Integrins are cell surface receptors involved in numerous pathological processes such as metastasis invasion and abnormal angiogenesis. To target these receptors, the epidermal growth factor (EGF)-like domain of human complement protease C1r was used as a natural scaffold to design chimeric modules containing the RGD motif. Here we report a high yield bacterial expression system and its application to the production of two such modules, EGF-RGD and V2, the latter variant mimicking the RGD-containing domain of disintegrins. These modules were characterized chemically, and their biological activity was investigated by cellular assays using various Chinese hamster ovary cell lines expressing β1 and β2 integrins and by surface plasmon resonance spectroscopy. Remarkably, the modifications leading to the V2 variant had differential effects on the interaction with β1 and β2 integrins. The disintegrin-like V2 module exhibited enhanced binding affinities compared with EGF-RGD, with \( K_d \) values of 7.2 nM for \( \alpha v \beta 3 \) (a 4-fold decrease) and 3.5 nM for \( \alpha v \beta 1 \) (a 1.5-fold decrease), comparable with the values determined for natural integrin ligands. Analysis by NMR spectroscopy also revealed a differential dynamic behavior of the RGD motif in the EGF-RGD and V2 variants, providing insights into the structural basis of their relative binding efficiency. These novel RGD-containing EGF modules open the way to the design of improved variants with selective affinity for particular integrins and their use as carriers for other biologically active modules.

Targeting the appropriate cell type is a major challenge in cancer and cell therapy. Integrins, a family of cell receptors for plasma proteins, extracellular matrix proteins, and cell surface ligands (1–3), are potential targets for this purpose, because they are expressed by all cell types and are involved in many physiological functions including cell adhesion, proliferation, or differentiation, and pathological processes such as metastasis invasion, abnormal angiogenesis and vascularization, or thrombosis (4). In mammals, 18 \( \alpha \) and eight \( \beta \) subunits assemble into 24 different non-covalent \( \alpha \beta \) heterodimers that mediate bidirectional signals through the cell membrane. In response to cell activation, “inside-out” signals control the level of binding affinity for the ligand. In turn, ligand binding induces rearrangements in integrin structure that trigger “outside-in” signaling pathways (5). Extensive cross-talk also takes place between integrin and growth factor receptor signaling pathways (6, 7), influencing most integrin functions.

The \( \beta \) integrin subset, comprising the major fibronectin receptor \( \alpha v \beta 1 \), is the most widely expressed. The \( \beta 3 \) subset includes the fibrinogen receptor \( \alpha v \beta 3 \) present on platelets and megakaryocytes, and \( \alpha v \beta 3 \), originally described as the vitronectin receptor, which also binds a variety of matrix proteins including fibronectin and fibrinogen. Integrin \( \alpha v \beta 3 \) is expressed on vascular cells during angiogenesis (8, 9) and is also present in the adult on activated leukocytes, osteoclasts, and macrophages, where it participates in bone resorption and ingestion of apoptotic cells. The expression of various integrins has been shown to be deregulated in a number of cancer cells and invasive tumors and is thought to participate in the malignancy phenotypes (10–14). Thus, a decreased expression of \( \alpha v \beta 3 \) appears to increase the degree of tumorigenicity. In contrast, the level of \( \alpha v \beta 1 \) is correlated with the survival and metastatic activity of tumor cells (10, 15) and has an important role in angiogenesis in tumors (11). Integrins, particularly \( \alpha v \beta 3 \), are therefore considered as appropriate targets for cancer therapy. Intracellular drug or DNA delivery by integrin-targeted vectors has been shown to use the endocytic pathway (16).

Many integrins, particularly \( \alpha v \beta 1 \) and \( \alpha v \beta 3 \), recognize the RGD motif as the critical determinant in their ligands (17). The structural basis of this interaction has been documented recently (18, 19) from the crystal structure of the extracellular domain of \( \alpha v \beta 3 \) alone or in complex with an RGD ligand. Among the natural ligands of integrins, disintegrins (20, 21), which are snake venom polypeptides related to the multifunctional AD-AMs protein family (22), are potent inhibitors of integrin function. They block platelet aggregation through interaction with \( \alpha I b \beta 2 \) and also inhibit \( \alpha v \beta 3 \) and \( \alpha v \beta 1 \) functions. Disintegrins are cysteine-rich peptides of 48–84 amino acid residues, and their activities are directly related to the presence of an RGD or a related sequence in a large flexible loop (23, 24) and mimic natural integrin ligands such as fibronectin, fibrinogen, or vitronectin. Integrin-targeted molecules developed in the recent years include antibodies, cyclic peptides, peptidomimetics, viruses, and small molecules (25). With the aim to develop a molecule able to interact with high affinity with integrins, we chose to introduce the critical RGD sequence in a stable, structurally compatible small protein scaffold. The EGF-like mod-

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§ The abbreviations used are: EGF, epidermal growth factor; CHO, Chinese hamster ovary; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; NOE, nuclear Overhauser effect; Trx, thioredoxin; HSQC, H-detected heteronuclear single-quantum coherence; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy; D-RGDW, the RGDW peptide with the D-isomer of arginine.
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ule of human complement protease C1r appeared as a good
candidate for this purpose, because it is stabilized by three
disulfide bridges and features a large, structurally independ-
ent, and mobile loop between its first two cysteine residues (26,
27). A further advantage of EGF-like modules is their natural
ability to associate with a variety of other module types, as seen
in many natural proteins (25, 29), which opens the possibility of
developing multimodular chimeric proteins.

In a previous study (30), we showed, using chemical synthe-
sis, that insertion of the GRGDSP motif of fibronectin into the
human C1r EGF module resulted in a chimeric molecule with the
ability to mediate attachment of CHO cells expressing the
β1 class of integrins. In the present study we describe an
efficient bacterial expression system that allows high yield
production of chimeric EGF-like modules. This strategy was
applied to the production of two modules, including a new
variant designed to mimic the RGD-containing domain of dis-
integrins. Functional characterization of the recombinant
modules by various methods including cell adhesion and surface
plasmon resonance analysis indicates preferential binding to
β1 integrins, whereas NMR spectroscopy provides insights into
the structural basis of their relative binding efficiency.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, Peptides, and Reagents

The cell lines CHO (ATCC number CCL-61), β1-CHO, and α1β1-CHO
(stably established by transfection with human megakaryocyte β1
and/or α1a cDNA (31)) were cultured in Glutamax Dulbecco's modified
Eagle's medium (Invitrogen) containing 7.5% (v/v) fetal calf serum, 1%
penicillin, and 1% streptomycin. The medium was supplemented
with 800 μg/ml of G418 (Invitrogen) when necessary. Peptides
D-RGDW and RGES were supplied by M.-H. Charon (Commissariat à l'Energie Atomique, Grenoble, France). Polyclonal anti-α1 antibodies
(AB1930) were from Chemicon International (Temecula, CA). Poly-
clonal anti-C1r antibodies recognizing native human C1r were raised in
rabbits (32). Integrins α1β1 from human placenta and α1β1 from human
smooth muscle and placenta tissue were purchased from Chemicon in
the octyl-β-D-glucopyranoside formulation. The purity and integrity of
each integrin sample were checked by SDS-PAGE analysis.

Preparation of the Trx Fusion Proteins

Cloning—The cDNA sequence coding for the human C1r EGF module
was amplified by PCR using the complete C1r cDNA (33) as a
template. A NdeI site and codons for the amino acid sequence EGR
(factor Xa cleavage site) were introduced at the 5' end. The 3' end
was cloned into a stop codon and an EcoRI site. The PCR product was then
ligated to the NdeI and EcoRI sites of the pET28b vector (Novagen,
Madison, WI). The codons for the GRGDSP amino acid sequence
were then introduced by the PCR overlap extension method (34, 35) to
produce the EGF-RGD vector and V2 modules (see Fig. 1). A SacII restriction
site was introduced into the PCR primers to allow mutation screening.
The NcoI/XhoI fragments isolated from the pET28b recombinant plas-
mids were then introduced into the same sites of the linearized pET32a
vector (Novagen). The resulting vectors, pET32-C1r EGF, pET32-EGF-
RGD, and pET32-V2 predicted a protein containing the 109 amino acids
of the Trx module fusions.

Protein Expression and Isolation—Recombinant pET32 vectors were
transformed by transformation into Escherichia coli BL21 (DE3) (Nova-
gen), and bacteria were cultured at 37 °C in a Luria-Bertani medium
containing 1 μM isopropyl-1-thio-β-D-galactopyranoside for 5 h at 37 °C. Bacteria were collected
by centrifugation, suspended in 1/25 culture volume of 20 mM MnCl2,
0.5 mM NaCl, 50 mM imidazole, pH 7.4, containing an anti-protease
mixture (Roche Molecular Biochemicals), sonicated, and then centri-
fuged at 40,000 × g for 30 min at 4 °C. The clarified fraction was added to a
histidine-binding nickel-Sepharose Fast Flow resin (Amersham Biosciences)
packed in an Econo-Pak column (Bio-Rad). After exten-
sive washing with 50 mM imidazole, 20 mM Na2HPO4, pH 7.4, elution
was achieved by 100–500 mM imidazole solutions in the same buffer.

The eluted samples were pooled and dialyzed against 0.05% (w/v) trif-
fluoroacetic acid and freeze-dried. For production of the recombinant mod-
ules isotopically labeled with 15N, freshly transformed bacteria were
grown in an M9 minimal medium complemented with 1 g/liter 15NH4Cl,
4 g/liter glucose and supplemented with 0.1 mM MnCl2, 0.05 mM ZnCl2,
0.05 mM FeCl3, and a vitamin solution according to Jansson et al. (36).

Factor Xa Cleavage, Protein Purification, and Analytical Methods

The recombinant EGF modules were released from thioredoxin
digestion of the fusion proteins (10 μg in 50 ml Tris-HCl, 100 mM NaCl,
5 mM CaCl2, pH 8.0) with factor Xa (1 units/50 μg) for 16 h at 25 °C.
The cleavage mixture was then analyzed and purified by reverse-phase
HPLC using an analytical (0.46 × 25 cm) or a semi-preparative (2.2 ×
25 cm) Vydac C18 column as described previously (30). Fractions were
assayed for enzymatic activity by electrospray mass spectrometry, and the
final puriﬁcation was controlled by N-terminal sequence analysis (30). The chimeric EGF
modules were freeze-dried and stored at 4 °C until use. Protein concen-
trations were determined by absorbance measurement at 280 nm using
molar extinction coefficients of 18,260 M⁻¹ cm⁻¹ for Trx module fusions
and of 4,200 M⁻¹ cm⁻¹ for the cleaved modules.

Adhesion Assays

Adhesion assays were performed in plastic microtitration plates
(Nunc, Inc.). Wells were coated overnight at 4 °C with the recombinant
protein modules (1–100 μg/ml in PBS) or ﬁbronectin (BD Biosciences)
(25 μg/ml in PBS), and free binding sites were blocked with 3% bovine
serum albumin. Cells were harvested at 60–80% of conﬂuence using
trypsin–EDTA (Invitrogen) and then allowed to attach for 1 h at 37 °C
using 5 × 10² cells in 100 μl of medium/well. Non-adherent cells were
removed by three washes in PBS, and the amount of attached cells was
determined using a cell quantiﬁcation kit by adding 20 μl of MTS reagent
(Promega) to each well. After color development, plates were read on a
Dynatech reader at 490 nm. Assays were run in triplicate, and nonspeciﬁc
attachment to BSA alone (less than 5% of total adhesion) was subtracted from all measurements. Control experiments done with
the Trx-C1r EGF or C1r EGF constructs showed no signiﬁcant adhe-
sion. For inhibition experiments, competitors at various concentrations
were preincubated with the cells for 15 min at 4 °C before attachment.
For inhibition by the soluble V2 module, ﬁbrinogen (Stago, Asnières,
France) and ﬁbronectin were used at concentrations yielding half-max-
imal adhesion, namely 5 and 16 μg/ml, respectively.

Staining of Actin Fibers

Glass slides were coated overnight at 4 °C with ﬁbronectin (25 μg/ml)
or the EGF-RGD and V2 modules (100 μg/ml) and then coated with
3% bovine serum albumin for 2 h at 37 °C. Harvested cells were suspended
in medium with serum and then plated onto the coated slides for 2 h at 37 °C.
After the incubation period, cells were ﬁxed for 10 min in PBS containing
3% paraformaldehyde and 2% saccharose and then permeabilized for
20 min in PBS containing 1% Triton X-100 (w/v). After three washes in PBS, actin staining was
performed using rhodamine-labeled phalloidin (Sigma). All incubations
were performed at room temperature. Stained cells were observed under
a laser confocal ﬂuorescence microscope (Leica). Polylysine (70
μg/ml) was used as a negative control for stress ﬁber formation.

Surface Plasmon Resonance Spectroscopy and Data Evaluation

Surface plasmon resonance measurements were performed using a
BIACore 3000 instrument (BIACore AB, Uppsala, Sweden). The running
buffer for protein immobilization was 145 mM NaCl, 5 mM EDTA, 10 mM
Hepes, pH 7.4. The recombinant EGF-RGD and C1r EGF modules were immobilized onto the carboxymethylated dextran surface of a CM5 sensor
chip (BIACore AB) using the amine coupling chemistry (BIACore AB
amine coupling kit). Binding of the puriﬁed α1β1 and α1β1 integrins was measured over 2,500 resonance units of immobilized EGF-RGD and
C1r EGF modules. For Trx immobilized V2 modules, a signal ﬂow rate of 20 μl/min
was used, and after 100 resonance units of a negative control were obtained.
In the running buffer (15 mM NaCl, 25 mM Tris-HCl, pH 7.4, 0.1 mM
CaCl2, 1 mM MgCl2, 10 μM octyl-β-D-glucopyranoside). This buffer was
identical to that of the purchased integrins, including divalent cations
and detergent, to allow sample modiﬁcation. Regeneration of the
surfaces was achieved by injection of 10 μl of a 10 mM solution of the
speciﬁc peptide. The speciﬁcity of binding was obtained by subtracting the background signal, routinely obtained by injection of the
protein sample over an activated-deactivated surface. Immobilized bovine serum albumin or the unmodiﬁed C1r EGF module (30) showed
no integrin binding. The data were analyzed by global ﬁtting to a 1:1

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Langmuir binding model of both the association and dissociation phases for several concentrations simultaneously, using the BIAevaluation 3.2 software (BIAcore AB). The apparent equilibrium dissociation constants (K_d) were calculated from the ratio of the dissociation and association rate constants (k_d/k_a). The data presented for both α_β and α_β correspond to a representative series of binding studies performed on the same sensor chip. In each case, similar results were reproduced from at least three independent experiments, using different integrin batches and different sensor chips.

Polyacrylamide Gel Electrophoresis and Immunoblotting

SDS-PAGE analysis was performed as described by Laemmli (37). Western blot analysis and immunochemical detection of the recombinant proteins were carried out as described by Rossi et al. (38). Chemiluminescence revelation was performed using the ECL kit (Amersham Biosciences) and an anti-rabbit IgG coupled to horseradish peroxidase.

NMR Spectroscopy

The EGF-RGD and V2 modules were dissolved at a final concentration of ~1 mM in 50 mM sodium phosphate, pH 6.7, containing 10% D_2O. All NMR experiments were acquired on Varian INOVA 400 and 800 spectrometers. For the assignment of the amide proton and nitrogen chemical shifts, 15N-edited HSQCC and three-dimensional NOESY-HSQC experiments, as well as two-dimensional, 15N-decoupled TOCSY and NOESY experiments were acquired at 900 MHz. TOCSY and NOESY mixing times were 70 and 150 ms, respectively. All chemical shifts were referenced with respect to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt using the 1H/15N frequency ratio of the zero point of 0.101329118 according to Markley et al. (39). Relaxation measurements were performed at 400 MHz. The 15N heteronuclear R1 and R2 relaxation rates and the 1H/15N NOE were measured using standard pulse sequences (40). Two-dimensional 1H-15N correlation spectra were acquired with 1024 (1H) and 100 (15N) complex points and 16 (R1 and R2) or 64 scans (1H/15N NOE) per t1 increment. The relaxation-caused magnetization decay was sampled at nine different time points for R1 (0.014, 0.072, 0.131, 0.189, 0.248, 0.306, 0.393, 0.466, and 0.598 s) and seven time points for R2 (0.008, 0.04, 0.072, 0.104, 0.152, 0.219, and 0.252 s). The first time point was repeated at the end to check for sample and measurement stability and to evaluate experimental errors. For the steady-state (1H/15N NOE) measurements, two spectra were acquired with and without proton saturation in an interleaved manner. The recycle delays were set to 1 s (R1 and R2) and 5 s (1H/15N NOE). Data processing and peak picking were performed using the FELIX 2000 software (Accelrys Inc.). The 15N R1 and R2 relaxation rates were determined from peak intensities rather than from peak volumes, using a non-linear least-squares fit to a two-parameter single exponential function I(t) = I(0)exp(-R1t), where I(t) is the peak intensity after a relaxation delay of time t, and I(0) is the initial peak intensity. Uncertainties in the resulting R1 and R2 values were estimated by Monte-Carlo simulations with 1000 random Gaussian noise iterations, taking into account the experimental noise from the spectra. The transverse relaxation rate constants R2 were calculated from R1 rate constants using the relation, R_2 = cos^2(θ) * R_1 + sin^2(θ) * R_1, where θ = tan-1(1/2π/ω, γ, R_1), and ω is the frequency difference between the 15N carrier and the frequency of the observed nitrogen. The steady-state heteronuclear NOE were determined from the ratio of the peak intensities measured in experiments acquired with and without proton saturation.

Interaction between Integrins and Nickel-Sepharose-Trx Modules

Cells were lysed on the culture plate for 1 h at 4 °C in PBS containing 1 mM CaCl_2, 1 mM MgCl_2, 1% Triton X-100, and an anti-protease cocktail (Roche Molecular Biochemicals). The cell extract was clarified by centrifugation at 13,000 × g for 15 min at 4 °C. Proteins were quantified using a micro BCA assay (Pierce). Fractions of the cell extract, containing 1 mg of total proteins, were incubated for 2.5 h at room temperature with an equal volume of a nickel-Sepharose-Trx module resin prepared by mixing 1 mg of Trx module fusion protein with 400 μl of chelating Sepharose. After extensive washes using PBS containing 1 mM CaCl_2, 1 mM MgCl_2, and 0.05% (w/v) Tween 20, bound proteins were recovered by elution with 1 ml of PBS containing 10 mM EDTA and 50 mM NaCl. Following trichloroacetic acid precipitation, samples were analyzed by SDS-PAGE under non-reducing conditions and Western blotting using anti-α_β antibodies and chemiluminescence detection.

RESULTS

Engineering, Production, and Biochemical Characterization of Recombinant EGF-RGD Modules—Our previous data based on a synthetic EGF-RGD module (30) led us to develop a recombinant strategy to overproduce the original module and a variant designed to mimic the structure of disintegrins (Fig. 1A). Based on the alignment of the RGD-containing loop of the EGF-RGD module with the consensus sequence of the corresponding region of disintegrins (Fig. 1B), a variant termed V2 was designed by shortening the loop to the same length as disintegrins. To produce soluble and well folded recombinant proteins we chose the pET 32/E. coli BL21 Trx B (DE3) system, which uses thioredoxin (TrxA) as a gene fusion partner. As shown in Fig. 1B, a factor Xa cleavage site was introduced between the fusion protein and the EGF module. As illustrated in the case of Trx-EGF-RGD, both fusion proteins were overproduced after induction with isopropyl-1-thio-β-D-galactopyranoside (Fig. 2A). Purification was carried out from clarified lysates obtained after sonication of the cell pellets. The Trx-EGF-RGD and Trx-V2 fusion proteins bound to nickel-Sepharose columns and were subsequently eluted using 200–300 mM imidazole (Fig. 2B). Western blotting realized on the different fractions using polyclonal anti-Clr antibodies revealed a major band of around 30 kDa in both cases (Fig. 2C), consistent with the calculated values (25,508.2 Da for Trx-EGF-RGD and 25,564.9 Da for Trx-V2). Two bands of about 66 and 94 kDa were observed in each case, likely corresponding to dimeric and trimeric forms of the recombinant proteins.

Cleavage with factor Xa efficiently released the recombinant EGF modules from thioredoxin. The isolated modules were then purified to homogeneity by reverse-phase HPLC (Fig. 2D), and their disulfide bridges were checked by peptide mapping as described previously (30), establishing a Cys1-Cys3, Cys2-Cys4, Cys5-Cys6 disulfide bridge pattern characteristic of EGF-like modules (28). Analysis by electrospray mass spectrometry confirmed the
identity and homogeneity of the recombinant EGF-RGD and V2 modules, yielding reconstructed molecular masses of 5,627.35 ± 0.53 and 5,384.59 ± 0.53 Da, respectively, consistent with the calculated values (5,628.0 and 5,384.8 Da, respectively). The yields obtained were routinely 50–70 mg per liter of culture for the Trx fusion proteins, resulting in 2–4 mg of the HPLC-purified isolated modules.

**EGF-RGD Modules Promote Cell Adhesion of CHO Cell Lines Expressing β1 and β3 Integrins**—To evaluate the ability of the chimeric EGF modules to promote integrin-mediated cell adhesion, we used a panel of related CHO cell lines expressing different integrins. Wild-type CHO cells express α5β1 as a major integrin and do not express β3 or β2 integrins; β3-CHO cells produce a hybrid (hamster/human) α1/β3 heterodimer; α1β2-CHO (31, 41, 42) express endogenous α5β1, weakly the hybrid (hamster/human) α1/β3, and predominantly the human α1β3 integrins. Preliminary experiments indicated that the Trx fusion proteins and the isolated chimeric EGF modules supported cell adhesion in a similar manner, indicating that thioredoxin did not alter the adhesion properties of the EGF modules. Adhesion assays were performed on plates coated with the isolated modules and indicated that both β3-transfected cell lines had the ability to bind to the chimeric modules, whether in the presence or absence of serum. In contrast, whereas wild-type CHO cells bound to fibronectin in the absence of serum, their adhesion to the EGF-RGD and V2 modules was strictly dependent on the presence of serum. Subsequent comparative adhesion assays were therefore performed using a serum-supplemented medium. As shown in Fig. 3A, wild-type CHO cells and the β3-transfected CHO cell lines bound specifically to the coated chimeric modules in a dose-dependent manner. The EGF-RGD module induced only weak
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Inhibition by D-RGDW of cell adhesion to the EGF-RGD and V2 modules

Table I

|          | IC50 (µg/ml) |
|----------|-------------|
| α1β1β2-CHO | 5.2         |
| β2-CHO     | 13.8        |
| α1β1β2-CHO | 6.7         |
| β2-CHO     | 25          |

Cells were preincubated for 15 min at 4 °C with D-RGDW peptide at various concentration and then allowed to attach for 1 h at 37 °C onto microwells coated with Trx-EGF-RGD or Trx-V2. Adhesion was measured as described under “Experimental Procedures.” IC50, was determined as the concentration of D-RGDW yielding 50% of the maximum inhibition.

EGF-RGD and V2 modules to induce cellular signaling, as assessed by the visualization of actin stress fibers by immunostaining and confocal microscopy. Wild-type and β3-transfected CHO cells plated on fibronectin all showed characteristic cell spreading and organization of actin stress fibers (Fig. 4, A, D, and G). When bound to the chimeric modules, wild-type CHO cells adopted a less spreading morphology, and their actin filaments did not form organized stress fibers (Fig. 4, B and C). In contrast, α1β1β2-CHO cells and β2-CHO cells attached to EGF-RGD or V2 both displayed a typical spreading morphology along with a characteristic network of parallel actin fibers (Fig. 4, E, F, H, and I), similar to the morphology observed on fibronectin. These results provided evidence for the ability of the chimeric modules to support both cell adhesion and spreading. Based on the differential behavior of β3-transfected cells compared with wild-type cells, it was likely that cellular signaling involved β3 integrins as major receptors.

Inhibitory Effect of the V2 Module on Cell Adhesion

To further investigate its functional properties, the chimeric V2 module was used as a soluble competitor for cell adhesion to natural ligands. As described under “Experimental Procedures,” these experiments were performed in serum-free medium to avoid inhibition by serum matrix proteins. The different CHO cell lines used in this study express αβ1, the major receptor for fibronectin, αβ2, which also binds to fibronectin, αβ3, specific to fibronectin, and αβ3, which recognizes a variety of ligands including fibronectin and fibrinogen, and αβ3, which in its resting state binds to surface-coated fibrinogen. Parallel inhibition assays were conducted using fibronectin and fibrinogen as ligands. Control experiments showed that the unmodified C1r EGF module had no effect on cell adhesion to fibronectin and fibrinogen (Fig. 5). When cells were plated on fibronectin, inhibition by V2 reached a maximal level of about 20% (Fig. 5A). On fibrinogen (Fig. 5B) V2 had a dose-dependent and more pronounced inhibitory effect, reaching at 2 µM concentration maximum inhibition levels of 60% for β2-CHO and wild-type CHO cells and of 40% for α1β1β2-CHO cells. These results demonstrated the ability of the recombinant V2 module to prevent interaction of integrins with their natural ligands. The weak inhibition observed on fibronectin and the strong inhibition observed on fibrinogen provided further support for a major involvement of β3 integrins, compared with β1 integrins, in the recognition of the V2 module. This conclusion was also consistent with the observed differential behavior of wild-type CHO cells which, in both systems, were less sensitive to V2 concentration than β3-CHO and α1β1β2-CHO cells (Fig. 5, A and B). Similar experiments were performed using EGF-RGD, yielding comparable inhibition patterns, although with much lower inhibitory effects, reaching maximal values of 13% in the case of fibronectin and 21% in the case of fibrinogen (data not shown).

Surface Plasmon Resonance Analysis of the Interaction between Purified Integrins and the Chimeric EGF Modules—

Fig. 3. Comparative adhesion of different cell lines to the EGF-RGD and V2 modules. Wild-type (wt) CHO cells, β2-CHO cells, and α1β1β2-CHO cells were allowed to attach to fibronectin, the EGF-RGD, and V2 modules for 1 h at 37 °C. The amount of adherent cells was determined as described under “Experimental Procedures.” Non-specific adhesion on bovine serum albumin was subtracted. A, adhesion of wild-type CHO (white lines), β2-CHO (gray lines), and α1β1β2-CHO cells (black lines) were measured on EGF-RGD (■) or V2 (●) immobilized at different concentrations. The data shown represent the mean value ± S.D. of triplicate experiments. B, adhesion was performed at a saturating concentration of the chimeric modules (100 µg/ml) and compared with fibronectin. Results are expressed relative to the adhesion achieved on fibronectin for each cell line. The data shown represent the mean value ± S.D. of three independent experiments.

binding of wild-type CHO cells, whereas adhesion of β2-CHO and α1β1β2-CHO cells reached much higher levels. The V2 variant yielded a similar pattern but with higher adhesion values for the three cell lines. Further experiments were conducted at a saturating concentration (100 µg/ml) of modules, and binding was compared with fibronectin (Fig. 3B). Similar results were obtained under these conditions, with adhesion values on V2 reaching about 80% relative to fibronectin in the case of β2-CHO and α1β1β2-CHO cells. Cell adhesion was totally inhibited by a 20 µM concentration of a RGDW peptide and insensitive to a RGES peptide. In addition, the unmodified C1r EGF module showed no significant cell binding activity, indicating that the observed cell adhesion was strictly RGD-dependent. It was concluded from these data that (i) V2 is a better ligand than EGF-RGD, especially in the case of wild-type CHO cells, which only express β3 integrins; (ii) adhesion involves both β3 and β3 integrins, the latter being clearly responsible for the enhanced adhesion achieved with β3-CHO and α1β1β3-CHO cells; (iii) as α1β1β3-CHO cells predominantly express α1β3 and only weakly αβ3 adhesion by these cells likely involves mainly the former of these integrins. Further support for this hypothesis came from the inhibitory effect of peptide D-RGDW, known to block α1β3 preferentially to αβ3 (43), which was three-to-four times more efficient in inhibiting adhesion of α1β1β3-CHO cells than of β3-CHO cells to the EGF-RGD and V2 modules (Table I).

Stress Fiber Formation on CHO Cell Lines Plated on Recombinant EGF-RGD Modules—We next tested the ability of the
Detailed information on the binding properties of the EGF-RGD and V2 modules was obtained by surface plasmon resonance spectroscopy, using the modules as immobilized ligands and purified αβ3 and αβ1 integrins as soluble analytes, as described under “Experimental Procedures.” Initial experiments showed that the integrins did not bind to an unrelated protein (bovine serum albumin) or to the native C1r EGF module. As illustrated in Fig. 6, αβ3 and αβ1 both readily bound to the immobilized EGF-RGD and V2 modules in the presence of MgCl2 and CaCl2. No binding was observed in the presence of 1 mM EDTA (data not shown). Each integrin showed comparable association and dissociation curves on both modules, with a much faster dissociation phase for αβ3 (Fig. 6, A and B) than for αβ1 (Fig. 6, C and D). The kinetic parameters of the interactions were determined by recording sensograms at different integrin concentrations. The association (k_on) and dissociation (k_off) rate constants, and the resulting apparent equilibrium constant K D are shown in Table II. αβ3 exhibited higher k_on values for both modules whereas, conversely, αβ1 showed lower k_off values. For both integrins, V2 was found to be a better ligand than EGF-RGD, because of decreased k_off values and, in the case of αβ3, to an increased k_on value. The resulting K D range from 31.5 nM for the interaction between αβ3 and EGF-RGD to 3.5 nM for the interaction between αβ1 and V2. As discussed below, these values are comparable with those determined for the binding of these integrins to their natural ligands.

**α3 Integrin from a CHO Cell Lysate Binds to a Nickel-Sepharose-Trx-V2 Column**—We next tested whether integrins from a total α3β1-CHO cell lysate, containing α3, α3b1, and α3 integrins, were able to interact with the Trx-V2 fusion protein non-covalently coupled on a nickel-Sepharose resin. The fusion protein was first incubated with the nickel-Sepharose resin and then the resin was suspended in a crude α3b1β1-CHO cell lysate for 2.5 h at room temperature. After extensive washes, bound proteins were eluted with EDTA and analyzed by SDS-PAGE and Western blotting using monoclonal anti-α3 antibodies. As shown in Fig. 7, α3 was specifically detected in the sample eluted from the Trx-V2 resin (Fig. 7, lane 9) but not from a control sample eluted from a Trx-C1r EGF resin (Fig. 7, lane 8).

The nickel-Sepharose resin alone showed no binding (Fig. 7, lane 7). Similar experiments designed to detect specifically the α3 or α3b1 integrins were both unsuccessful (data not shown). These data provided direct evidence of the ability of the α3β3 integrin expressed by the α3b1β1-CHO cells to associate with the V2 module in such a way that allows formation of a stable complex on the nickel-Sepharose resin. Such a stability is likely rendered possible by the low dissociation rate constant of the interaction, as observed by surface plasmon resonance analysis (Fig. 6).

**Comparative Analysis by NMR of the EGF-RGD and V2 Modules**—NMR spectroscopy was used to obtain detailed structural and dynamic information on the two modules. Assignment of the 1H and 15N frequencies of the backbone amides was performed using two-dimensional TOCSY and NOESY, as well as a three-dimensional NOESY-HSQC experiments. For both modules, assignment could be obtained for all residues except Gly19 and a stretch from Pro13 to Cys19. Comparison of the 1H frequencies of EGF-RGD and V2 with those of the native C1r EGF module (27) revealed significant similarity in the C-terminal part and indicated that the loop between the first two cysteines was also disordered in the RGD-containing modules. Further structural analysis of the EGF-RGD and V2 modules based on a detailed comparison of their NOE patterns in the three-dimensional NOESY-HSQC experiments showed no significant variation in peak intensity or cross-peak pattern. It was concluded therefore that the overall EGF module fold was maintained in both modules, the loop harboring the RGD se-
integrins were measured simultaneously to immobilized EGF-RGD which were immobilized onto the carboxymethylated dextran surface of a CM5 sensorchip using the amine coupling chemistry. Binding of the purified protein dynamics at the residue level by measuring different sequence being flexible in both cases.

The association ($k_a$) and dissociation ($k_d$) rate constants were determined by global fitting of the data using a 1:1 Langmuir binding model (BLAevaluation 3). The dissociation constants $K_D$ were determined from the $k_d/k_a$ ratio.

| Ligand       | $k_a$     | $k_d$     | $K_D$     | $k_a$     | $k_d$     | $K_D$     |
|--------------|-----------|-----------|-----------|-----------|-----------|-----------|
| EGF-RGD      | $0.8 \times 10^5$ | $2.56 \times 10^{-2}$ | 31.5      | $2.2 \times 10^5$ | $1.1 \times 10^{-3}$ | 5.1       |
| V2           | $1.5 \times 10^6$ | $1.09 \times 10^{-2}$ | 7.2       | $1.7 \times 10^5$ | $0.6 \times 10^{-3}$ | 3.5       |

The association ($k_a$) and dissociation ($K_D$) rate constants were determined by global fitting of the data using a 1:1 Langmuir binding model (BLAevaluation 3). The dissociation constants $K_D$ were determined from the $k_d/k_a$ ratio.

Fig. 6. Surface plasmon resonance analysis of the binding of $\alpha_\beta_2$ and $\alpha_\beta_3$ to the EGF-RGD and V2 modules. The chimeric modules were immobilized onto the carboxymethylated dextran surface of a CM5 sensorchip using the amine coupling chemistry. Binding of the purified integrins was measured simultaneously to immobilized EGF-RGD or V2 or to an activated-deactivated surface in the running buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM octyl-$\beta$-D-glucopyranoside) at a flow rate of 20 $\mu$L/min using a BIACORE 3000 apparatus. Association and dissociation curves are shown for different $\alpha_\beta_2$ or $\alpha_\beta_3$ integrin concentrations. The specific binding signal shown was obtained by subtracting the background signal (obtained by injection of the protein sample over a activated-deactivated surface).

Fig. 7. The $\alpha_\v$ integrin subunit from a $\alpha_\v\beta_2$-CHO cell lysate binds to a nickel-Sepharose-Trx-V2 resin. Samples eluted from a free nickel-Sepharose resin (lanes 1, 4, and 7), a nickel-Sepharose-Trx-C1r EGF resin (lanes 2, 5, and 8), or a nickel-Sepharose-Trx-V2 resin (lanes 3, 6, and 9) were separated on a 7.5% polyacrylamide SDS-PAGE under non-reducing conditions and then analyzed by Western blotting using polyclonal antibodies directed against the $\alpha_\v$ integrin subunit. Unbound fractions (lanes 1–3), washes (lanes 4–6), and EDTA/NaCl elution fractions (lanes 7–9) are shown for the three resin types. Positions of molecular mass markers are shown. The $\alpha_\v$ integrin subunit (apparent molecular mass 150,000 Da) is detected in the unbound material from the free nickel-Sepharose (lane 1) and nickel-Sepharose-Trx-C1r EGF resins (lane 2), as well as in the fraction eluted from the nickel-Sepharose-Trx-V2 resin (lane 9).

NMR spectroscopy also offers the possibility to characterize protein dynamics at the residue level by measuring different $^{15}$N relaxation rates ($R_1$, $R_2$) and the $[^1H]^{15}$N NOE, which are dependent on the mobility of the $^{15}$N-$^1$H vectors in the picosecond nanosecond time scale. In addition, the $R_2$ relaxation rate can also include a contribution from a local chemical or conformational exchange. Relaxation rates could be determined for 38 of the 43 assigned residues in the case of EGF-RGD and for 34 of the 41 assigned residues for V2. This confirmed that both modules comprise a relatively flexible N-terminal part, characterized by low $[^1H]^{15}$N NOE values (Fig. 8A) and low $R_1$ and/or $R_2$ relaxation rates (Fig. 8B) and a more rigid C-terminal part, in keeping with our previous analysis of the native C1r EGF module (27). Detailed comparative analysis of these data for the two modules revealed very similar values for the $R_1$ rate constants and the $[^1H]^{15}$N NOE values, which both sample internal motion at the pico- to nanosecond time scale. In contrast, significant differences in the $R_2$ rate constants can be observed, notably at the level of the RGD motif (Fig. 8B), which shows significantly higher values in V2 than in EGF-RGD. It was not possible to precisely measure the overall rotational correlation time of each module, a prerequisite for a detailed motional analysis based on the model-free approach proposed by Lipari and Szabo (44). Thus, although our data clearly suggest that the different affinities of the modules for integrins are related to a differential motional behavior, it cannot be concluded whether this difference arises from a slow
conformational reorientation of the RGD motif in V2 or a higher mobility in EGF-RGD.

DISCUSSION

The present study describes an efficient method for the production in a bacterial expression system of engineered EGF-like modules containing an RGD sequence and their use as high affinity ligands for cell surface integrins. The system used is based on expression of Trx fusion proteins and allows production of recombinant EGF-like modules in a soluble form, at a high and with a conformation that retains the natural folding of the human C1r EGF module used as a protein scaffold, namely the Cys1-Cys3, Cys2-Cys4, Cys5-Cys6 disulfide bridge pattern characteristic of EGF-like modules. Based on the analogy with the RGD-containing domain of snake disintegrins, our original EGF-RGD module was modified by decreasing the length of the RGD-containing loop and thereby changing the position of the RGD motif within the loop, leading to the V2 variant.

The EGF-RGD and V2 modules have been characterized functionally using various methods aimed at measuring their ability to promote cell adhesion, to inhibit cell adhesion to natural ligands, to induce signal transduction, and to associate with particular integrins using a resin binding assay and direct analysis by surface plasmon resonance spectroscopy. Based on these different assays, it may be concluded that the EGF-RGD and V2 modules both exhibit preferential binding to \( \beta_3 \) integrins, compared with \( \beta_1 \) integrins. This conclusion is based on the following observations. (i) When coated on a surface both modules yielded high adhesion levels in the case of \( \beta_3 \)-CHO cells (expressing \( \alpha_\text{v}\beta_3 \)) and \( \alpha_\text{IIb}\beta_3 \)-CHO cells (predominantly expressing \( \alpha_\text{IIb}\beta_3 \)). In contrast, significantly lower adhesion levels were observed in the case of wild-type CHO cells (only expressing \( \beta_1 \) integrins), and adhesion in this case was strictly dependent on the presence of serum, suggesting the requirement of one or more additional factors from serum, possibly endogenous plasmatic ligands or growth factors. (ii) As revealed by visualization of actin stress fibers, cell adhesion led to intracellular signaling for both \( \beta_3 \)-CHO and \( \alpha_\text{IIb}\beta_3 \)-CHO cells but not for wild-type CHO cells. (iii) In competition assays, the soluble V2 module strongly inhibited cell adhesion to fibronectin (the major ligand of \( \alpha_\text{v}\beta_3 \) and \( \alpha_\text{IIb}\beta_3 \) and only poorly inhibited cell adhesion to fibronectin (the major ligand of \( \alpha_\text{v}\beta_1 \)). In addition, for both ligands, wild-type CHO cells were significantly less sensitive to V2 concentration than \( \beta_3 \)-transfected cells (see Fig. 5). (iv) As shown by direct surface plasmon resonance analysis using purified integrins, the EGF-RGD and V2 modules exhibit significantly higher affinities for \( \alpha_\text{v}\beta_3 \) than for \( \alpha_\text{v}\beta_1 \), essentially because of a lower dissociation rate constant. In this respect, however, it should be emphasized that the affinity of the V2 variant for purified \( \alpha_\text{v}\beta_1 \) (\( K_D = 7.2 \text{ nM} \)) is not strikingly lower than that for \( \alpha_\text{IIb}\beta_3 \) (\( K_D = 3.5 \text{ nM} \)). Despite this similarity, V2 efficiently triggers intracellular signaling in \( \beta_3 \)-transfected cell lines and has no detectable effect on wild-type CHO cells (see Fig. 4), consistent with the fact that, unlike \( \beta_3 \) integrins, signaling by \( \beta_1 \) integrins not only involves binding to an RGD motif but also requires recognition of a synergy sequence, such as the Pro-His-Ser-Arg-Asn motif present on fibronectin (45).

Another conclusion that can be drawn from our data is that the modification converting EGF-RGD into V2 has more impact on \( \alpha_\text{v}\beta_3 \) than on \( \beta_3 \) integrins. Thus, analysis by surface plasmon resonance spectroscopy clearly indicates that this modification results in a 4-fold decrease in \( K_D \) for \( \alpha_\text{v}\beta_3 \), compared with only 1.5 in the case of \( \alpha_\text{IIb}\beta_3 \). This 4-fold \( K_D \) decrease is indeed very close to the increase in adhesion observed for wild-type CHO cells (Fig. 5B), providing strong support for the hypothesis that, in the case of \( \beta_3 \)-transfected cells as well, the observed increased adhesion on V2 may be due, for a large part, to the better affinity of this module for \( \alpha_\text{v}\beta_3 \) integrins.

Other observations appear consistent with the hypothesis that, in their soluble form, the recombinant modules recognize \( \alpha_\text{v}\beta_3 \) preferentially to \( \alpha_\text{IIb}\beta_3 \). Thus, binding assays using the Trx-V2 construct attached to a nickel-Sepharose resin resulted in the specific capture of the \( \alpha_5 \) subunit from a \( \alpha_\text{IIb}\beta_3 \)-CHO cell lysate and showed no evidence for binding of the \( \alpha_\text{Ib} \) or \( \alpha_5 \) subunits. This is in agreement with the fact that \( \alpha_\text{IIb}\beta_3 \)-CHO cells express the \( \alpha_\text{Ib}\beta_3 \) integrin in a resting state with only low affinity for soluble ligands, including fibronectin (46, 47). Further support for this hypothesis arises from the observation that, in competition experiments, compared with \( \beta_3 \)-CHO cells, adhesion of \( \alpha_\text{IIb}\beta_3 \)-CHO cells to fibronectin was slightly but consistently less sensitive to inhibition by the soluble V2 mod-
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counter with other carrier systems such as peptides, phages, and viruses, (16, 25, 57, 58).

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