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Arg-Gly-Asp-containing peptides expose novel collagen receptors on fibroblasts: implications for wound healing

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Integrins are a family of cell-surface receptors intimately involved in the interactions of cells with their extracellular matrix. These receptors comprise an α and β subunit in noncovalent association and many have been shown to recognize and bind an arginine-glycine-aspartate (RGD) sequence contained within their specific extracellular matrix ligand. Fibroblasts express integrin receptors belonging to two major subfamilies. Some of the members within the subfamily defined by β1 (VLA) are receptors for collagen but, perhaps surprisingly, the other major subfamily of integrins on fibroblasts—that defined by the α chain of the vitronectin receptor, αv—all appear to bind primarily vitronectin and/or fibronectin. In the present study we show that RGD-containing peptides expose cryptic binding sites on the αv-associated integrins enabling them to function as collagen receptors. The addition of RGD-containing peptides to fibroblasts cultured on type I collagen induced dramatic cell elongation and, when the cells were contained within collagen matrices, the peptides induced marked contraction of the gels. These processes were inhibited by Fab fragments of a monoclonal antibody against an αv integrin. Also, αv-associated integrins from cell lysates bound to collagen I affinity columns in the presence, but not in the absence, of RGD-containing peptides. These data suggest a novel regulatory control for integrin function. In addition, because the cryptic collagen receptors were shown to be implicated in the contraction of collagen gels, the generation of such binding forces suggests that this may be the major biological role for these integrins in processes such as wound healing.

Introduction

The biological mediators responsible for the reorganization of extracellular matrix by tissue fibroblasts include cell adhesion molecules acting through an array of cell membrane receptors, the best characterized of which is a family of transmembrane molecules termed integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Albelda and Buck, 1990; Gani and Burns, 1990). Each of the individual receptors consists of an α and β subunit in noncovalent association, and the heterodimer complex thus formed binds, in most cases, an arginine-glycine-aspartate (RGD) sequence contained within the specific extracellular matrix ligand (Pytela *et al.*, 1986; Ruoslahti and Pierschbacher, 1987). The affinity of integrins for specific ligands controls many aspects of cellular behavior, including migration, invasiveness, cytoskeletal organization, and adhesion.

Mesenchymal cells are capable of simultaneously expressing several integrins, and these can be broadly divided into two main groups. The first group is defined by the β1 subunit that can form associations with several α subunits. This group, also known as the VLA subfamily (very late antigens; Hemler, 1990), contains receptors for collagen, laminin, and fibronectin (Hynes, 1987; Albeda and Buck, 1990; Gani and Burns, 1990). The other subfamily is defined by an α subunit known as the α chain of the vitronectin receptor, or αv, which can associate with several different β subunits (Cheresh *et al.*, 1989; Bodary and McLean, 1990; Freed *et al.*, 1989; Krissansen *et al.*, 1990; Vogel *et al.*, 1990). At least some members of the αv subfamily have a relatively high affinity for the RGD sequence (Ruoslahti and Pierschbacher, 1987) and, perhaps in consequence, they have a somewhat relaxed specificity in being able to bind the RGD-

containing matrix glycoproteins fibrinogen, vitronectin, von Willebrand factor, and thrombospondin without apparent selectivity (Cheresh, 1987; Lawler *et al.,* 1988; Albeda and Buck, 1990). Other members of the αv subfamily are less well characterized, but their ligand specificity appears to be restricted to fibronectin and/or vitronectin (Cheresh *et al.,* 1989; Bodary and McLean, 1990; Freed *et al.,* 1990; Krissansen *et al.,* 1990; Vogel *et al.,* 1990). Individual fibroblasts are able to express concurrently multiple VLA and αv integrins all able to bind fibronectin, but at the present time there is no explanation that accounts for this apparent redundancy (Fingerman and Hemler, 1988; Bates *et al.,* 1991).

We examined the role of these two major integrin subfamilies in the process of wound healing using cell spreading on collagen and collagen gel contraction as representative in vitro model systems (Guidry and Grinnell, 1985; Agrez and Chua, 1990; Gullberg *et al.,* 1990). In our attempts to inhibit fibroblast function with RGD-containing peptides known to inhibit integrin-mediated cell attachment to several ligands (Cheresh, 1987; Ruoslahti and Pierschbacher, 1987; Lawler *et al.,* 1988; Krissansen *et al.,* 1990), we unexpectedly found that these peptides induced cell spreading and collagen gel contraction in a dose-dependent manner. Investigation of this phenomenon produced evidence that the peptides exposed previously cryptic epitopes on several of the integrin molecules, epitopes that functioned as collagen receptors. These results therefore call for a reappraisal of the primary function of RGD-containing matrix glycoproteins and of the integrins themselves.

Results

RGD-containing peptides promote fibroblast stretching on collagen and contraction of collagen lattices

Human diploid colon fibroblasts were seeded on collagen type I gels under serum-free conditions in the presence or absence of RGD-containing peptides. At relatively low concentrations of the peptide a dramatic increase in cell stretching was observed (Figure 1A). This effect on stretching appeared to be dose related until high concentrations of peptide were used, when complete inhibition was seen to occur (data not shown). Because the phenomenon of cell stretching parallels DNA synthesis (Folkman and Moscona, 1978; Ingber, 1990; Agrez, unpublished data), we quantitated the effect of increasing concentration of peptide on fibroblast cultures by measuring the incorporation of tritiated thymidine. A dose-related uptake of thymidine was observed in the presence of RGD peptide in this quantitative assay (Figure 1B). High concentration of peptide (500 μg/ml) resulted in inhibition of cell stretching and reduced thymidine uptake to levels below that of cells in the absence of peptide (Figure 1B). The phenomenon of cell stretching is linked to the ability of fibroblasts to contract collagen matrices in vitro, and this process of collagen lattice contraction has been considered analogous to the process of wound contractions (Guidry and Grinnell, 1985; Agrez and Chua, 1990; Gullberg *et al.,* 1990). Therefore, we also measured directly the effect of RGD-containing peptides on fibroblast-induced collagen lattice contraction. In these experiments RGD peptide-induced collagen gel contraction and a dose-related effect was observed both visually and quantitatively (using tritiated water) to a concentration of peptide (250 μg/ml), whereas higher concentration of peptide (500 μg/ml) inhibited contraction to less than that seen in the absence of peptide (Figure 2, A and B). Particularly in the cell stretching assay but also in gel contraction assays, the arginine-glycine-glutamate-serine (RGES) peptide caused a smaller but significant biological effect, but high-dose inhibition did not occur (Figures 1B and 2B). We have not investigated this effect of RGES peptide in detail, but it does appear to be specific because random tetrapeptides did not induce cell stretching or gel contraction, and preliminary experiments with arginine-glycine peptides indicate that the glutamate at least is required to be present (data not shown).

Inhibition of fibroblast spreading was assessed visually in the presence of antibodies to αv (LM142, 13C2), β3 (SZ21 and rabbit polyclonal antibody), β1 (QE2E5), α2 (AK7), and the αvβ3 complex (LM609, 23C6), as well as antibodies to a platelet collagen receptor, GPIV (IA7, IE8). Only antibodies to αv, β1, and the αvβ3 complex were inhibitory (data not shown). Their effectiveness in inhibiting RGD-mediated effects was confirmed in the gel contraction assay with selected antibodies (Figure 3A), and the Fab fragment of one of the antibodies so tested (23C6) was found to be equally effective in a dose-dependent manner (Figure 3B). At high concentration the antibody QE2E5 directed against the β1 subunit was more effective in inhibiting spontaneous contraction than the antibody 23C6 to the αvβ3 complex (which very effectively inhibited peptide-induced contraction) (Figure 3C).

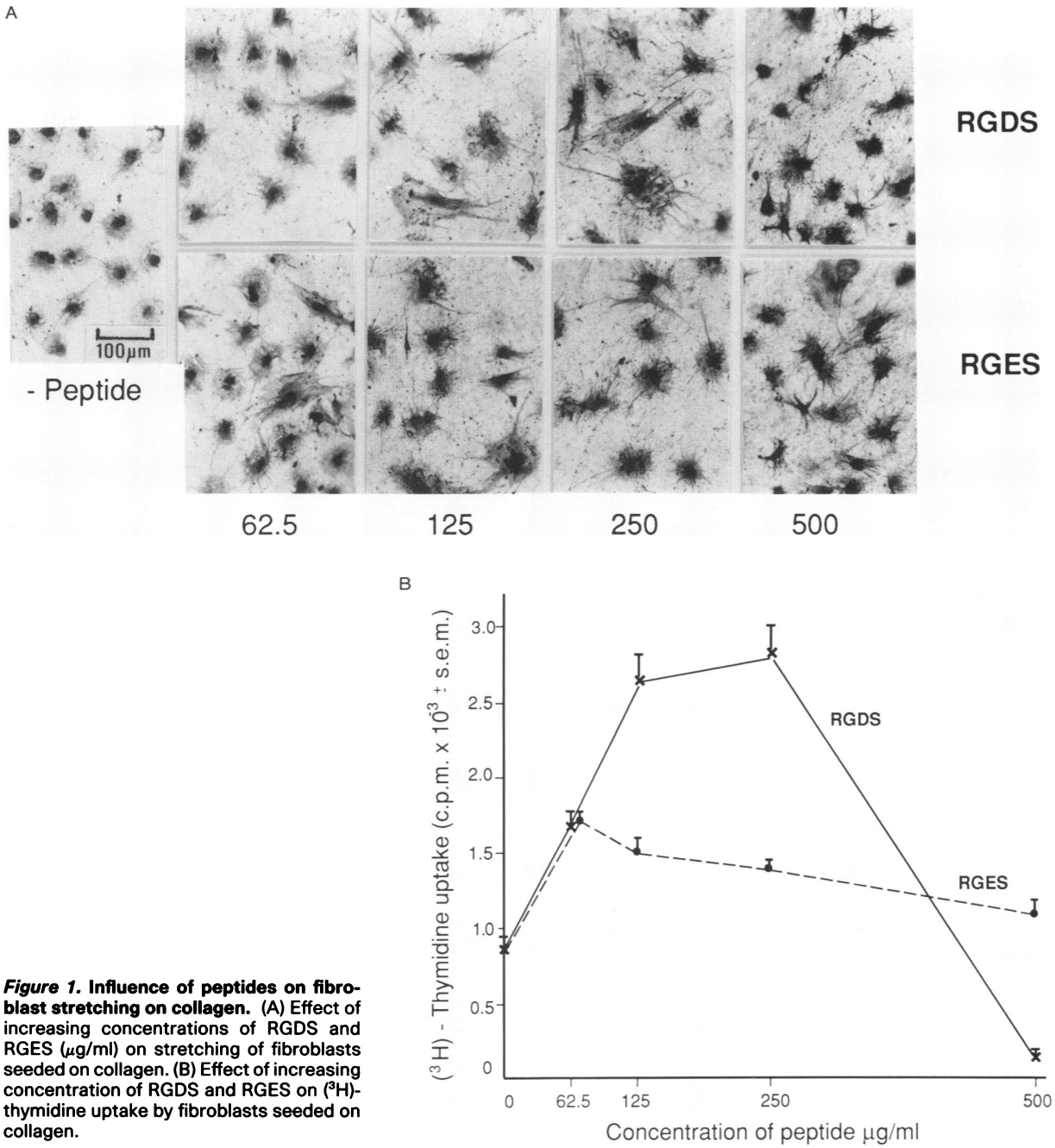

***Figure 1.* Influence of peptides on fibroblast stretching on collagen.** (A) Effect of increasing concentrations of RGDS and RGES (μg/ml) on stretching of fibroblasts seeded on collagen. (B) Effect of increasing concentration of RGDS and RGES on (^{3}H)-thymidine uptake by fibroblasts seeded on collagen.

RGD peptides induce collagen binding by αv integrins

To determine directly the polypeptides involved in collagen binding, we carried out affinity chromatography with collagen type I columns. In the absence of peptide the collagen binding proteins presented a profile that was essentially identical to that described by others on a number of cell types (Santoro, 1986; Kirchhofer *et al.,* 1990), and by immunoprecipitation with monoclonal antibodies (not shown) we were able to identify the two major integrin bands as α2 and β1. In the presence of arginine-glycine-aspartate-serine (RGDS) several additional bands were seen to bind to the collagen column (Figure 4). One of these bands was clearly identified as αv by immunoprecipitation, and there was an obvious increase in the amount of β1 subunit without a corresponding increase in α2. There was also a diffuse band observed at

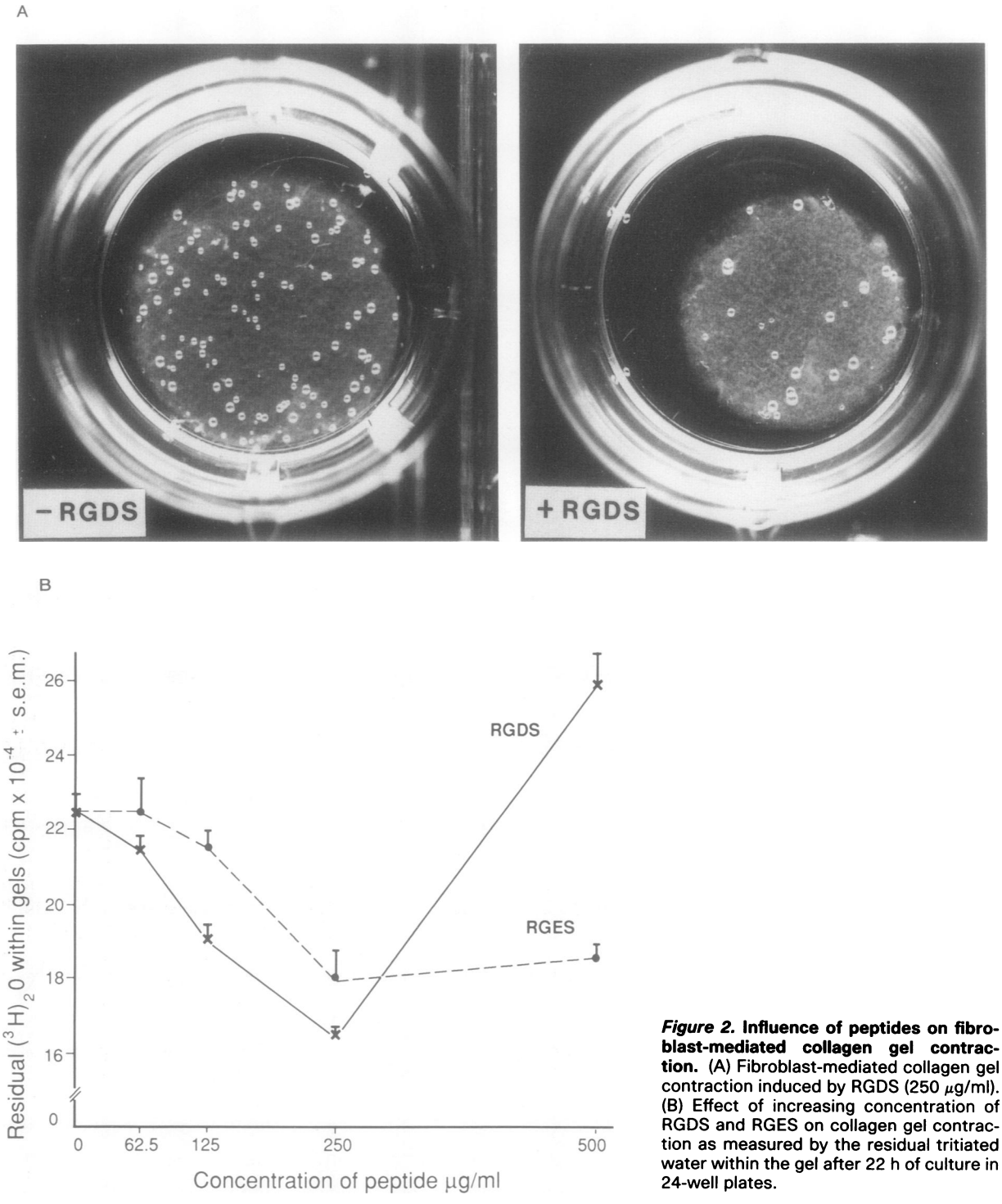

***Figure 2.* Influence of peptides on fibroblast-mediated collagen gel contraction.** (A) Fibroblast-mediated collagen gel contraction induced by RGDS (250 μg/ml). (B) Effect of increasing concentration of RGDS and RGES on collagen gel contraction as measured by the residual tritiated water within the gel after 22 h of culture in 24-well plates.

around 90 kDa (Figure 4) that showed an increase in apparent molecular weight on reduction (data not shown); this band showed the same migration pattern as β3 but, because it appears to be a β chain associated with αv, the possibility that it might be β5 cannot be excluded (Cheresh *et al.*, 1989; Freed *et al.*, 1990). In three experiments the amount of β3/β5 band recovered from the collagen affinity columns varied considerably but it was always much less prominent than the bands corresponding to αv and β1 (data not shown).

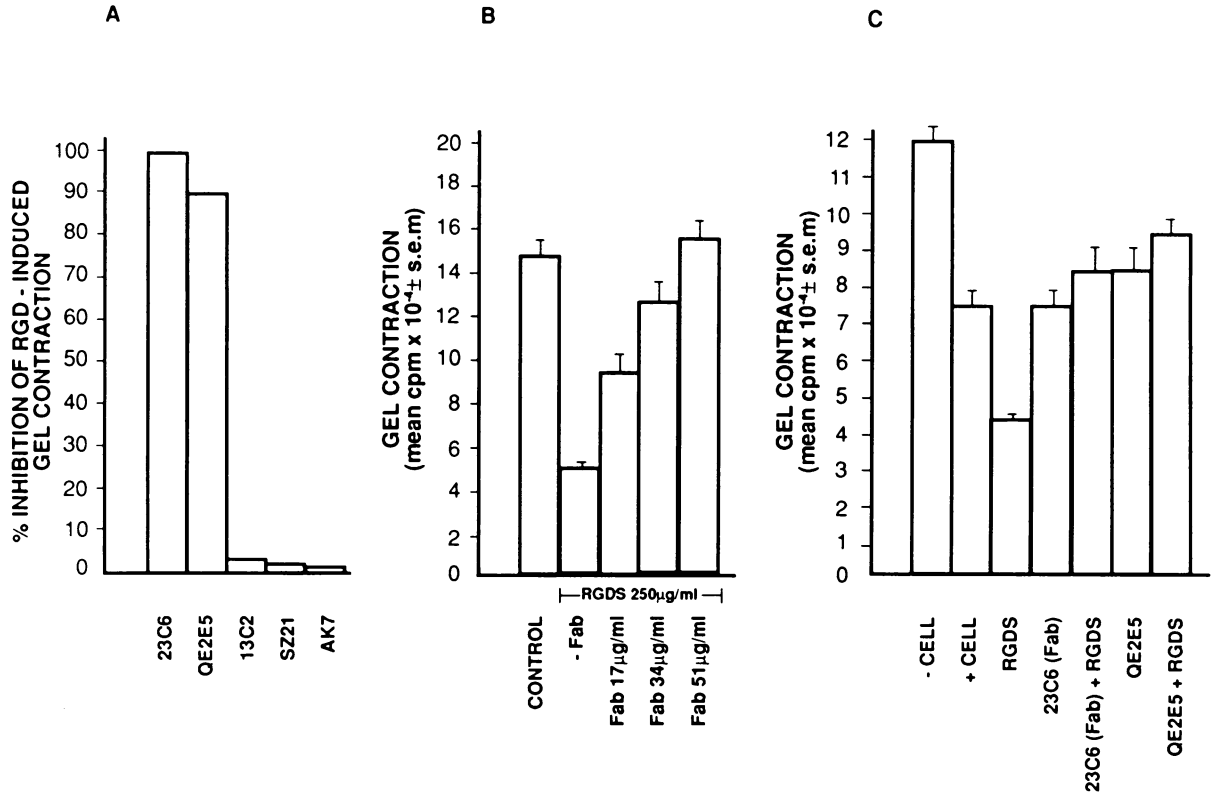

Figure 3. **Antibody-mediated inhibition of collagen gel contraction.** (A) Effect of selected mAbs on RGDS-induced fibroblast-mediated collagen gel contraction in microtiter wells. The inhibitory effect, if any, is expressed as percentage inhibition of RGD-induced gel contraction relative to uncontracted cell-free control gel volumes. Final concentrations of purified mAbs within wells were 23C6, 30 μg/ml; QE2E5, 13 μg/ml; 13C2, 29 μg/ml; SZ21, 36 μg/ml; and AK7, 8 μg/ml. (B) Dose-response effect of 23C6 Fab fragment on RGDS-induced fibroblast-mediated collagen lattice contraction. The inhibitory effect of Fab fragment on RGDS (250 μg/ml)-induced contraction is expressed as residual $(^3H)_2O$ within gels. (C) Effect of the mAbs 23C6 (Fab) and QE2E5 on nonpeptide and RGDS-induced (250 μg/ml) collagen gel contraction. Antibody concentrations: 23C6 (Fab), 45 μg/ml; QE2E5, 18 μg/ml. The inhibitory effects of the antibodies on gel contraction are expressed as residual $(^3H)_2O$ within gels.

An apparent anomaly between the data from collagen chromatography and those from the inhibition studies is that the inhibitory antibodies LM609 and 23C6 specifically identify the $\alpha v\beta 3$ complex (Cheresh and Spiro, 1987; Cheresh *et al.*, 1989; Davies *et al.*, 1989; Krissansen *et al.*, 1990) yet this complex is not prominent among the collagen binding proteins. This apparent contradiction is perhaps resolved by the data shown in Figure 4A. In the absence of RGD, peptide 23C6 specifically immunoprecipitates $\alpha v\beta 3$, but in the presence of peptide the antibody precipitates αv together with multiple β chains. In parallel immunoprecipitation experiments (data not shown) one of these associated β chains could be identified as $\beta 1$ and another as $\beta 3$, but we did not have a good precipitating antibody to $\beta 5$ available for this study and the presence of this subunit cannot be excluded. Further immunoprecipitation experiments with antibodies to $\beta 1$ and $\beta 3$ also served to show that the additional bands precipitated by 23C6 in the presence of peptide were not caused by RGD-induced association between the integrin subunits; thus, immunoprecipitations performed with anti-$\beta 3$ did not contain $\beta 1$ and anti-$\beta 1$ immunoprecipitates did not contain $\beta 3$ (Figure 4B). The additional bands seen at ~200 kDa in all of the affinity chromatography and immunoprecipitation experiments in the presence of RGD-containing peptide were not identified and may represent peptide-induced integrin aggregation, but although these were seen in all immunoprecipitation experiments, the multiple β chains seen to associate with αv were precipitated only by 13C2 (to αv) and 23C6 (Figure 4, A and B).

Discussion

Contraction of the extracellular matrix by mesenchymal cells is thought to play an important

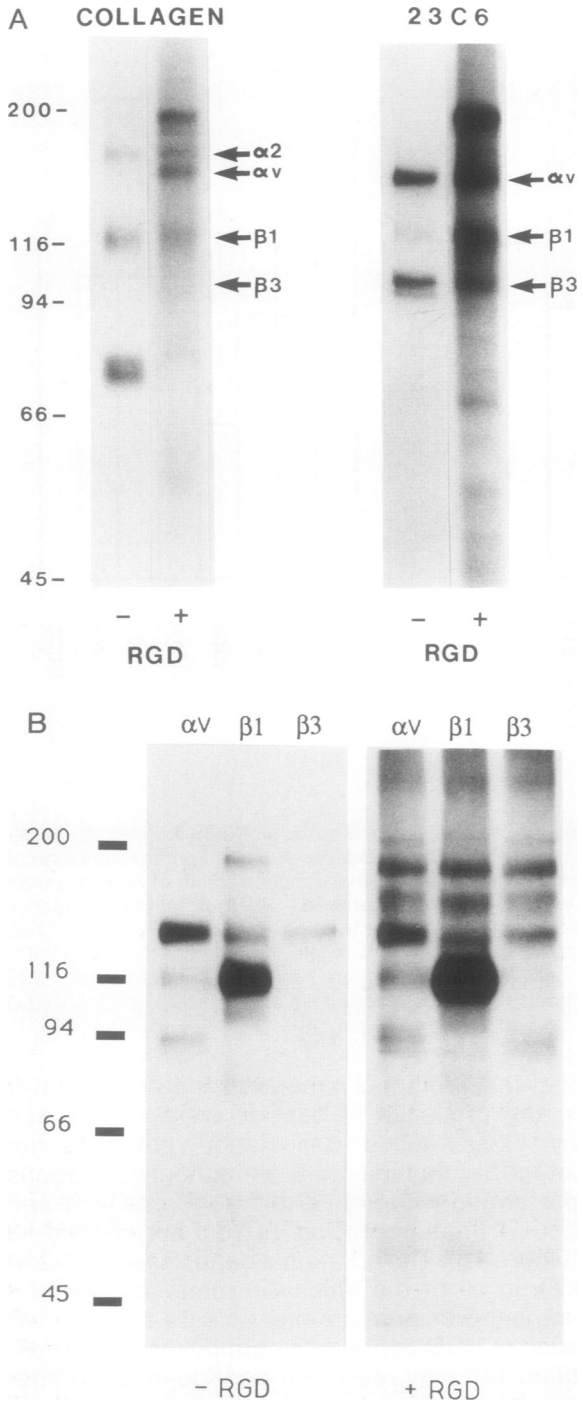

***Figure 4.* SDS-PAGE analysis showing the effects of RGD peptide on integrin binding to collagen and precipitated by monoclonal antibodies.** (A) Autoradiographs of SDS-PAGE of cell lysates labeled with I^{125}. Left tracks (collagen), collagen binding proteins induced by exposure of cell lysates to RGD-peptide. Right tracks (23C6), integrins precipitated by the 23C6 antibody from RGD-exposed cell lysates. The arrowheads indicating the relative molecular weights of αv, $\beta 1$, and $\beta 3$ are derived from parallel immunoprecipitation experiments using the same cell lysates. (B) Immunoprecipitations of cell lysates precipitated with antibodies to αv (13C2), $\beta 1$ (QE2E5), and $\beta 3$ (SZ21) in the absence (−RGD) and presence (+RGD) peptide.

role in the process of wound repair, and populated collagen gels have been used as a model system to study this process in vitro (Guidry and Grinnell, 1985; Agrez and Chua, 1990; Gullberg *et al.,* 1990). RGD-containing peptides induce fibroblast-mediated collagen lattice contraction by exposing cryptic binding sites on the αv-associated integrins, enabling them to function as collagen receptors.

Nesbitt and colleagues (1989) have shown that the complex-specific epitope bound by the monoclonal antibody 23C6 is on the αv subunit of the integrin vitronectin receptor and its expression is dependent on complex formation with $\beta 3$. We can postulate here that expression of this cryptic epitope on αv can be induced by association with other β subunits in the presence of RGD peptide. In this regard the 23C6 epitope appears to be very similar to the ligand-induced binding sites described by Frelinger *et al.* (1990). These authors reported that the addition of RGD-containing peptides to the platelet integrin glycoprotein (GP)IIb-IIIa (αIIbβ3) exposed novel antibody binding sites, and some antibodies directed to these cryptic epitopes were able to inhibit fibrin clot retraction (Frelinger *et al.,* 1990). Hence, the exposure of cryptic binding sites within integrins consequent on RGD binding may be a more universal phenomenon of general biological significance to the processes controlling tissue damage and wound repair.

The mechanism by which the RGD peptides induce collagen binding by the αv integrins remains to be established. The presence of peptide may cause some receptor aggregation because high molecular weight bands were observed to bind to the collagen column in affinity chromatography and were immunoprecipitated by all of the antibodies tested in the presence of peptide. We have not analyzed these nonspecific bands but their presence alone is insufficient to account for the specific immunoprecipitation of αv-associated β chains by the 23C6 antibody because these subunits were coprecipitated by an antibody to αv but not by antibodies to $\beta 1$ or $\beta 3$. Further, the highly specific inhibition of peptide-induced cell stretching and collagen gel contraction by the 23C6 antibody (and Fab fragments) argues against a simple model of peptide-induced integrin aggregation accounting for the phenomenon. Cell elongation and collagen gel contraction were also induced by RGES peptide (Figures 1 and 2), and this was also inhibitable by the 23C6 antibody (data not shown). Preliminary experiments indicate that such RGES potentiation may be specific and that this peptide

induces the same αv integrins to bind to a collagen affinity as do RGD-containing peptides (Bates, unpublished data). However, in agreement with published reports (Santoro, 1986; Dedhar *et al.,* 1987), the RGES peptide had no inhibitory effect whatever on cell attachment to collagen, even at very high concentrations. Hence, peptide-induced exposure of cryptic epitopes has different requirements from inhibition of cell attachment, apparently being less stringent. The proposal can be made that only very low affinity binding is required to the RGD binding domain on the integrin to induce the receptor to undergo a conformational change that exposes the collagen binding site and the 23C6 epitope.

The integrin α2β1 (VLA-2) has been clearly demonstrated to function as a collagen receptor in a number of cell types (Santoro, 1986; Kirchhofer *et al.,* 1990). Collagen binding mediated by this receptor on platelets, however, is relatively resistant to displacement by RGD peptides, and high concentrations of free peptide (100 μg/ml) caused only a 20% reduction in platelet adhesion to collagen (Santoro, 1986). This may be a reflection of the fact that the β1-associated integrins exhibit a 10- to 100-fold lower affinity for the peptide ligand than does the β3-associated vitronectin receptor (Ruoslahti and Pierschbacher, 1987), although Staatz et al. (1990) have shown the α2β1 receptor binds to a collagen peptide that does not contain an RGD sequence. It is possible therefore that the small amount of cell spreading seen on collagen and the collagen lattice contraction observed by us in the absence of low-dose RGD peptide might be attributable to constitutively expressed collagen receptors in addition to α2β1 integrin, and affinity chromatography on collagen I columns supported this notion. In the absence of peptide the only integrin seen to bind was α2β1, but this was accompanied by a band at ~70 kDa (Figure 4A). Dedhar et al. (1987) have identified three collagen binding polypeptides of 250, 70, and 30 kDa on the surface of human MG-63 osteosarcoma cells. Binding of these receptors was shown to be inhibited by RGD-containing peptides and by calcium chelation with EDTA. In our assays, cell spreading on collagen was inhibited only by high concentration of RGDS peptide, and arginine-glycine-aspartate-threonine inhibited spontaneous (nonpeptide-induced) gel contraction to a greater degree than RGDS peptide (data not shown), which are findings consistent with the known higher affinity of these particular polypeptides for RGDT than RGDS (Dedhar *et al.,* 1987). Thus, the 70-kDa band eluted from the collagen column may represent one of the molecules identified by Dedhar *et al.* (1987), and this may be initiating nonpeptide-induced binding to collagen. However, at high concentration the antibody QE2E5 directed against the β1 integrin subunit effectively inhibited spontaneous collagen gel contraction, and therefore it is likely that α2β1 is also involved in this process.

Our findings suggest a novel regulatory role for the subfamily of integrins defined by αv. At the sites of wound repair matrix glycoproteins are produced transiently by a number of cell types in response to cytokines or tissue damage. It is reasonable to speculate that the presence of such RGD-containing glycoproteins induces a conformational change on the αv-associated integrins. It also appears that fibroblast viability is not a prerequisite for this conformational change because the collagen binding proteins in tumor cell lysates exposed to RGD-containing peptide were identified as αv in association with several β subunits. This notion is consistent with the recent observations made by Ginsberg's group that activation of the platelet integrin glycoprotein (GP)IIb-IIIa can occur in fixed cells that presumably lack physiological signal transduction mechanisms (O'Toole *et al.,* 1990). We postulate from our fibroblast model that this conformational change enables hitherto unexposed collagen receptors to generate the matrix binding forces necessary to restore organ integrity through the process of wound contraction. This may explain some of the previously documented wound-promoting effects of fibronectin (Grinnell *et al.,* 1987), and in our recent experiments we have found that the RGD-containing glycoproteins, thrombospondin and vitronectin as well as fibronectin, also promote fibroblast stretching on collagen in vitro (Agrez, unpublished data). The generation of such matrix binding forces suggests that this may be the major biological role for these integrins in the process of wound healing. Furthermore, the observation that these important functional binding sites on integrins remain cryptic until exposed by RGD binding provides a further mode of regulation for integrin receptors.

Materials and methods

Cells

CCD-18 (human colon) fibroblasts were purchased from the American Type Culture Collection (Rockville, MD). The cells were adapted to monolayer growth in tissue culture flasks using standard culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories,

McLean, VA) supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% fetal calf serum. Chemically defined serum-free medium was used in all matrix experiments and consisted of DMEM supplemented with glutamine, hydrocortisone, nonessential amino acids, mercaptoethanol, insulin, transferrin, and selenium. Before experiments the cells growing in standard culture medium were harvested by exposure to 0.05% trypsin/0.02% EDTA (Flow Laboratories) and washed once in standard medium. The cell preparations were then washed three times in serum-free medium before resuspension in serum-free medium and estimation of cell viability with 0.4% trypan blue solution.

Cell spreading and stretching

Three-dimensional collagen type I gels were prepared essentially as described using collagen extracted from rat tail tendons (Agrez, 1989; Agrez and Chua, 1990). In brief, the collagen (2.5 mg/ml) was mixed with a ×2 concentrate of chemically defined serum-free medium and 100 μl aliquoted into microtiter wells (Linbro 96-well microtitration plates; Flow Laboratories). The gels were allowed to set at 37°C before the addition of cells. Cells suspended in serum-free medium were seeded onto the collagen base in triplicate wells at a cell density of 2×10^3 cells per well. Cultures were incubated at 37°C in 5% CO_2 for 48 h, and exposure of the cells to peptides was continuous for the duration of the experiment (RGDS and RGES in phosphate-buffered saline [PBS] added as 30 μl of peptide per well; peptides were purchased from Auspep, Melbourne, Australia). Visual assessment of fibroblast stretching in the presence of peptides was performed after fixation and staining of 48-h gel cultures with Coomassie blue (0.1% solution in 10% acetic acid and 40% methanol). Photomicrography was performed with a 35mm camera attachment fitted to a Leitz Labovert (Wild Leitz, Switzerland) inverted microscope using technical pan film (Kodak, Rochester, NY) at 100 ASA setting. To quantitate the degree of cell stretching in the presence of peptides, DNA synthesis was estimated by pulsing individual wells with 1 μCi of (^{3}H)-thymidine 24 h after the initiation of cultures. Cells were harvested at 48 h by dissolution of the collagen matrix with 25 μl of collagenase (Cat. No. C5138; 15 mg/ml in PBS, Sigma, St. Louis, MO) before automated cell harvesting and measurement of radioactivity.

Collagen matrix contraction

To examine the effect of RGD-containing peptides on fibroblast-induced collagen matrix contraction, a modification of the method described by van Bockxmeer and Martin (1982) was employed as described previously (Agrez and Chua, 1990). Collagen gels (600 μl) were prepared in 24-well plates (Linbro, Flow Laboratories) in an identical manner to that described for the microtiter system, except for incorporation of $(^3H)_2O$ and fibroblasts into the collagen-medium mixture. CCD-18 fibroblasts were seeded into triplicate gels at a cell density of 50×10^3 cells per gel. Gelled cultures were overlain with 600 μl of serum-free medium containing the peptide and rimmed at the plastic interface with a fine needle at the initiation of experiments. The gels were incubated at 37°C in 5% CO_2 and retrieved after 22 h to quantitate gel volume by measuring the residual $(^3H)_2O$ within contracted gels (Agrez and Chua, 1990). Before recovery of gels for scintillation counting, each gel was photographed using technical pan film (Kodak) at 25 ASA setting.

Antibody inhibition assays

To determine which receptors were involved in RGD-induced cell stretching and gel contraction, the matrix cultures were exposed to a panel of monoclonal antibodies. Monoclonal antibodies (mAb) (13C2) to the αv subunit and 23C6 to the αvβ3 complex were a kind gift from Dr. Michael Horton of the Imperial Cancer Research Fund, London, and anti-β3 subunit (SZ21), anti-α2 (AK7), and rabbit anti-β3 antibodies were kind gifts from Dr. Michael Berndt of the Department of Medicine, Westmead Hospital, Sydney, Australia. The LM609 and LM142 antibodies to αvβ3 complex and αv subunit were kindly supplied by Dr. David Cheresh, Scripps Clinic and Research Foundation, CA, the IA7 and IE8 antibodies to platelet glycoprotein IV have been described previously (Biggs *et al.*, 1990), and the antibody to the β1 subunit (QE2E5) was a kind gift from Dr. Graeme Russ of the Queen Elizabeth Hospital, Adelaide, Australia.

In these experiments preparation of collagen gels and estimation of gel contraction was performed in an identical manner to that described for the 24-well plates with the exception that 150 μl gel volumes were cast into quadruplicate microtiter wells for each antibody tested in the presence of RGDS (250-μg/ml final well concentration). Fibroblast density was in the range of $12–14 \times 10^3$ cells per gel. Fab fragments from one of the antibodies (23C6) were also tested. In brief, ascites fluid containing the mAb 23C6 (IgG_1) was precipitated with 45% ammonium sulphate and resuspended in 100 mM sodium acetate (pH 5.5) to a final concentration of 5 mg/ml. This was dialyzed to remove ammonium sulphate and cysteine and EDTA added (final concentrations: 50 mM cysteine, 1 mM EDTA). The antibody was exposed to 10 μg papain (Sigma) per milligram of antibody at 37°C for 12 h. Iodoacetamide was then added to a final concentration of 75 mM with further incubation at room temperature for 30 min. Fab fragments were separated from Fc fragments and any residual mAb on a protein A-Sepharose column (CL-4B, Pharmacia, Sydney, Australia) using PBS (pH 7.0). The purity of Fab fragments was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

Affinity chromatography and immunoprecipitation analysis

The polypeptides involved in fibroblast-collagen binding were identified by means of affinity chromatography using collagen type I columns. Colon fibroblasts were ^{125}I-labeled on their cell surface, and the lactoperoxidase method used for labeling, as well as the methods used for immunoprecipitation and SDS-PAGE analysis, was as described previously (Krissansen *et al.*, 1990; Bates *et al.*, 1991). Cells were lysed in cation-supplemented octylglucoside buffer (10 mM tris(hydroxymethyl)aminomethane (Tris), 150 mM NaCl, octylglucoside 100 mM containing 1 mM $CaCl_2$, 1 mM $MgCl_2$) and protease inhibitors (2 mM phenylmethylsulfonyl fluoride; 20 mM iodoacetamide; 50 μg/ml soybean trypsin inhibitor; Sigma). The cell lysate was centrifuged and one half incubated with fibronectin-related peptide 1 (−GRGDSP-, Auspep) at 1 mg/ml concentration for 30 min before addition to collagen columns. One-milliliter collagen columns were prepared by coupling collagen type I to CNBr-activated Sepharose 4B beads (Pharmacia), and the columns were prewashed with 10 volumes of 50 mM octylglucoside lysis buffer with added $MgCl_2$, $CaCl_2$, and protease inhibitors (as above). The cell lysates with or without RGD peptide (each of 1-ml volume) were applied to the columns and allowed to bind at 4°C for 18 h. Columns were then washed with 10 volumes of 50 mM octylglucoside lysis buffer with additives followed by elution of integrins with 50 mM EDTA in

50 mM octylglucoside lysis buffer. Precipitation of integrins by the 23C6 antibody and by antibodies to αv (13C2), β1 (QE2E5), and β3 (SZ21) was performed with ^{125}I-labeled cells lysed in cation-supplemented octylglucoside buffer as above. One half of each lysate (+RGD) was incubated together with GRGDSP for 30 min, and this and the rest of the lysate (−RGD) was immunoprecipitated with the appropriate antibody coupled to Sepharose 4B beads (Pharmacia). The antigen-antibody complexes were dissociated by boiling in SDS-PAGE sample buffer (62 mM Tris, 100 mM SDS, 10% glycerol, pH 6.8). Eluate fractions and immunoprecipitates were analyzed reduced (not shown) and nonreduced by electrophoresis in 7.5% SDS-PAGE followed by autoradiography.

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References

Agrez, M.V. (1989). A collagen matrix microassay for use in tumour-stromal cell cocultures. Immunol. Cell Biol. *67,* 101–105.

Agrez, M.V., and Chua, F.K. (1990). The role of colon fibroblasts in malignant large bowel obstruction—an experimental in vitro model. Br. J. Cancer *62,* 567–572.

Albelda, S.M., and Buck, C.A. (1990). Integrins and other cell adhesion molecules. FASEB J. *4,* 2868–2879.

Bates, R.C., Rankin, L.M., Lucas, C.M., Scott, J.L., Krissansen, G.W., and Burns, G.F. (1991). Individual embryonic fibroblasts express multiple β chains in association with the αv integrin subunit: loss of β3 expression with cell confluence. J. Biol. Chem. (*in press*).

Biggs, B.-A., Gooze, L., Wycherley, K., Wilkinson, D., and Boyd, A.W. (1990). Knob-independent cytoadherence of Plasmodium falciparum to the leukocyte differentiation antigen CD36. J. Exp. Med. *171,* 1883–1892.

Bodary, S.C., and McLean, J.W. (1990). The integrin β1 subunit associates with the vitronectin receptor αv subunit to form a novel vitronectin receptor in a human embryonic kidney cell line. J. Biol. Chem. *265,* 5938–5941.

Cheresh, D.A. (1987). Human endothelial cells synthesise and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. Proc. Natl. Acad. Sci. USA *84,* 6471–6475.

Cheresh, D.A., Smith, J.W., Cooper, H.M., and Quaranta, V. (1989). A novel vitronectin receptor integrin αvβx is responsible for distinct adhesive properties of carcinoma cells. Cell *57,* 59–69.

Cheresh, D.A., and Spiro, R.C. (1987). Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. J. Biol. Chem. *262,* 17703–17711.

Davies, J., Warwick, J., Totty, N., Philp, R., Helfrich, M., and Horton, M. (1989). The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. J. Cell Biol. *109,* 1817–1826.

Dedhar, S., Ruoslahti, E., and Pierschbacher, M.D. (1987). A cell surface receptor complex for collagen type I recognizes the Arg-Gly-Asp sequence. J. Cell Biol. *104,* 585–593.

Fingerman, E., and Hemler, M.E. (1988). Regulation of proteins in the VLA cell substrate adhesion family: influence of cell growth conditions on VLA-1, VLA-2, and VLA-3 expression. Exp. Cell Res. *177,* 132–142.

Folkman, J., and Moscona, A. (1978). Role of cell shape in growth control. Nature *273,* 345–349.

Freed, E., Gailit, J., van der Geer, P., Ruoslahti, E., and Hunter, T. (1989). A novel integrin β subunit is associated with the vitronectin receptor α subunit αv in a human osteosarcoma cell line and is a substrate for protein kinase C. EMBO J. *8,* 2955–2965.

Frelinger, A.L., III, Cohen, I., Plow, E.F., Smith, M.A., Roberts, J., Lam, S.C., and Ginsberg, M.H. (1990). Selective inhibition of integrin function by antibodies specific for ligand-occupied receptor conformers. J. Biol. Chem. *265,* 6346–6352.

Gani, J.S., and Burns, G.F. (1990). Integrins and accessory adhesion molecules. Life Sci. *2,* 32–36.

Grinnell, F., Toda, K., and Takashima, A. (1987). Activation of keratinocyte fibronectin receptor function during cutaneous wound healing. J. Cell Sci. Suppl. *8,* 199–209.

Guidry, C., and Grinnell, F.J. (1985). Studies on the mechanism of hydrated collagen gel reorganization by human skin fibroblasts. J. Cell Sci. *79,* 67–81.

Gullberg, D., Tingstrom, A., Thuresson, A-C., Olsson, L., Terracio, L., Borg, T.K., and Rubin, K. (1990). Beta 1 integrin-mediated collagen gel contraction is stimulated by PDGF. Exp. Cell Res. *186,* 264–272.

Hemler, M.E. (1990). VLA proteins in the integrin family: structures, functions, and their role on leukocytes. Annu. Rev. Immunol. *8,* 365–400.

Hynes, R.O. (1987). Integrins: a family of cell surface receptors. Cell *48,* 549–554.

Ingber, D.E. (1990). Fibronectin controls capillary endothelial cell growth by modulating cell shape. Proc. Natl. Acad. Sci. USA *87,* 3579–3583.

Kirchhofer, D., Languino, L.R., Ruoslahti, E., and Pierschbacher, M.D. (1990). Alpha 2 beta 1 integrins from different cell types show different binding specificities. J. Biol. Chem. *265,* 615–618.

Krissansen, G.W., Elliott, M.J., Lucas, C.M., Stomski, F.C., Berndt, M.C., Cheresh, D.A., Lopez, A.F., and Burns, G.F. (1990). Identification of a novel integrin beta subunit expressed on cultured monocytes (macrophages). Evidence that one alpha subunit can associate with multiple beta subunits. J. Biol. Chem. *265,* 823–830.

Lawler, J., Weinstein, R., and Hynes, R.O. (1988). Cell attachment to thrombospondin: the role of Arg-Gly-Asp, calcium, and integrin receptors. J. Cell Biol. *107,* 2351–2361.

Nesbitt, S., Hart, I., and Horton, M.A. (1989). Epitope analysis of the vitronectin receptor (CD51). In: Leucocyte Typing IV, ed. W. Knapp, Oxford, England: Oxford University Press, 1037.

O'Toole, T.E., Loftus, J.C., Du, X., Glass, A.A., Ruggeri, Z.M., Shattil, S.J., Plow, E.F., and Ginsberg, M.H. (1990). Affinity modulation of the $\alpha IIb\beta_3$ integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor. Cell Regul. *1,* 883–893.

Pytela, R., Pierschbacher, M.D., Ginsberg, M.H., Plow, E.F., and Ruoslahti, E. (1986). Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. Science *231,* 1559–1562.

Ruoslahti, E., and Pierschbacher, M.D. (1987). New perspectives in cell adhesion: RGD and integrins. Science *238,* 491–497.

Santoro, S.A. (1986). Identification of a 160,000 dalton platelet membrane protein that mediates the initial divalent cation-dependent adhesion of platelets to collagen. Cell *46,* 913–920.

Staatz, W.D., Walsh, J.J., Pexton, T., and Santoro, S.A. (1990). The $\alpha 2\beta 1$ integrin cell surface collagen receptor binds to the $\alpha 1$ (I)-CB3 peptide of collagen. J. Biol. Chem. *265,* 4778–4781.

van Bockxmeer, F.M., and Martin, C.E. (1982). Measurement of cell proliferation and cell mediated contraction in three-dimensional collagen matrices. J. Tissue Culture Methods *7,* 163–167.

Vogel, B.E., Tarone, G., Giancotti, F.G., Gailet, J., and Ruoslahti, E. (1990). A novel fibronectin receptor with an unexpected subunit composition $\alpha v\beta 1$. J. Biol. Chem. *265,* 5934–5937.