B). Under the conditions used, adhesion reached plateau levels at a coating concentration of about 5 μg/ml for both substrates, although on a molar base full sized EMILIN-1 resulted at least 10 times more effective than the isolated gC1q-1 domain. However, the coating efficiency of the two ligands was verified to be similar on the type of plastic used for the CAFC data (not shown). Divalent cations such as Ca^{2+} and Mg^{2+} are generally necessary for integrin-ligand recognition (29). To evaluate the integrin dependence of the EMILIN-1 and gC1q-1 adhesive activities, cell adhesion was determined in the absence of cations and found to be negligible (data not shown). Furthermore, cations regulated cell adhesion in a concentration-dependent manner, with Mg^{2+} showing a higher effect (data not shown).

The cell adhesion assay used in this study permits the estimation of the relative adhesion strength displayed to a given substrate by varying the cell detachment force applied. Thus, we next examined the profile of cell attachment to EMILIN-1 and gC1q-1 compared with the adhesion strength obtained on FN (Fig. 3C). The force necessary to detach 50% SW982 cells was ≈500 μg, 250 μg, and 270 μg for FN, EMILIN-1, and gC1q-1, respectively. Taken together these results indicate that the constitutively active receptors involved confer to these cells the capability of recognizing EMILIN-1 and gC1q-1 with a similar but lower relative avidity compared with the levels attained with FN.

Cell Adhesion to gC1q-1 Is β1 Integrin-dependent —The above results suggested the potential for integrin-gC1q-1 interactions. In fact, consistent with the inhibition of cell adhesion by EDTA and with the cation dependence of cell attachment (data not shown), the constitutive attachment of SW982 cells to gC1q-1 but also to EMILIN-1 was entirely mediated by a β1 subunit-containing integrin (Fig. 4). In fact, cell adhesion was completely blocked by 4B4, a neutralizing β1 integrin subunit mAb, whereas the αβ2-blocking mAb failed to inhibit adhesion.

C1q-C' has been shown to support adhesion and spreading of endothelial cells (30, 31), to induce chemotaxis and chemokinesis of mast cells (32), and to promote eosinophil migration (33). Cell adhesion to C1q-C' could be blocked by RGD-containing peptides (34), supporting the notion that C1q-C'-mediated adhesion and spreading may involve, in addition to the various nonintegrin C1q receptor(s) (35), the participation of integrin(s). Whether C1q-C' promoted cell adhesion in our assay system and whether this activity could be abolished by the 4B4 β1-blocking mAb were then investigated in analogy with the present findings with both EMILIN-1 and gC1q-1. C1q-C' strongly promoted cell adhesion of SW982 cells; however, treatment with 4B4 mAb was totally ineffective (Fig. 4). As expected, this mAb displayed no inhibition of the αβ2-dependent VN-mediated cell adhesion.

Cell Adhesion to EMILIN-1/gC1q-1 Is αβ1-dependent—Because the results described above indicate that SW982 cells bound to EMILIN-1 or gC1q-1 domain via a β1 integrin, we next examined the effects of a subunit function-blocking mAbs in inhibiting the gC1q-1 or the EMILIN-1 binding activity.

### Table I

| Cells          | FN (%) | EMILIN-1 (%) | gC1q-1 (%) |
|----------------|--------|--------------|------------|
| Hs913T        | 69     | 15           | 40         |
| SW982         | 90     | 90           | 95         |
| SK-UT-1       | 96     | 92           | 97         |
| HT-1080       | 99     | 97           | 45         |
| RAMOS         | 70     | 97           | ND         |
| Jurkat        | 86     | 93           | 97         |
| FLG 29.1      | 92     | 7            | 5          |
| HUVEC         | 81     | 63           | 91         |
| NIH3T3        | 92     | ND           | 9          |
| NQ22          | ND     | 91           | 93         |
| NQ29          | ND     | 86           | 95         |

* ND, not determined.

**Fig. 3. Cell attachment to EMILIN-1 and gC1q-1.** Kinetics of cell attachment (A) and dose-response relationship (B) are shown. Recombinant gC1q (open circles) was purified under conditions favoring refolding and plated at the indicated concentrations. Recombinant EMILIN-1 (closed circles) was purified from the cell culture medium of 293-EBNA cells. BSA (open squares) and human FN (closed squares) were used as negative and positive control ligands. Bars represent the S.D. of triplicate assays. C, assessment of the relative strength of cell attachment to EMILIN-1, gC1q-1, and FN by varying the centrifugal force applied to dislodge the cells. SW982 cells were allowed to attach to the substrates coated with the various ligands at 3 μg/ml, in the presence of 1 mM Ca^{2+} or 1–3 mM Mg^{2+}.

![Figure 3](http://www.jbc.org/Downloadedfrom)
Treatment of cells with inhibitory antibodies indicated that the α subunit was neither α1, α2, α3, α5, α6, nor αv because blocking mAbs to these subunits had no effect on cell attachment. In separate experiments their function blocking activity for their respective ligands, i.e. FN, VN, and LNs, was confirmed with various cell lines (data not shown). In contrast, only the anti-α4 mAb P1H4 fully abrogated cell adhesion to gC1q-1 and up to 70% to EMILIN-1. SW982 cells express consistent levels of α4 integrin chains as well as α1, α3, α5, αv, and β1 as evaluated by fluorescence-activated cell sorter analysis (data not shown). That α4β1 really participates in cell adhesion to gC1q-1 and the inhibition detected is not the mere consequence of the expression levels of this integrin were demonstrated also by the dose dependence of inhibition of adhesion (Fig. 5) and by the finding that a control anti-α4 mAb, P4G9, directed against epitope A (not related to cell attachment to ECM) on the α chain (36), was ineffective (Fig. 5A) as was the function-blocking mAb PS2 recognizing the murine α4 (data not shown). To investigate further the specificity of the recognition by α4β1 integrin, additional experiments were performed. Binding between EMILIN-1 and α4β1 integrin was evaluated in a solid phase assay (Fig. 5B, inset). Binding to α4β1 was twice as high as that to α2β1, and it was specifically inhibited by anti-α4 function-blocking mAb. Second, EMILIN-1 and FN were adsorbed on the plastic substrate, and cell adhesion was evaluated in the presence or in the absence of purified α4β1 or α4β4 integrins. Although α4β1 had no effect, a 40% reduction in cell adhesion was detected only when soluble α4β1 was added (Fig. 5).

![Figure 4](http://www.jbc.org/)

**Figure 4.** The function-blocking β1 mAb perturbs cell attachment to EMILIN-1 and gC1q-1 but not to C1q-C. The miniplate wells were coated with 10 μg/ml EMILIN-1, gC1q-1, C1q-C, or VN. The antibodies were added at 5 μg/ml just before cell plating. Results shown are the average ± S.D. of triplicate experiments.

![Figure 5](http://www.jbc.org/)

**Figure 5.** Cell attachment to gC1q-1 is α4 integrin-dependent. A, attachment of SW982 cells in the presence of different function-blocking mAbs. The miniplate wells were coated with 10 μg/ml gC1q-1. Antibodies were added at 5 μg/ml just before cell plating: 4B4 (anti-β1), TS2/7 (anti-α1), 12/171 (anti-α2), F4 (anti-α3), P1H4 (anti-α4), LBS5 (anti-αv), B2121 (anti-β1), and LM609 (anti-αv). B, attachment of Jurkat cells to gC1q-1 (circles) and to FN (squares) coated at 10 μg/ml was performed in the presence of varying concentrations of the function-blocking mAb P1H4 (closed symbols) and a non-function-blocking mAb (open symbols). C, attachment of Jurkat cells in the presence of soluble integrins. The miniplate wells were coated with 10 μg/ml EMILIN-1 or FN. Integrins were added at 5 μg/ml just before cell plating. Inset, protein-protein interaction in a solid phase assay. Wells were coated with 10 μg/ml purified integrins; soluble EMILIN-1 or FN was added at 10 μg/ml for 60 min in the presence or in the absence of function-blocking mAbs. The wells were then incubated with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. D, attachment of K562 and αv/K562 cells in the presence of 10 ng/ml 12-O-tetradecanoylphorbol-13-acetate. The miniplate wells were coated with 10 μg/ml gC1q-1. Antibodies were added at 5 μg/ml just before cell plating: 4B4 (anti-β1) and P1H4 (anti-α4). Results shown are the average ± S.D. of triplicate experiments.
5C). As a third approach, adhesion of $\alpha_4$K562 cells to EMILIN-1 was analyzed. Adhesion to EMILIN-1 reached 45% (Fig. 5D). In contrast, wild type K562 cells did not adhere at all.

**SW982 Cells Do Not Form Focal Adhesion Plaques or Stress Fibers after Attachment to EMILIN-1 and gC1q-1**—To distinguish between cell tethering to and cell spreading on the substratum, the cell morphology of adherent cells was examined by confocal microscopy and double immunofluorescence staining with phalloidin and anti-paxillin antibodies. In accord with the different strengths of cell adhesion, there was a pronounced qualitative difference in the morphology of cells attached to EMILIN-1 compared with the cells plated on FN or VN. As expected, cells attached to FN and VN appeared well spread and displayed wide ruffles, extending in multiple direction (Fig. 6A). Phalloidin staining of cells spread on FN and VN revealed a high number of actin-containing stress fibers. In addition, paxillin localized to large focal contacts at the tips of stress fibers. In contrast, phalloidin staining of cells attached to EMILIN-1 revealed that the actin organization was mainly along the cell periphery at the level of extended cell protrusions, reflecting a different organization status of the actin cytoskeleton compared with the cells on FN or VN; paxillin was distributed evenly in the cell cytoplasm without any apparent focal contact formation.

Because SW982 cells express several integrins that can recognize FN or VN, whereas cell adhesion to EMILIN-1 depends only upon $\alpha_4\beta_1$, the morphology of cells attached to gC1q-1 and CS-1 was then compared. Under these conditions, the $\alpha_4\beta_1$-dependent adhesion to CS-1 was different compared with adhesion to FN, with the cells displaying a smaller size and lacking clearly visible phalloidin-positive stress fibers and focal contacts (Fig. 6B). In analogy with the pattern detected on EMILIN-1, adhesion to gC1q-1 was accompanied by the formation of several cellular protrusions positive for colocalized actin and paxillin, but again with no detectable stress fibers nor focal contacts. Cells attached to C1q-C', shown for comparison, also were poorly spread with no visible stress fibers or focal contacts. $\alpha_5\beta_1$ is not localized in focal adhesions in most cell types, and it has been reported that the $\alpha_4$ cytoplasmic tail confers a migratory activity (37) and that it promotes broad lamellipodia protrusions (38). The pattern of cell adhesion to EMILIN-1 and/or gC1q-1 was suggestive of a promigratory function, cells were added to the upper side of Transwell inserts. As seen in Fig. 7, cell migration extensively toward the migration was fully abrogated by the addition of anti-$\beta_1$ or anti-$\alpha_4$ function-blocking mAbs.

**FIG. 6**. SW982 cell attached onto EMILIN-1 and gC1q-1 do not form stress fibers or focal adhesions. A, cells were plated for 30 min on substrate-coated coverslips, fixed, and stained with Texas Red phalloidin and anti-paxillin antibody followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG. The overlays clearly demonstrate stress fibers and focal adhesion formation only on cells attached to FN and VN. B, cells were plated on CS-1, gC1q-1, and C1q-C' and stained as in A.

**FIG. 7**. Cell migration assay. The underside of the insert membrane was coated with 20 mg/ml EMILIN-1 or gC1q-1 at 4°C in bicarbonate buffer and blocked with 1% (w/v) BSA for 1 h at room temperature. HT-1080 cells (105) were aliquoted to the upper side in the presence or in the absence of blocking mAbs. The migratory ability of HT-1080 cells was evaluated as the number of cells migrated/field after 4 h.

**DISCUSSION**

In this study we have demonstrated that the gC1q-1 domain of EMILIN-1 is recognized by the integrin $\alpha_4\beta_1$. In fact, cell adhesion and receptor binding were inhibited by function-blocking anti-$\beta_1$ and anti-$\alpha_4$ mAbs. This is the first description of $\alpha_4\beta_1$ recognition of a gC1q domain and indicates that EMILIN-1 appears to be distinct from several of the elastic fiber-associated constituents that use mainly $\alpha_5\beta_1$, but also $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_8\beta_1$, or $\alpha_9\beta_1$ for cell adhesion and spreading (18–22). Recognition of the gC1q-1 domain did not require exogenous activation of $\alpha_4\beta_1$ by the addition of Mn2+ or anti-$\beta_1$-activating mAbs, but the presence of Ca2+ or Mg2+ was necessary because EDTA treatment fully abolished cell adhesion.

The finding that gC1q-1 maintains full capability of cell attachment, although at a higher molar ratio compared with EMILIN-1, suggests that the rest of the molecule does not impose conformation constraints at least to affect its adhesive function. The differential activity in molar terms between EMILIN-1 and gC1q-1 is not without precedent for other ECM adhesion proteins. For example, the central cell binding domain of FN showed a 100–200-fold reduced adhesion activity compared with intact FN (39), and the recombinant globular domain 3 of LN-5 was about 250-fold less effective than LN-5 in molar terms (40). However, in those cases synergistic cell binding sites contributing to cell adhesion are present (41, 42) or hypothesized (40). EMILIN-1-dependent cell adhesion was apparently fully accounted for by the gC1q-1 domain because a mAb against gC1q-1 could totally abrogate cell adhesion to EMILIN-1, and the integrin $\beta_1$ subunit-blocking mAb could block cell adhesion to gC1q-1 as well as EMILIN-1. Thus, the trimeric nature of gC1q-1 and the packing geometry of these globular domains represent a sufficient structural organization...
to promote cell attachment even at moderately low ligand densities. The few previous studies on cell adhesion using gC1q domain-containing substrates such as type VIII collagen have indicated that integrins of the αβ₁ type were involved (43, 44); however, because in both reports the pepsin-resistant collagenous part of the molecule devoid of the globular C1q domain was used, the cell adhesive function was not attributable to the gC1q domain.

C1q-C⁺, the prototype of the C1q/tumor necrosis factor superfamily, is a promiscuous protein, and its function is not limited to the recognition and triggering of the classical complement pathway, but following interaction with cells it can directly mediate several immune effector functions such as phagocytosis, chemotaxis, and the generation of procoagulant activity (45). C1q-C⁺ binds via either of its two structurally and functionally different domains to a large number of proteins among which cell-associated receptors/binding proteins with quite diverse molecular structure and function were identified on several cell types (35, 46). Although the significance of those cell-associated molecules has been undermined by the controversy surrounding each of the identified molecules, among the various receptors/binding proteins gC1q-R, a predominantly mitochondrial protein (47) that can also be expressed on the cell surface, was shown to interact with the globular C-terminal domain of C1q-C⁺ (48) and promote cell adhesion (35). A recent study supports the hypothesis that C1q-C⁺-mediated endothelial cell adhesion and spreading require cooperation between gC1q-Rs and β₁ integrins (34). This conclusion was based on the finding that anti-β₁, 4B4 blocked both cell adhesion and spreading and that anti-α₁ mAb blocked spreading. In addition, cell spreading on and in part cell adhesion to C1q-C⁺ were also inhibited by RGDS-containing peptides (34). The present data on C1q-C⁺ are apparently not in accord with those of Feng et al. (34), but in that study cell adhesion to and spreading on C1q-C⁺ were evaluated at 1–2 and 6–7 h, respectively, and not at 30 min as in the present study. For this reason the reported involvement of integrins might well have been a secondary phenomenon. Furthermore, that cell attachment to EMILIN-1 and gC1q-1 was fully dependent upon a β₁ integrin, αβ₁, whereas cell attachment to C1q-C⁺ was unaffected by a β₁-blocking mAb, highlights the presence of different cell adhesion mechanisms to account for interaction with gC1q domains.

αβ₁ integrin mediates tethering, rolling, and firm arrest on VCAM-1, which is expressed on endothelial cells at sites of inflammation (49). αβ₁ also binds to alternatively spliced variants of FN (50) which contain connecting segment CS-1. The αβ₁ integrin shows a broader ligand binding specificity than most other members of the integrin family (51) because overlapping but distinct binding mechanisms exist for different ligands, and distinct conformational changes are induced upon engagement by different ligands (52). In fact, several additional naturally occurring ligands for αβ₁ have been reported recently (53–59), and there are no sequence homologous to the known αβ₁ recognition motifs QIDSP (VCAM-1), EILDV (FN), MLDG (EC3 disintegrin peptide) within the gC1q-1 domain of EMILIN-1 (1).

Because αβ₁ exhibits a predominantly leukocyte expression pattern, the finding that nonhematopoietic cells could attach very efficiently to gC1q-1 via αβ₁ was not expected. The present finding that both hematopoietic and nonhematopoietic cells attach to gC1q-1 via αβ₁ without any prior artificial cellular activation or immunological manipulation of the integrin receptor complex suggests that, irrespective of the cell-specific constraints, the constitutive activation status of αβ₁ is sufficient to determine cell attachment to gC1q-1. Furthermore, and in accord with the literature (60), only αβ₁/K562 needed cellular activation to display cell adhesion. Melanoma cells were reported to bind to CS-1 peptide according to αβ₁ density (61). In addition, in the developing human aorta αβ₁ was detected on smooth muscle cells at 10 weeks, but its expression was reduced within the 24th week of gestation and disappeared in the adult aortic media (62). However, smooth muscle cells from intimal atherosclerotic thickening of adult aorta reexpress αβ₁ (62), suggesting a possible role in the induction of smooth muscle differentiation. In accord with this notion is the finding that αβ₁ activates the L-type calcium channels in vascular smooth muscle and causes arteriole vasoconstriction, pointing to an involvement in the modulation of vascular tone and in vascular responses to mechanical signals, such as pressure and flow (63). The elevated expression of EMILIN-1 detected in vascular tissues (3) is in keeping with the above results and hypothesizes a role also for EMILIN-1 in development as well as pathological processes of large vessels.

Cell spreading is a complicated phenomenon that requires active remodeling of adhesion sites to enable cells to extend processes subsequent to attachment. A current model for cell adhesion is that there is a hierarchical mechanism for the formation of focal adhesions in which paxillin accumulation and a small cluster of ligand-bound integrins favor the nucleation of additional signaling and structural molecules joining the complex (64). After the integrin-substrate interaction, cells increase their surface contact area with the ECM through formation of actin microfilaments and initial cell spreading. This attachment stage is considered an intermediate stage between that of the weak initial contact and the strong adhesion to the appropriate ECM ligands. Most ECM constituents including FN and VN promote cell adhesion and cause cytoskeletal reorganization as described above. However, αβ₁-dependent interactions that were studied extensively in hematopoietic cells have shown that the initial and intermediate stages of cell adhesion, i.e. attachment and spreading, were supported, whereas focal adhesion and stress fiber formation, characteristic of strong cell adhesion, are rarely if ever observed (65). The distribution pattern of actin and paxillin suggests that attachment to EMILIN-1 and gC1q-1 leads to an accumulation of ruffles-inducing signals; this would explain the lack of polarization and of stress fiber formation. In an elegant study using αβ₁/green fluorescent fusion proteins to evaluate the role of α4 in lamellipodia protrusions in response to scratch-wounding, Yang and collaborators (38) demonstrated that αβ₁ forms transient puncta that do not colocalize with paxillin-positive focal adhesion complexes. It has been suggested that intermediate states of adhesion favor cell motility and that cell migration is diminished in cells exhibiting strong adhesion (66). Thus, whereas αβ₁ in focal complexes mediates cell substratum adhesion stabilizing it (67), αβ₁ promotes lamellipodia formation independent of focal adhesion complexes (38). Accordingly, αβ₁-dependent migration toward gC1q-1 was demonstrated in the present study. The formation of focal adhesion stabilized by stress fibers is disadvantageous for cell detachment, and the absence of mature focal adhesions has long been associated with a motile phenotype. The lack of stress fibers and focal adhesions in cells attached to EMILIN-1 and gC1q-1 indicates that cells, by binding via αβ₁ to these ligands, are preferentially stimulated to migrate rather than to adhere firmly.

Acknowledgments—We thank Drs. R. Pytel, A. Sonnenberg, V. Woods, and L. Zardi for providing function-blocking mAbs, Dr. Martin J. Humphries for purified αβ₁ integrin, Dr. J. Teixido for the αβ₁/K562 cell line, and Dr. V. Gattei for the FLG 29.1 cell line.
β₁ Integrin-dependent Cell Adhesion to EMILIN-1 Is Mediated by the gC1q Domain

Paola Spessotto, Marta Cervi, Maria Teresa Mucignat, Gabriella Mungiguerra, Ida Sartoretto, Roberto Doliana and Alfonso Colombatti

J. Biol. Chem. 2003, 278:6160-6167.
doi: 10.1074/jbc.M208322200 originally published online November 26, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208322200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 67 references, 39 of which can be accessed free at http://www.jbc.org/content/278/8/6160.full.html#ref-list-1