Fibronectin receptor overexpression and loss of transformed phenotype in a stable variant of the K562 cell line

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A variant of the K562 erythroleukemia cell line, FA-K562, was selected by cycles of adhesion to solid-phase plasma fibronectin (FN). FA-K562 expresses fourfold more cell-surface $\alpha_5\beta_1$ fibronectin receptor (FNR) than parental K562. In addition to expected differences in adhesion to FN, other differences between FA-K562 and K562 implicate this FNR in the regulation of cell growth and morphology. FA-K562 proliferates slowly in liquid culture, its cloning efficiency in soft agar is only ~10% compared with ~85% for parental K562, and it is nontumorigenic in nude mice. The reduced soft agar growth potential of FA-K562 involves FNR function, because either glycine-arginine-glycine-aspartate-serine (GRGDS) or monoclonal anti- α_5 antibody in the agar medium increased cloning efficiency of FA-K562 about fivefold. Morphologically, FN-adherent FA-K562 become fibroblastoid in appearance, assemble filamentous actin, and differ from K562 in vimentin staining intensity and pattern. Soluble GRGDS peptide inhibits both FA-K562 adhesion to FN and the associated cytoskeletal changes. These findings link the $\alpha_5\beta_1$ FNR to both the transformed phenotype and morphology of FA-K562.

Introduction

The $\alpha_5\beta_1$ fibronectin receptor (FNR)¹ is one member of a group of heterodimeric glycoproteins termed integrins that mediate cell-cell and cell-substrate adhesion. Virtually all cells examined to date express at least one surface integrin-like molecule (Hemler, 1990). Nu-

merous distinct alpha and beta subunits have been identified, with significant sequence similarity found among homologous integrin subunits from different species (reviewed in Hynes, 1987; Hemler, 1990). Many integrins, the FNR among them, recognize and bind an arginine-glycine-aspartic acid (RGD) sequence present in fibronectin (FN), fibrinogen, and several other extracellular matrix proteins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). The ligand-binding specificity of integrins seems to depend on the particular alpha/beta combination expressed and the cell in which the integrin is expressed (Hemler, 1990).

The β_1 family of integrins, of which the high-affinity FNR ($\alpha_5\beta_1$) is a member, is involved in cell migration during wound healing and embryogenesis (Duband *et al.*, 1986; Savagner *et al.*, 1986). The potential role of the FNR in neoplasia was suggested by the findings that the FNR is phosphorylated in *v-src* transformed cells (Hirst *et al.*, 1986); that transforming agents affect the structure, function, and expression levels of the $\alpha_5\beta_1$ FNR (Plantefaber and Hynes, 1989; Symington *et al.*, 1989); and that glycine-arginine-glycine-aspartate-serine (GRGDS) peptide or anti- β_1 antibodies can prevent pulmonary metastases of B16 melanoma cells (Humphries *et al.*, 1986). Our interest in the potential role of the FNR in tumor metastasis prompted efforts to generate $\alpha_5\beta_1$ expression variants of established tumorigenic cell lines. Described here is the isolation and characterization of one FNR overexpressing cell line, termed FA-K562. FA-K562 was derived from the human erythroleukemia cell line K562 by selection for strong adhesion to human plasma FN. Exposure of FA-K562 cells to FN reversibly regulates cell morphology and cytoskeletal organization. FA-K562 displays slower growth under standard culture conditions and fails to grow in soft agar or nude mice. Both GRGDS and anti- α_5 antibodies specifically stimulate the anchorage-independent growth of FA-K562 in a dose-dependent fashion. These results define a nonstructural role for the high-affinity FNR, $\alpha_5\beta_1$.

¹ Abbreviations used: BSA, bovine serum albumin; FCS, fetal calf serum; FN, fibronectin; FNR, fibronectin receptor; GRGDS, glycine-arginine-glycine-aspartate-serine; PBS, phosphate-buffered saline; RGD, arginine-glycine-aspartic acid; TGF-beta, transforming growth factor-beta.

Results

Selection of FA-K562

An objective of the present study was to learn whether variant cells stably expressing high levels of the $\alpha_5\beta_1$ FNR could be selected by growth on immobilized ligand. K562 was chosen for this study because it is a well-characterized tumorigenic cell line that expresses only the $\alpha_5\beta_1$ integrin (Lozzio and Lozzio, 1979; Hemler *et al.*, 1987; Symington *et al.*, 1989). Parental K562 cells bind weakly to FN without spreading (Symington *et al.*, 1989). After 72 h of growth on FN-coated plastic, ~40% of plated K562 attach, but <1% of these cells spread. Attached cells were expanded into a distinct K562 subline, termed FA-K562 (Fibronectin Adherent), by removal of nonadherent and weakly adherent cells during medium changes. FA-K562 cells displayed a fibroblastic morphology when grown on FN (Figure 1, A and B) but were morphologically indistinguishable from parental K562 cells when grown on uncoated plastic (Figure 1C). FA-K562 cells could not be dislodged from FN-coated dishes by pipetting, but could be released by incubation with

trypsin or 10 mM EDTA in phosphate-buffered saline (PBS). FA-K562 began to reattach and spread on FN-coated dishes within 10 min at 37°C after detachment. The adhesive phenotype of FA-K562 has proved stable, in the absence of selective pressure, for >2 y.

The role of the cell binding domain of FN in FA-K562 adhesion was demonstrated by the ability of 1 mM GRGDS to inhibit attachment and spreading of FA-K562 cells (Figure 1E). Therefore, FA-K562 appears to require specific interaction with the RGD-containing, cell-binding domain of FN. Additional FN-specific sequences or conformation were also required, as FA-K562 did not adhere to plastic coated with thrombin; GRGDS peptide; or the RGD-containing proteins laminin, collagen, or fibrinogen (Figure 1F and data not shown). Although transforming growth factor- β (TGF- β) has been detected in some FN preparations (Fava and McClure, 1987) and could be responsible for morphologic changes, no bioactive TGF- β was found in the FN preparation used in this study (personal communication, Dr. A. Purchio). The dramatic morphologic changes induced in FA-K562 by FN-binding made it important to

Figure 1. Morphology of FA-K562 and K562 grown on different substrates. (A and B) Low- and high-power views, respectively, of FA-K562 growing on plastic coated with human fibronectin. (C) Low-power view of FA-K562 grown on tissue culture plastic. (D) Parental K562 grown on human FN. (E) FA-K562 grown on FN-coated plastic in the presence of 1 mM GRGDS (compare with A). (F) FA-K562 grown on laminin. Bar indicates 10 μ m.

Figure 2. Cell-surface expression of FNR and CD36 by FA-K562 and K562. (A) Autoradiograms of $\alpha_5\beta_1$ immunoprecipitated from equal numbers of [^{35}S]-methionine-labeled parental (K) and FA-K562 (F) cells using the anti- β_1 subunit-specific mAb A1A5 or the anti- α_5 subunit-specific antibodies P1D6 and P1F8, as indicated, and separated by nonreducing SDS-PAGE. Migration positions of molecular weight markers are indicated. (B and C) Fluorescence histogram of K562 (—) and FA-K562 (···) reacted sequentially with saturating amounts of A1A5 (anti- β_1 , in B) or 5F1 (anti-CD36, in C), as indicated, and FITC-conjugated goat anti-mouse IgG. Y-axis represents cell number, X-axis represents log fluorescent intensity.

demonstrate that FA-K562 and K562 were related. The identical patterns generated when genomic DNA from the two cell lines was digested with Taq1 and probed with the polymorphic DNA marker p1-79 (Buroker *et al.*, 1987) demonstrate the relatedness of K562 and FA-K562 (data not shown).

Characterization of FA-K562 receptor expression

One explanation of FA-K562's adhesive phenotype would be that the FNR of FA-K562 differs from that of parental K562. To examine this possibility, FNR was immunoprecipitated from [^{35}S]-methionine labeled K562 and FA-K562 cells with antibodies against distinct α_5 and β_1 epitopes. The A1A5 antibody recognizes the β_1 subunit shared by the Very Late Activation antigens, including the $\alpha_5\beta_1$ FNR (VLA-5). A1A5 immunoprecipitates a complex of α_5 , β_1 , and β_1 precursor subunits from parental K562 cells (Hemler *et al.*, 1987; Symington *et al.*, 1989). P1D6 and P1F8 recognize distinct α_5 epitopes and immunoprecipitate the complex of α_5 and β_1 , without the precursor β_1 (Wayner *et al.*, 1988). The sodium dodecyl sulfate-polyacryl-

amide gel electrophoresis (SDS-PAGE) mobilities of α_5 , β_1 , and 110-kDa β_1 precursor immunoprecipitated from FA-K562 and K562 were identical (Figure 2A). Differences in subunit phosphorylation or glycosylation between K562 and FA-K562 were not detected (data not shown). Densitometric analysis of autoradiograms revealed that FA-K562 expresses four times more immunoprecipitable FNR than does parental K562 using all three antibodies. In addition, fivefold increases in expression of the 110 kDa β_1 precursor were detected with A1A5. The different $\alpha_5\beta_1$ staining intensities detected by A1A5 compared with P1D6 or P1F8 may reflect differences in antibody affinity for, or accessibility to, the $\alpha_5\beta_1$ antigen. In each case, irrelevant, isotype-matched control antibody staining (with 10-3.6-27 for A1A5 or P1B5 for P1D6 and P1F8), was identical for K562 and FA-K562 (Figure 3 and data not shown). The increased cell surface $\alpha_5\beta_1$ expression by FA-K562 was confirmed by immunofluorescence experiments using A1A5 (Figures 2B and 4, E and F). Again, no differences in staining were detected with the isotype-matched irrelevant control antibody 10-3.6-27. Using flow cytometric analysis of cells stained with saturating concentrations of spe-

Figure 3. Cell-surface expression of adhesion receptors. Fluorescence histograms of K562 (—) and FA-K562 (···) sequentially incubated with saturating amounts of the indicated primary and fluorescein-conjugated goat anti-mouse IgG. Antigens stained are (a and b) $\alpha_2\beta_1$, (c) $\alpha_3\beta_1$, (d) ECMR-III/CD 44, (e and f) $\alpha_4\beta_1$, (g) vitronectin receptor ($\alpha_5\beta_3$), and (h) irrelevant IgG.

Figure 4. Distribution of vimentin, actin, and FNR in FA-K562 versus K562. FA-K562 and K562 were stained with antibodies to vimentin (A and B) or to FNR (E and F) or with phalloidin (C and D) and photographed with equal exposure times. Cells in A–D were fixed and permeabilized before staining. Cells in E and F were viable at the time of staining with A1A5. Arrows in C point to areas of actin corresponding to cell processes visible by phase microscopy. Bar indicates 10 μ m.

cific antibodies, we detected no differences in expression levels of $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_3$, extracellular matrix receptor III (ECMR-III), or $\alpha_{IIb}\beta_3$ in K562 and FA-K562 (Figures 3, A–F, and 5A). Staining of these antigens was approximately equal to background Fc receptor staining detected with an irrelevant, isotype-matched antibody (Figure 3G), as previously reported for parental K562 (Hemler *et al.*, 1987; Symington *et al.*, 1989; and unpublished observations). Finally, the above-mentioned receptors could not be immunoprecipitated from [35 S]-labeled K562 or FA-K562 (data not shown). These results were consistent with the lack of cell adhesion to collagen, laminin, or fibrinogen. Thus, the adhesion, spreading, and morphologic changes observed when FA-K562 contacts FN are mediated by overexpression of the prototype FNR, $\alpha_5\beta_1$, and are not due to increases in expression of the alternate FNRs $\alpha_4\beta_1$ and $\alpha_3\beta_1$, vitronectin receptors, or surface proteoglycans

(Wayner and Carter, 1987; Wayner *et al.*, 1989). Cells with similar phenotypes and FNR expression levels have been independently generated three times.

Fourfold elevations in FA-K562 surface CD36 immunofluorescence were detected with the use of the 5F1 antibody (Figure 2C). This antigen is predominantly expressed by adherent hematopoietic cells, such as megakaryocytes, erythroblasts, platelets, and adherent monocytes (Andrews *et al.*, 1983). Although its function is unknown, it is intriguing that CD36 expression parallels the acquisition of adhesiveness by blood cells and the FA-K562 cell line.

Adhesive properties of FA-K562

Short-term adhesion to FN was quantitated using a centrifugal force-based assay (Symington *et al.*, 1989). Approximately twice as many FA-K562 as parental K562 adhered to FN in inde-

pendent assays (Table 1). Although ~40% of K562 did adhere to FN, no spreading or morphologic changes were observed during the 90-min assay. In contrast, approximately twice as many FA-K562 attached to FN and all of the adherent FA-K562 spread and assumed a fibroblastoid morphology. The increased FN adhesion of FA-K562 relative to K562 was observed over a range of FN concentrations (1–100 μ g/ml), incubation times (10–240 min), or centrifugal forces used to remove unattached cells (10–750 $\times g$).

Ligand-induced cell spreading and cytoskeletal organization

Because the ability to spread on FN was a major difference between K562 and FA-K562, cytoskeletal organization seemed likely to differ in the two cell lines. When cultured on FN, only the actin-containing cell cortex of attached parental K562 cells stains with rhodamine-conjugated phalloidin (Figure 4D). In contrast, the cell cortex, leading edges, and pseudopods of FN-adherent FA-K562 stained with phalloidin (Figure 4C). Viable cell immunofluorescence using A1A5 confirmed the increased surface FNR staining of FA-K562 observed by flow cytometric and biochemical data (Figure 4, E and F). FA-K562 displayed a filamentous vimentin staining pattern in contrast with the perinuclear staining pattern observed in K562 (Figure 4, A and B). In each of these experiments, parallel vimentin or phalloidin staining of the mesenchymal cell line MG63 was used as a positive control. Flow cytometric analysis of fixed, permeabilized cells stained with saturating amounts of anti-vimentin antibodies demonstrated low-level staining of FN-adherent K562 or FA-K562 grown in suspension (Figure 5H, —), high-level staining of the mesenchymal cell line MG63 (Figure 5H, — — —), and intermediate-level staining of FA-K562-grown adherent to FN at the time of staining (Figure 5H, · · · ·). Thus, both vimentin staining pattern and levels correlate with the degree of cell spreading in FA-K562.

FA-K562 does not differentiate in response to FN

It has been suggested that FN plays a role in the erythroid differentiation of hematopoietic

Table 1. Specific adhesion of K562 and FA-K562 to FN*

Assay	K562	FA-K562	Ratio (FA-K562/K562)
1	29	72	2.5
2	48	84	1.8
3	35	83	2.4
4	48	86	1.8

10⁴ [³⁵S]-methionine-labeled FA-K562 or K562 cells were plated per well of 96-well plates precoated with FN (30 μ g/ml) or BSA (1 mg/ml). After 90 min at 37°C, wells were washed to remove nonadherent cells, and bound cells were solubilized and counted.

* Percentage specific adhesion, defined as percentage of input cells adhering to FN minus percentage of input cells adhering to BSA (<5%). Results presented are the averages of duplicate wells.

cells (Patel and Lodish, 1987). Because parental K562 cells are multipotential (Lozzio and Lozzio, 1979), the role of differentiation in the adhesive phenotype of FA-K562 was investigated. No evidence of differentiation of FA-K562 compared with K562 was detectable by the use of two independent methods. First, flow cytometric analysis of cells stained with antibodies to a panel of megakaryocytic (Figure 5, A and B) or myeloid (Figure 5, C–F) differentiation antigens were similar (Andrews *et al.*, 1983; Reinherz *et al.*, 1985). No differences were observed in intracellular cytochemical staining with myeloperoxidase (granulocytes) or periodic acid Schiff reagent (megakaryocytes) (Wintrobe, 1981) (data not shown). Finally, constitutive and hemin-inducible fetal globin expression by FA-K562 was similar to that observed in K562 by indirect immunofluorescence (data not shown), indicating that FA-K562 maintains an early erythroid phenotype. These findings, in conjunction with the observations that FA-K562 could proliferate and grow indefinitely on FN without detaching, suggest that the distinguishing features of FA-K562 are not due to differentiation.

Reduced anchorage-independent growth of FA-K562

Transformed fibroblasts and suspension cell lines typically can grow in soft agar, unlike pri-

Figure 5. Surface differentiation marker and intracellular vimentin staining of K562 and FA-K562. K562 (—) and FA-K562 (· · ·) were stained with saturating amounts of primary antibodies (as indicated) and fluorescein-conjugated goat anti-mouse IgG. Panels a–g represent surface staining of viable cells, whereas panel h represents intracellular staining of permeabilized cells. Antigens stained in panels a and b are megakaryocytic, and panels c–f are granulocytic differentiation markers. Panel g is the irrelevant antibody control for panels a–f. Panel h represents the vimentin staining of K562 (—), FA-K562 (· · ·) or MG63 (— — —). Irrelevant antibody staining is indicated. Staining of FA-K562 grown in suspension was superimposable on K562 staining and is depicted by the same line.

Figure 6. Soft agar colony formation and proliferation. (A) Representative colonies of FA-K562 (a and b) or K562 (c and d) growing in soft agar. (a and c) show high-magnification views; (b and d) show low-magnification views. Bars in panels a and b indicate 10 μm . (B) Growth curves for parental (○) and FA-K562 (▲) cells. X-axis represents days in culture, Y-axis represents cell number ($\times 10^5$). Graph depicts one representative experiment of cells grown on noncoated plastic, although similar results were obtained when cells were grown on FN-coated plastic.

many cells and adherent cell lines (MacPherson and Montagnier, 1964; Metcalf, 1977). The ability of fibroblastoid FA-K562 cells to grow in soft agar was therefore evaluated. Parental K562 cells readily formed large colonies (>50 cells) in soft agar, as was previously described (Lozzio and Lozzio, 1979). In contrast, few ($\sim 10\%$) FA-K562 formed colonies, and most ($\sim 70\%$) FA-K562 failed to proliferate in soft agar (Figure 6A, Table 2). Overall, 85% of parental and only 10–15% of FA-K562–formed colonies were estimated to contain >50 cells in soft agar. Although FA-K562 proliferated more slowly than parental cells in suspension culture (Figure 6B), this did not account for its inability to grow in soft agar. Neither the size nor the number of colonies formed in soft agar by FA-K562 cells increased noticeably during prolonged culture (4–6 wk). Finally, soft agar colonies of both parental and FA-K562 could be picked from agar and grown in suspension, suggesting that the poor growth of FA-K562 in soft agar was not due to cell death. Tumor formation in nude mice correlated with anchorage-independent growth. All of five mice injected with parental cells and none of five mice injected with FA-K562 developed tumors at the injection site (at 2 and 6 wk of observation, respectively).

To test whether anchorage-independent growth was linked to FNR expression, we plated cells in soft agar in the presence of GRGDS peptides or the anti- α_5 antibody P1D6. Both GRGDS peptide and P1D6 stimulated soft agar growth of FA-K562, but had little or no effect on K562 growth (Table 3). These effects were

Table 2. Soft agar colony formation

	Colonies*	Single cells
K562	80 $\pm$ 4† 87 $\pm$ 2	23 $\pm$ 4 14 $\pm$ 2
FA-K562	15 $\pm$ 4 11 $\pm$ 3	67 $\pm$ 6 74 $\pm$ 10

Equal numbers of parental and FA-K562 were plated in RPMI with 20% FCS. Colonies were counted at day 14 and 21 and expressed as a percentage of input cells to correct for minor differences in plating number.

* Colonies defined as >50 cells.

† Results are expressed as percentage ($\pm$ SE) of plated cells growing in colonies or clusters. Each value represents the mean of four wells. Of note, most of the plated parental cells formed large colonies with cells too numerous to count. In contrast, most of subline remained as single cells (see Figure 6A).

Table 3. GRGDS or Anti-FNR stimulate anchorage-independent growth of FA-K562

	Concentration	GRGDS	TKPG
Peptides	100 μ g/ml	69 $\pm$ 5	8 $\pm$ 1
	50 μ g/ml	48 $\pm$ 4	14 $\pm$ 2
	5 μ g/ml	33 $\pm$ 1	21 $\pm$ 3
	0 μ g/ml	13 $\pm$ 3	
		P1D6	5F1
Antibodies	5 μ g/ml	72 $\pm$ 2	17 $\pm$ 4
	1 μ g/ml	59 $\pm$ 3	14 $\pm$ 4
	0.1 μ g/ml	46 $\pm$ 4	28 $\pm$ 4
	0 μ g/ml	13 $\pm$ 3	

Results are presented as the mean percentage of FA-K562 plated ($\pm$ SE for quadruplicate samples) that formed colonies in the presence of peptide or purified antibodies, as indicated. Equivalent concentrations of test peptide or antibody (GRGDS or P1D6, anti- $\alpha_5\beta_1$, respectively) and irrelevant peptide (threonine-lysine-proline-glycine, TKPG) or cell-binding antibody (5F1, anti-CD36) were used. Colony formation by K562 was uniformly high under all conditions ($>85 \pm 4$).

specific and dose dependent, as neither the negative control peptide (threonine-lysine-proline-glycine) nor the cell-binding, negative control antibody 5F1 stimulated soft agar growth of FA-K562. Thus, ligand or antibody binding to the receptor can reverse the growth inhibitory effects of $\alpha_5\beta_1$ overexpression. These results suggest a complex interaction between surface FNR levels, FNR occupation, and growth control.

Discussion

This report demonstrates that a cell line overexpressing a single integrin can be generated by growth on immobilized ligand without mutagenesis. FA-K562 expressed four- to fivefold higher levels of the prototype FNR ($\alpha_5\beta_1$) without detectable increases in expression levels of other integrins. Of particular interest, expression of "alternate" FNRs, such as $\alpha_4\beta_1$ or $\alpha_3\beta_1$, was not induced (Wayner *et al.*, 1989). In addition, prolonged growth on immobilized laminin, collagen, or vitronectin did not result in cell adhesion or selection of cells overexpressing integrins specific for these ligands. Finally, no selective pressure was required to maintain FNR overexpression during 2 y of suspension culture. These data suggest that inductive receptor expression does not occur and favor a mechanism involving selection of preexisting FNR overexpressors. Sol-

uble GRGDS was used previously to select stable FNR overexpressors of the adherent cell line MG63 (Dedhar *et al.*, 1987). Interestingly, although the MG63 cells originally expressed more than one RGD-binding integrin, only FNR overexpressors were selected. Both studies demonstrate that environmental pressure alone can result in selection of stable $\alpha_5\beta_1$ overexpressors.

No increases were observed in expression levels of two different vitronectin receptors (gpIIb/IIIa or $\alpha_v\beta_3$) (Figures 3G and 5A). This was potentially important to rule out, as vitronectin is considered the serum factor responsible for cell spreading (Holmes, 1967; Hayman *et al.*, 1983). No increases in surface expression of the collagen-binding heparan sulfate proteoglycan, CD44/ECMR-III, were detected by immunoprecipitation or immunofluorescence (Figure 3D and data not shown) (Wayner and Carter, 1987). Finally, because FA-K562 adhesion to FN is trypsin insensitive and RGD inhibitable, syndecan is unlikely to be the receptor mediating FA-K562 adhesion to FN (Saunders and Bernfield, 1988).

Cells selected for high-level FNR expression coincidentally were found to express high levels of CD36, independent of the continued presence of immobilized FN. Among the phenotypic markers analyzed, only CD36 expression was elevated in FA-K562. Although CD36 was originally identified as a differentiation antigen, its expression correlates more with the adhesive phenotype than the differentiation state of cells (Andrews *et al.*, 1983). CD36 is the monomeric, 88-kDa transmembrane receptor expressed by monocytes, endothelial cells, and melanoma cell lines, mediating binding of *Plasmodium falciparum*-infected erythrocytes to these cells (McGregor *et al.*, 1989; Oquendo *et al.*, 1989). Although its normal function is unknown, CD36 does not bind FN and has no sequence homology to previously identified adhesion receptors (McGregor *et al.*, 1989; Oquendo *et al.*, 1989). FA-K562 may prove helpful in identifying the CD36 ligand and in examining the regulation of CD36 expression.

Insofar as K562 express $\alpha_5\beta_1$ and bind to but do not spread on FN, they are mechanicochemically uncoupled. FA-K562, expressing modestly elevated surface $\alpha_5\beta_1$, can both bind to and spread on FN in an RGD-sensitive manner. How could increased FNR expression confer the ability to spread on FN? Extracellular FN, by virtue of its large size, two RGD sequences, and tendency to aggregate, may allow adherence of cells with low surface

levels of FNR. The low-affinity interactions between FNR and the smaller, cytoskeletal proteins involved in spreading (Burrige, 1986) may require multivalent binding provided by a critical plasma membrane FNR concentration.

Cell spreading on FN was accompanied by several features distinguishing FA-K562 from other integrin overexpressors. Whereas FA-K562 and K562 were morphologically indistinguishable when grown in suspension, exposure to solid-phase FN rapidly resulted in acquisition of fibroblastic morphology, cytoskeletal organization, and increased vimentin immunoreactivity by FA-K562 only (Figures 4, A and B, and 5H). Flow cytometric analysis of permeabilized cells stained with anti-vimentin antibodies showed that vimentin staining levels were dependent on the degree of cell spreading. In addition, spreading induced organization of vimentin into filaments. FA-K562 thus provides a model for examining how matrix regulates cell morphology and gene/protein expression (Bissell and Varcellos-Hoff, 1987; Menko and Boettiger, 1987; Werb *et al.*, 1989).

The present findings link $\alpha_5\beta_1$ overexpression by FA-K562 cells with loss of transformed phenotype. We propose to call this retrotransformation. Unlike parental K562, FA-K562 grew poorly in soft agar and was no longer tumorigenic in nude mice. Utilizing DNA transfection instead of selection of natural variants, Giancotti and Ruoslahti (1990) recently showed that 16- to 33-fold increases in $\alpha_5\beta_1$ expression result in retrotransformation of a fibroblast cell line. In FA-K562, more modest increases in $\alpha_5\beta_1$ were accompanied by loss of transformation. This may reflect a stronger influence of $\alpha_5\beta_1$ expression on transformation than previously suspected or cell-lineage-specific sensitivity to $\alpha_5\beta_1$ expression. In either case, both studies indicate that $\alpha_5\beta_1$ function is critical to growth control.

Both GRGDS peptide and anti- α_5 antibodies were shown to stimulate anchorage-independent growth of FA-K562 in a dose-dependent and specific manner. These findings suggest that $\alpha_5\beta_1$ has a direct role in growth control. Although $\alpha_5\beta_1$ could influence growth in numerous ways, any potential mechanistic explanation must account for the observations that 1) increased $\alpha_5\beta_1$ expression results in poor anchorage-independent growth and that 2) ligand or antibody binding by those cells overexpressing $\alpha_5\beta_1$ overcomes this inhibition. One hypothesis consistent with the data is that FN-binding by more

than a threshold number of surface FNR molecules inhibits anchorage-independent growth. Parental K562 lacks sufficient surface FNR to cross this threshold. In this model, GRGDS or anti- α_5 antibodies could act as competitive inhibitors of FN. A more speculative hypothesis is that $\alpha_5\beta_1$ expression above a threshold level allows cells to bind low-affinity, growth-inhibitory substances. Soluble ligand or antibody to $\alpha_5\beta_1$ may displace this inhibitory substance, allowing normal signal transduction to occur. A central role of $\alpha_5\beta_1$ in growth control may explain why diverse transforming agents have been found to affect the structure, expression, or function of this particular integrin (Hirst *et al.*, 1986; Plantefaber and Hynes, 1989; Symington *et al.*, 1989). The final common pathway shared by transforming agents may be the disruption of growth regulatory signals transmitted through $\alpha_5\beta_1$. Understanding the nature of the growth regulatory signals induced by FNR-ligand interactions will form the basis of future studies.

Methods

Materials

Purified human plasma FN was obtained from the New York Blood Center (New York, NY), Sigma Chemical (St. Louis, MO), Dr. H. Karges (Behringwerke, Marburg, Federal Republic of Germany), and Dr. W. Carter (Fred Hutchinson Cancer Research Center [FHCRC], Seattle, WA). Monoclonal antibodies A1A5 (specific for the β_1 subunit of the human VLA-5 antigen/FNR), P1D6, P1F8 (specific for α_5), P1H5, P4B4 (anti- $\alpha_2\beta_1$), P1B5 (anti- $\alpha_3\beta_1$), P1G12 (anti-ECMR-III), P3E3, and P4C2 (anti- $\alpha_4\beta_1$) were kindly provided by Drs. M. Hemler (Dana-Farber Cancer Institute, Boston, MA) and W. Carter (FHCRC, Seattle, WA), (Hemler *et al.*, 1987; Wayner and Carter, 1987; Wayner *et al.*, 1988). Monoclonal antibodies to the platelet glycoproteins (anti-IIb/III_a and I_b) and to myeloid differentiation antigens (5F1, 1G10, T5A7, 12-8, and L4F3) were generously provided by Dr. R. Andrews (FHCRC, Seattle, WA) (Andrews *et al.*, 1983; Reinherz *et al.*, 1985). Rhodamine-conjugated phalloidin was a gift from Dr. R. Manger (NeoRx, Seattle, WA). Affinity-purified mouse anti-human vimentin antibodies were purchased from Boehringer-Mannheim (Indianapolis, IN). Affinity-purified fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG was purchased from Tago Laboratories (Burlingame, CA). Bacto-Agar was purchased from Difco Laboratories (Detroit, MI). Hemin, GRGDS, threonine-lysine-proline-glycine, 12-O-tetradecanoylphorbol 13-acetate, laminin, bovine serum albumin (BSA), gelatin, purified human fibrinogen, and thrombin were purchased from Sigma. L-[³⁵S]-methionine (>800 Ci/mmol) was purchased from DuPont, New England Nuclear (NEN, Boston, MA).

Cell culture

The K562 cell line was obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) at 37°C under a

humidified 95% air, 5% CO₂ atmosphere. Medium was replenished every 3 d.

Subline selection

Polystyrene petri dishes or 96-well plates (Flow Labs, McLean, VA) were incubated with the specified concentrations of FN (1–30 µg/ml) in PBS containing 1 mM CaCl₂ and MgCl₂ (CM-PBS) for 2 h at 37°C. The plates were then rinsed with PBS and cells were added. Plates were washed daily to remove loosely adherent cells and allow expansion of the <1% strongly adherent cells. After expansion, ~90% of the selected subline was tightly FN adherent under limiting dilution cloning conditions. Cells cloned in this manner or recloned in soft agar had similar phenotypes.

Cell labeling and immunoprecipitation

Cell labeling and immunoprecipitation were performed as previously described (Symington *et al.*, 1989). Briefly, cells were labeled with [³⁵S]-methionine (100 µCi/ml) overnight in methionine-free RPMI; normalized for cell number (for cell-surface immunoprecipitation) or for trichloroacetic acid (TCA)-precipitable radioactivity (for lysates); and sequentially incubated with saturating amounts of primary antibody, secondary antibody, and protein A-Sepharose beads. The protein A-sepharose beads were washed and boiled in sample buffer, and the eluates were loaded onto 7.5% SDS-PAGE gels. Quantitation of bands seen on autoradiograms was performed by using the Visage 2000 computer-assisted integrating optical densitometer equipped with a Kodak 20-step gray gradient wedge.

Indirect immunofluorescence

Indirect immunofluorescence and flow cytometry of viable cells were performed by the use of saturating amounts of primary and FITC-conjugated secondary antibodies as previously described (Symington *et al.*, 1989). Briefly, cells were serially incubated at 4°C with primary and fluorescein-conjugated secondary antibodies, and the washed cells were then analyzed using a FACS II flow cytometer and a Zeiss (Thornwood, NY) immunofluorescence photomicroscope. Specific fluorescent staining intensities were determined by subtracting staining with an irrelevant, isotype-matched control antibody from staining with test antibody. The flow cytometer was calibrated by measuring the fluorescence intensity of beads coupled to known amounts of fluorescein (coefficient of linearity 0.998). Phalloidin or vimentin staining was performed on cells fixed for 20 min at room temperature with 4% paraformaldehyde in PBS, permeabilized for 3 min with 0.2% Triton X-100 in PBS, and rinsed in PBS. Rhodamine-conjugated phalloidin (100 ng/ml) was then added to cells on coverslips and incubated for 2 h at 37°C. For vimentin staining, cells on coverslips were fixed, incubated for 30 min at room temperature with fetal calf serum (FCS), rinsed, and incubated for 1 h at 37°C with 5 µg/ml of anti-vimentin followed by fluorescein-conjugated anti-mouse IgG. Rinsed coverslips were mounted in ice-cold Elvanol (Monsanto, Indian Orchard, MA). For these experiments, MG63 cells were used as a positive control for both actin and vimentin staining. Photographs of parental and adherent K562 cells were taken with identical exposure times.

Growth curves

Equal numbers of parental cells or FA-K562 were plated in triplicate in 96-well plates either coated or not coated with FN (10 µg/ml). Cells in each well were resuspended and

counted by the use of a hemacytometer at the designated times. FN-adherent cells were completely detached by trypsinization before counting.

Adhesion assays

These were performed as previously described (Symington *et al.*, 1989). Briefly, 10⁶ [³⁵S]-methionine-labeled cells (average incorporation 5–10 cpm/cell) were plated in serum-free RPMI 1640 in wells of plastic 96-well plates precoated with test proteins. After a 90-min incubation at 37°C, plates were inverted and spun at 200 × g for 2 min. Adherent cells (expressed as a percentage of total input cells) were solubilized in Biofluor (NEN) and radioactivity was measured in a scintillation counter.

Soft agar colony assays

Equal numbers of cells in double-strength RPMI containing 40% heat-inactivated FCS were mixed with an equal volume of 45°C, 0.6% aqueous Bacto-Agar before plating in petri dishes. In some experiments, peptides or purified antibodies were included in soft agar. Colonies were counted 10–30 d later.

Experimental metastases

Nude mice (Simonson Labs) were injected subcutaneously with 10⁶ viable K562 or FA-K562 cells, maintained in micro-isolator cages, and examined for tumor formation.

Restriction fragment length polymorphism analysis

Genomic DNA was isolated from the two cell lines by phenol-chloroform extraction, incubated with Taq 1 restriction enzyme, separated on 0.8% agarose gels, transferred to nitrocellulose, and probed with ³²P-labeled p1-79 (a polymorphic DNA marker) (Buroker *et al.*, 1987) and exposed to film.

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