THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 270, No. 37, Issue of September 15, pp. 21612–21618, 1995
Printed in U.S.A.

Requirement for the Synergy Site for Cell Adhesion to Fibronectin Depends on the Activation State of Integrin $\alpha5\beta1$*

(Received for publication, April 5, 1995, and in revised form, July 11, 1995)

Erik H. J. Danen‡§, Shin-ichi Aota¶, Annemieke A. van Kraats‡, Kenneth M. Yamada¶, Dirk J. Ruiter‡, and Goos N. P. van Muijen‡

From the ‡Department of Pathology, University Hospital, 6500 HB Nijmegen, The Netherlands and the ¶Laboratory of Developmental Biology, NIDR, National Institutes of Health, Bethesda, Maryland 20892

We investigated the influence of the activation state of integrin $\alpha5\beta1$ on its dependence on the PHSRN synergy site for binding to RGD in fibronectin. K562 and MV3 cells lacked $\alpha v\beta3$ expression and adhered to fibronectin through $\alpha5\beta1$. Mel57 cells adhered through $\alpha v\beta3$ and $\alpha5\beta1$. A recombinant fibronectin polypeptide, containing five type III repeats from the central cell binding domain 3Fn6–10, and a mutated polypeptide lacking the synergy site were equally effective in promoting Mel57 adhesion. For K562 and MV3, the mutated polypeptide was not or poorly active compared to the control polypeptide. Expression of $\alpha v\beta3$ in MV3 induced strong adhesion to the mutated polypeptide. TS2/16 stimulatory $\beta1$-integrin antibodies or Mn^{2+} induced $\alpha5\beta1$-mediated adhesion of K562 and MV3 to GRGDSP. In the presence of TS2/16 or Mn^{2+}, $\alpha5\beta1$-mediated MV3 adhesion to the mutated polypeptide was equally strong as adhesion to the control polypeptide. Mn^{2+} or TS2/16 induced weak K562 binding to the mutated polypeptide, and in the presence of a combination of phorbol 12-myristate 13-acetate, Mn^{2+}, and TS2/16, $\alpha5\beta1$-mediated K562 adhesion to the mutated and control polypeptide was equally strong. Our findings demonstrate that requirement for the PHSRN synergy site for $\alpha5\beta1$-mediated adhesion to RGD in fibronectin depends on the activation state of the integrin.

Fibronectin (Fn)[1] is an extracellular matrix glycoprotein that functions in cell adhesion and migration in wound healing, embryonic development, and malignant transformation (1, 2). The Fn molecule is composed of three types of repeating modules, termed type I, II, and III repeats (3), which are organized into functional domains. Proteolytic cleavage yields several fragments containing domains that promote cell adhesion, including the carboxyl-terminal HepII domain (4), the alternatively spliced type III connecting segment (5), and the central cell binding domain (CCBD).

The CCBD consists of type III repeats, each containing approximately 90 amino acids (6). Cells bind to the CCBD via receptors of the integrin family (7). Integrins are $\alpha\beta$ heterodimeric transmembrane molecules mediating cell-cell adhesion and attachment of cells to the extracellular matrix (8). Integrins that bind the CCBD include $\alpha3\beta1$ (9), $\alpha5\beta1$ (10, 11), $\alpha v\beta1$ (12), $\alpha v\beta3$ (13), $\alpha IIb\beta3$ (14, 15), and $\alpha v\beta6$ (16).

The Arg-Gly-Asp (RGD) sequence in the 10th type III repeat (3Fn10) is the key attachment site for binding of these integrins to the CCBD, as demonstrated by inhibition of cell adhesion with synthetic RGD-containing peptides (17, 18). Furthermore, two synergistic regions in the CCBD besides RGD have been identified that are required for cell adhesion through $\alpha IIb\beta3$ (19) and $\alpha5\beta1$ (20–23). For $\alpha5\beta1$ binding to Fn, the synergy region in 3Fn9 is the most important of these two regions (21), and recently, a short amino acid sequence Pro-His-Ser-Arg-Asn (PHSRN) was identified in this repeat that synergistically enhances the cell adhesion promoting activity of the RGD sequence (24). This sequence is also present in an 11-amino acid integrin binding site from 3Fn9 that is recognized by $\alpha IIb\beta3$ (25).

Integrins do not always constitutively bind to their ligands with high affinity. Integrin adhesiveness can be stimulated by phorbol esters and other more physiologically relevant agonists (8, 26). In addition, antibodies have been described to integrin $\beta1$ (27–30), $\beta2$ (31), and $\beta3$ (32) subunits, which induce a high affinity state of the integrins. Studies with stimulatory $\beta1$ antibodies on hematopoietic cells have demonstrated modulation of binding to natural ligands (28–30), modulation of ligand specificity (33), modulation of binding to different regions in one ligand (34), and modulation of the minimal sequence of a binding site required for adhesion (35).

In the present study, we have investigated the role of the PHSRN synergy site in $\alpha5\beta1$- and $\alpha v\beta3$-mediated cell adhesion to the CCBD in Fn. We show that requirement for the PHSRN synergy site for cell adhesion to the CCBD depends on the integrins expressed and on the activity of the integrins involved.

MATERIALS AND METHODS

Fibronectin, Fragments, and Peptides—Plasma Fn was purchased from Sigma. A 120-kDa chymotryptic Fn fragment containing the CCBD (36, 37) was purchased from Life Technologies, Inc. Synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) was obtained from the Department of Organic Chemistry, Faculty of Science, University of Nijmegen (The Netherlands) and covalently bound to bovine serum albumin (BSA) as previously described (38).

Production of Recombinant Fibronectin Polypeptides—To avoid the artifactual losses of adhesive activity known to result from adsorbing short polypeptides on substrates (*e.g.* see Ref. 23), we used recombinant Fn polypeptides containing five type III Fn repeats from 3Fn6 through 3Fn10. The 3Fn6–10 wild type expression construct was generated based on the T7 phage promotor and a Fn cDNA fragment encoding Fn type III repeat numbers 6–10 produced using the polymerase chain reaction method (24); the PHSRN sequence was present in repeat 9 and RGD in repeat 10 (Fig. 2*A*). The amino-terminal sequence of 3Fn6 starts immediately after an initiation codon for methionine. To create

* This work was supported by Dutch Cancer Society Grant NUKC 91-09. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Pathology, University of Nijmegen, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands. Fax: 31-80-540520.

[1] The abbreviations used are: Fn, fibronectin; BSA, bovine serum albumin; CCBD, central cell binding domain; 3Fn10, 10th type III fibronectin repeat; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium.

TABLE I
Fibronectin-binding integrins on K562, MV3, and Mel57 cells

	Mean fluorescence							
	C	α3	α4	α5	αv	αIIb	β1	αvβ3
K562	4	4	4	46	7	3	52	3
MV3	4	69	31	62	13	3	107	2
Mel57	3	41	17	23	33	3	33	41

substitution mutants, two complementary oligonucleotides with appropriate sequences were synthesized, annealed, and then cloned between the *Bam*HI and *Eco*RI sites of 3Fn9. This yielded a mutated polypeptide 3Fn6–10(SPSDN) where the PHSRN sequence from 3Fn9 was substituted by SPSDN from 3Fn8 (Fig. 2, *A* and *B*).

Protein expression was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside treatment of *Escherichia coli* strain BL21 (DE3, pLysS) containing the expression plasmid. The expressed recombinant polypeptides were purified by sequential DEAE and hydroxyapatite column chromatography. The polypeptide was eluted from a DEAE column (DE52, Whatman) using a linear gradient of 0–0.5 M NaCl in 10 mM sodium phosphate (pH 7.4), 1 mM EDTA, 0.02% sodium azide, applied to a hydroxyapatite column (Bio-Rad), and eluted using a linear gradient from 5 mM sodium phosphate (pH 6.5), 0.4 mM EDTA, 0.02% sodium azide to 250 mM sodium phosphate (pH 6.5), 0.4 mM EDTA, 0.02% sodium azide. The fractions with peak absorbance were evaluated for purity by SDS-polyacrylamide gel electrophoresis, pooled, dialyzed against phosphate-buffered saline without Ca^{2+} or Mg^{2+} and with 0.02% sodium azide, and stored at −80 °C.

Cell Lines and Culture Conditions—The human melanoma cell lines used included Mel57 (39) and MV3 (40). The K562 erythroleukemic cell line was provided by Dr. Nancy Hogg. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Flow, Irvine, United Kingdom) supplemented with 10% fetal calf serum, penicillin, and streptomycin.

Antibodies—Anti-integrin antibodies included P1B5 anti-α3 (41), purchased from Telios Pharmaceuticals Inc. (San Diego); HP2/1 anti-α4 (42), provided by Dr. Francisco Sanchez-Madrid; NKI-Sam1 anti-α5 (30), provided by Dr. Carl Figdor; 4B4 anti-β1 (43), purchased from Coulter Immunology (Hialeah, FL); AJ2 anti-β1 (44), provided by Dr. Eberhard Klein; C17 anti-β3 (45), provided by Dr. Arnoud Sonnenberg; A109 polyclonal anti-αv (46), purchased from Life Technologies, Inc.; 10E5 anti-αIIb (47), provided by Dr. Barry Coller; and LM142 anti-αv and LM609 anti-αvβ3 (48), provided by Dr. David Cheresh. The stimulatory anti-integrin β1 mAbs were 8A2 (29), provided by Dr. Nicholas Kovach, and TS2/16 (49), provided by Dr. Francisco Sanchez-Madrid.

Cell Adhesion—Cell adhesion assays were performed as previously described (50). In short, polystyrene microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight with the appropriate adhesive ligands and blocked for 1 h at 37 °C with DMEM containing 0.5% (w/v) BSA. Subsequently, 1×10^4 ^{51}Cr-labeled cells in 50 μl of DMEM/BSA were added to the wells and incubated for 30 min at 37 °C in 5% CO_2. Unbound cells were removed by washing with DMEM/BSA, bound cells were lysed by detergent, and radioactivity of the lysate was measured in a gamma counter. Results are presented as the mean percentage of cell binding from triplicate wells. For induction of adhesion, radiolabeled cells were preincubated with TS2/16 or 8A2 mAbs for 30 min at 4 °C before seeding in the wells or 1 mM $MnCl_2$; or, 100 ng/ml phorbol 12-myristate 13-acetate (PMA) was added to the cells prior to seeding in the wells. For antibody inhibition studies, cells were preincubated with the appropriate mAbs for 30 min at 4 °C before seeding into the wells.

Flow Cytometry—Cells were incubated with mAbs in phosphate-buffered saline containing 0.5% (w/v) BSA and 0.02% (w/v) sodium azide for 30 min at 4 °C. After washing with phosphate-buffered saline/BSA/azide, the cells were incubated with fluorescein-isothyocyanate-labeled $F(ab')_2$ fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark) for 30 min at 4 °C. After washing, fluorescence was measured on an Epics Elite flow cytometer (Coulter, Mijdrecht, The Netherlands).

Transfection—The full-length cDNA for the integrin β3 subunit (51), a kind gift from Dr. Erkki Ruoslahti, was cloned in the polylinker of the mammalian expression vector pBJ1neo (52), kindly provided by Dr. René de Waal-Malefijt. 20 μg of this construct was used for stable transfection of MV3 cells according to the calcium phosphate precipitation method (53), using the calcium phosphate transfection system (Life Technologies, Inc.). After 48 h, stably transfected cells were selected by culturing in the presence of 1 mg/ml G418 (Life Technologies, Inc.) for 2 weeks. Cell populations were enriched for αvβ3 expression by cell sorting in an Epics Elite flow cytometer using LM609 mAbs. After three cycles of sorting, transfected cell lines contained >95% αvβ3 positive cells. Cells were maintained in culture in medium containing 200 μg/ml G418 and were regularly monitored for αvβ3 expression.

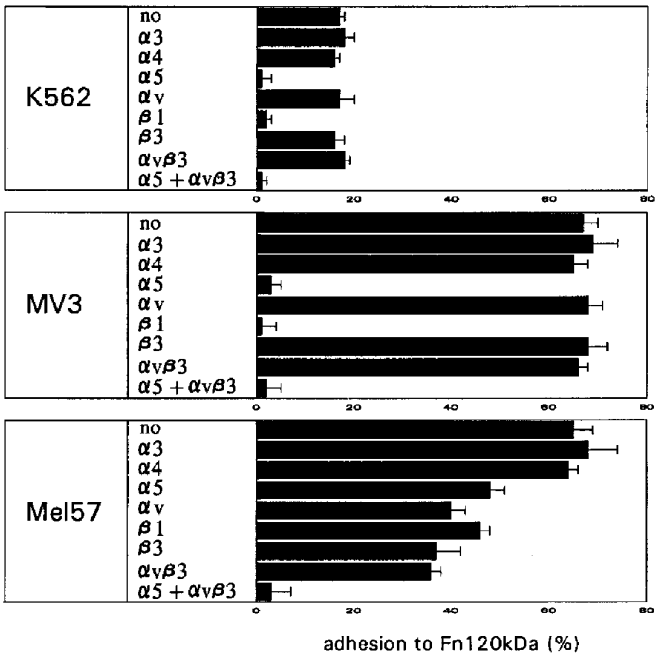

FIG. 1. **Inhibition of adhesion to Fn120 kDa with integrin mAbs.** Cells were allowed to adhere to wells coated with 20 μg/ml of a 120-kDa fragment of Fn (Fn120 kDa) in the absence (*no*) or in the presence of inhibitory mAbs to integrin subunits as indicated. Adhesion to BSA was less than 5%. One representative experiment of four is shown.

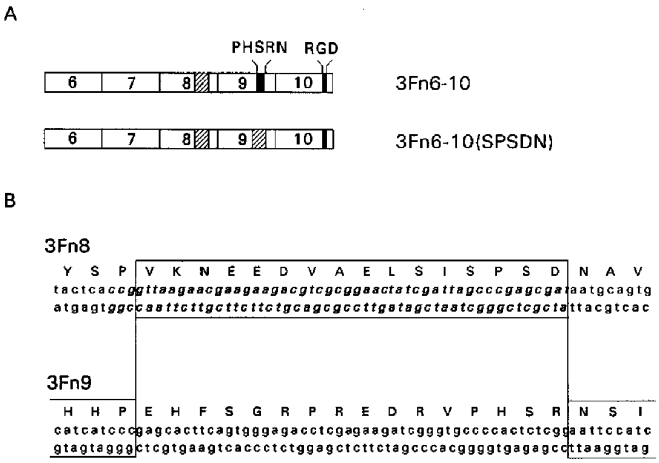

FIG. 2. **Recombinant FN polypeptides.** *A*, schematic representation of a recombinant Fn polypeptide 3Fn6–10 consisting of five type III Fn repeats from 3Fn6 through 3Fn10 and of a mutated Fn polypeptide 3Fn6–10(SPSDN) where the region containing PHSRN in 3Fn9 has been substituted by the corresponding region from 3Fn8 (*hatched bar*). *B*, in 3Fn6–10(SPSDN), 16 amino acid residues from 3Fn9 were substituted with the corresponding residues from 3Fn8 to disrupt the PHSRN site in 3Fn9. *Boxed sequences* are from 3Fn6–10(SPSDN). The region with *italicized nucleotides* differs from the original sequence in 3Fn8 for technical reasons to alter restriction sites, but this does not alter the amino acid sequence.

RESULTS

K562, MV3, and Mel57 Differentially Adhere to the CCBD—We investigated adhesion of K562 human erythroleukemic cells and MV3 and Mel57 human melanoma cells to the CCBD. Of the integrins known to be involved in adhesion to Fn,

K562 exclusively expressed $\alpha5\beta1$ (Table I). MV3 and Mel57 expressed $\alpha3\beta1$, $\alpha4\beta1$, and $\alpha5\beta1$. In addition, Mel57 but not MV3 expressed $\alpha v\beta3$. MV3 and Mel57 expressed other αv integrins including $\alpha v\beta5$ (54) and possibly $\alpha v\beta1$ that may bind to Fn.

To exclude influences from domains outside the CCBD that are known to have cell adhesive activity (HepII, IIICS), we used a 120-kDa Fn fragment that lacks the heparin-binding domain and the V region but includes the CCBD. K562 adhered weakly to Fn120 kDa, whereas MV3 and Mel57 both adhered strongly (Fig. 1). As expected from the surface expression data, adhesion of K562 was completely blocked by mAbs to $\alpha5$ or $\beta1$. Even though MV3 expressed several Fn-binding integrins, adhesion was fully blocked by mAbs to $\alpha5$, whereas mAbs to $\alpha3$ or $\alpha4$ or polyclonal anti-αv had no effect. Adhesion of Mel57 was inhibited approximately 35% by mAbs to $\alpha5$ or $\beta1$ and about 50% by

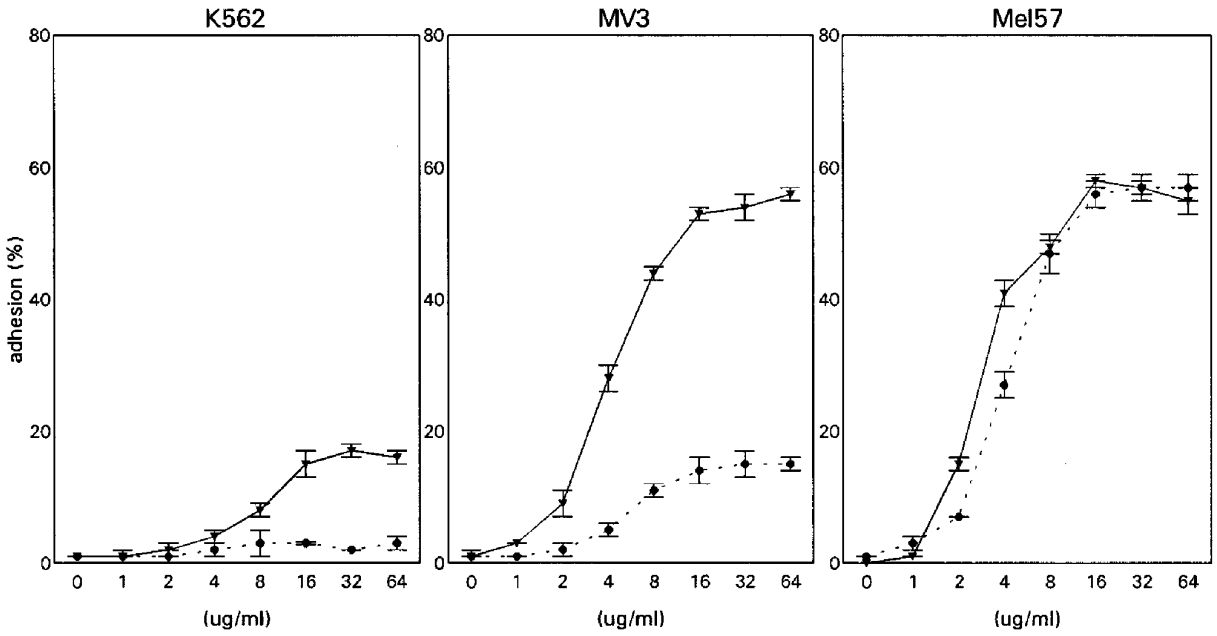

FIG. 3. **Adhesion to recombinant Fn polypeptides.** K562, MV3, or Mel57 cells were allowed to adhere to wells coated with increasing concentrations of 3Fn6–10(SPSDN) (*dotted line*) or 3Fn6–10 (*line*) as indicated. Adhesion to 0.1 mg/ml BSA was less than 4%. One representative experiment of four is shown.

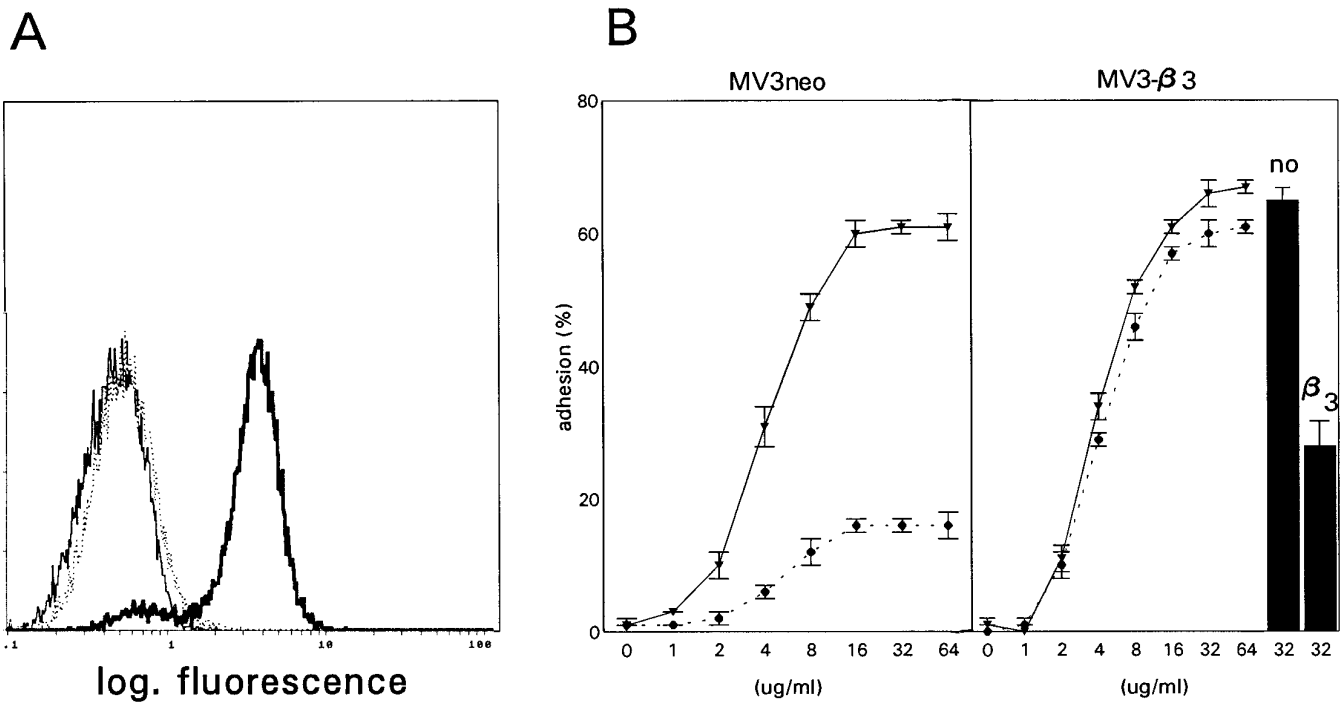

FIG. 4. **Expression of $\alpha v\beta3$ on MV3 induces adhesion to 3Fn6–10(SPSDN).** *A*, MV3 cells were untransfected (*dotted line*), transfected with pBJ1neo alone (*thin line*), or transfected with pBJ1neo including integrin $\beta3$ cDNA followed by sorting with LM609 anti-$\alpha v\beta3$ mAbs (*thick line*). Shown is the relative fluorescence after incubation with LM609 and a fluorescein-isothyocyanate-labeled second antibody. *B*, MV3neo or MV3-$\beta3$ cells were allowed to adhere to wells coated with increasing concentrations of 3Fn6–10(SPSDN) (*dotted line*) or 3Fn6–10 (*line*) as indicated. *Filled bars* represent remaining adhesion to wells coated with 32 μg/ml 3Fn6–10(SPSDN) in the presence of inhibitory anti-integrin mAbs as indicated. Adhesion to BSA was less than 3%. One representative experiment of three is shown.

mAbs to $\beta3$ or $\alpha v\beta3$ or by polyclonal αv. The combination of mAbs to $\alpha5$ and $\alpha v\beta3$ completely blocked adhesion of Mel57.

Thus, K562 adheres weakly to the CCBD through $\alpha5\beta1$, MV3 binds strongly through $\alpha5\beta1$, and Mel57 binds strongly through $\alpha5\beta1$ and $\alpha v\beta3$.

K562 and MV3 Require the PHSRN Synergy Site, Whereas for Mel57 RGD Is Sufficient—To study the mechanism of binding of these cells to the CCBD, we used a recombinant Fn polypeptide containing 3Fn6–3Fn10 and a mutated polypeptide lacking the recently described PHSRN synergy site (24) (Fig. 2). As shown in Fig. 3, K562 did not adhere to the mutated polypeptide and weakly to the control polypeptide. Only very low adhesion of MV3 cells was observed to the mutated polypeptide, whereas adhesion of MV3 to the control polypeptide was five times higher. In contrast, Mel57 cells adhered strongly to both polypeptides. To investigate if this difference was due to differential expression of $\alpha v\beta3$, we transfected MV3 cells with $\beta3$ cDNA, resulting in $\alpha v\beta3$ surface expression (Fig. 4*A*), and used these cells in adhesion assays. Expression of $\alpha v\beta3$ provided MV3 cells with the capacity to adhere to GRGDSP (not shown) and to the mutated polypeptide, and this adhesion could be inhibited by C17 anti-$\beta3$ mAbs (Fig. 4*B*). A similar level of inhibition was found with LM609 anti-$\alpha v\beta3$ (not shown).

From these results, we conclude that the differential requirement for the PHSRN synergy site for adhesion to RGD in the CCBD of MV3 *versus* Mel57 is due to the different binding mechanisms of $\alpha5\beta1$ *versus* $\alpha v\beta3$.

Stimulation of $\alpha5\beta1$-mediated RGD Binding with $\beta1$ mAbs, PMA, and Manganese—The fact that K562 did not adhere to the mutated polypeptide whereas MV3 did to a low extent (Fig. 3), even though both cell lines used $\alpha5\beta1$, suggested that binding of $\alpha5\beta1$ to RGD in 3Fn10 might depend on the activation state of the integrin. To investigate this, we treated both cell lines with 8A2 and TS2/16 stimulatory $\beta1$ mAbs, with Mn^{2+}, or with PMA prior to using them in adhesion assays to a GRGDSP peptide. PMA had no effect, 8A2 and TS2/16 induced weak adhesion of K562 to GRGDSP, and treatment of MV3 cells with these mAbs resulted in 25% adhesion to GRGDSP (Fig. 5*A*). A control $\beta1$-integrin mAb AJ2 had no effect (not shown). The strong binding of Mel57 was not enhanced by 8A2 or TS2/16. Mn^{2+} was less effective for K562 but induced adhesion of MV3 cells up to 35%. We performed adhesion inhibition assays to examine whether the effect of 8A2 and TS2/16 was due to activation of $\alpha5\beta1$ or to the recruitment of other integrins. Induced adhesion of K562 to GRGDSP in the presence of 8A2 (Fig. 5*B*) or TS2/16 (not shown) was blocked by mAbs to $\alpha5$ and not by any of the other mAbs. In addition, even though 8A2 and TS2/16 may activate $\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$, and possibly $\alpha v\beta1$ on MV3 cells, induced adhesion of MV3 to GRGDSP was completely blocked by mAbs to $\alpha5$, whereas mAbs to $\alpha3$, $\alpha4$, and αv had no effect (Fig. 5*B*). The fact that the 4B4 anti-$\beta1$ mAb did not inhibit adhesion in the presence of 8A2 is in line with the report that activating and inhibiting antibodies share a common epitope on the $\beta1$ subunit (55).

Thus, the strength of $\alpha5\beta1$ binding to RGD can be increased by Mn^{2+} and by activating $\beta1$ antibodies.

Requirement for the PHSRN Synergy Site Depends on the Activation State of $\alpha5\beta1$—As stimulatory $\beta1$ mAbs and Mn^{2+} induced $\alpha5\beta1$-mediated adhesion to GRGDSP, we hypothesized that the activation state of $\alpha5\beta1$ determines the requirement for the PHSRN synergy site for cell adhesion to the CCBD. Therefore, we treated K562 and MV3 cells with PMA, TS2/16, or Mn^{2+} and allowed them to adhere to the mutated and control Fn polypeptides. TS2/16 and, to a lesser extent, Mn^{2+} induced adhesion of K562 cells to the mutated polypeptide and enhanced adhesion to the control polypeptide (Fig. 6). PMA enhanced adhesion of K562 cells to the control polypeptide but had no effect on adhesion to the mutated polypeptide. For MV3 cells, no effect of PMA was observed, but the low adhesion to the mutated polypeptide was enhanced 5-fold by TS2/16 and Mn^{2+}, resulting in a level of adhesion that was similar to that observed with the fully active control polypeptide.

The fact that in the presence of TS2/16 or Mn^{2+} no difference was observed between the mutated and control polypeptide regarding adhesion of MV3 cells, whereas for K562 the mutated polypeptide was still poorly active, could suggest 1) that stimulation of MV3 cells resulted in recruitment of other RGD-binding integrins or 2) that $\alpha5\beta1$ on K562 cells was not maximally activated by these agents. To exclude possibility 1, we used mAbs to $\alpha3$, $\alpha4$, $\alpha5$, αv, $\beta1$, $\beta3$, $\alpha v\beta3$, or the combination of these mAbs in the absence of anti-$\alpha5$ for inhibition of TS2/16-stimulated adhesion of MV3 cells to the mutated polypep-

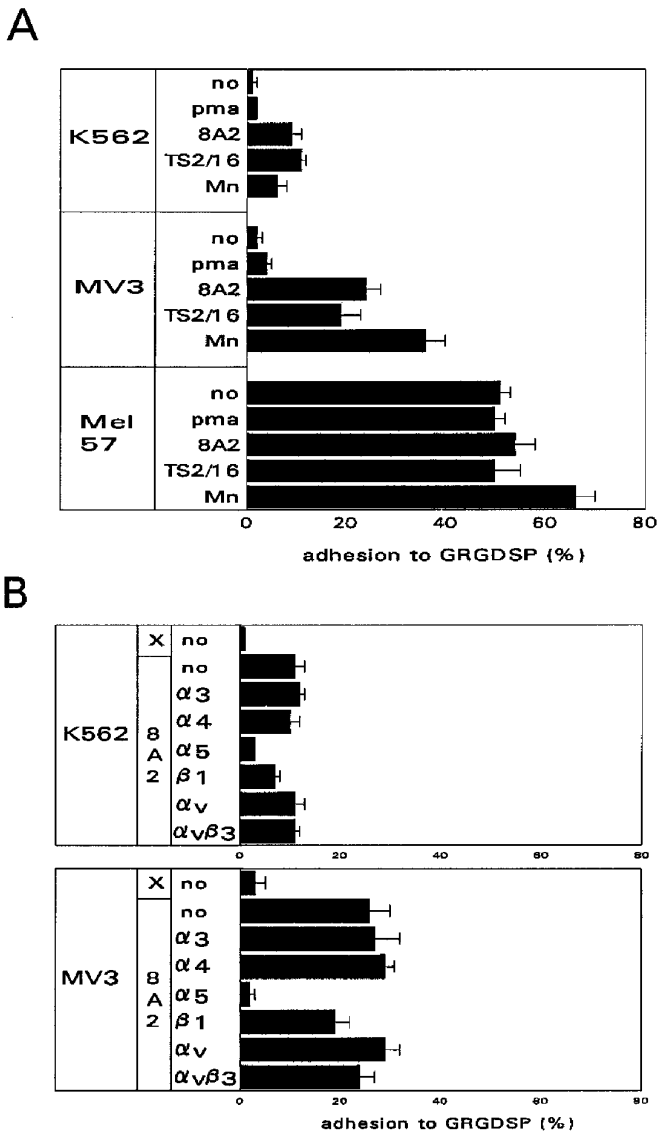

FIG. 5. **Stimulation of $\alpha5\beta1$-mediated adhesion to GRGDSP.** *A*, Cells were incubated in the absence (*no*) or in the presence of PMA, 8A2 or TS2/16 stimulatory $\beta1$ mAbs, or manganese (Mn) and allowed to adhere to wells coated with 20 μg/ml BSA-GRGDSP. *B*, cells that had been previously incubated in the absence (*x*) or in the presence of 8A2 stimulatory $\beta1$ mAbs (*8A2*) were incubated in the absence (*no*) or in the presence of inhibitory anti-integrin mAbs as indicated and allowed to adhere to wells coated with 20 μg/ml BSA-GRGDSP. Adhesion to BSA was less than 5%. One representative experiment of three is shown.

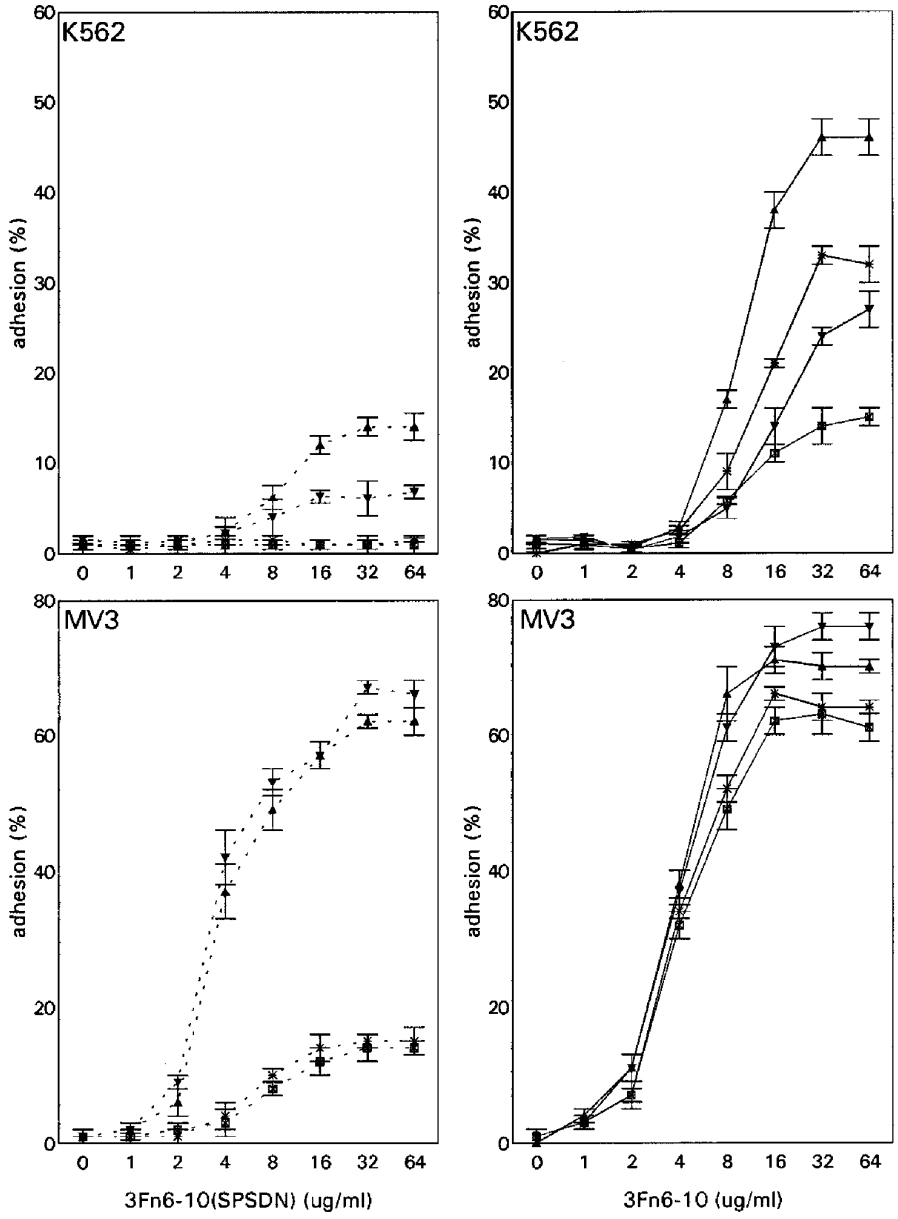

FIG. 6. **Stimulation of adhesion to recombinant Fn polypeptides.** Cells were incubated in the absence (☒) or in the presence of PMA (*), TS2/16 (▲), or manganese (▼) and allowed to adhere to wells coated with increasing concentrations of 3Fn6–10(SPSDN) (*dotted line*) or 3Fn6–10 (*line*) as indicated. Adhesion to BSA was less then 4%. One experiment of three is shown.

tide. Stimulated adhesion was blocked by the anti-α5 mAb and not by any of the other mAbs or their combination (Fig. 7), suggesting that induction of adhesion to the mutated polypeptide of MV3 by TS2/16 was due to activation of $\alpha5\beta1$ and not to recruitment of other integrins.

To investigate possibility 2, we incubated K562 cells with PMA, TS2/16, or Mn^{2+} and the various combinations and allowed the cells to adhere to the mutated and control polypeptide. In the presence of the combination of TS2/16 and Mn^{2+}, adhesion to the mutated polypeptide was more than half the level of adhesion to the control polypeptide (Fig. 8). PMA had no effect by itself on adhesion to the mutated polypeptide but enhanced adhesion to the control polypeptide more than 2-fold. Finally, in the presence of the combination of PMA, TS2/16, and Mn^{2+}, the control and the mutated polypeptide were equally effective in promoting adhesion of K562 cells. This adhesion was blocked by α5 mAbs (not shown).

From these results, we conclude that requirement of the PHSRN synergy site for $\alpha5\beta1$-mediated adhesion to RGD in the CCBD depends on the activation state of $\alpha5\beta1$.

DISCUSSION

In line with earlier reports, we find that $\alpha v\beta3$ does not require the PHSRN site. We base this conclusion on 2 observations. First, Mel57 cells express $\alpha v\beta3$ and adhere equally well to all molecules tested containing RGD, *i.e.* GRGDSP, the mutated polypeptide lacking the synergy site 3Fn6–10(SPSDN), the control polypeptide 3Fn6–10, and Fn120 kDa. Second, the $\alpha v\beta3$ negative MV3 cells do not adhere to RGD-containing ligands that lack the PHSRN site, and transfection with $\beta3$ cDNA resulting in $\alpha v\beta3$ surface expression leads to binding of these cells to GRGDSP and 3Fn6–10(SPSDN).

These findings confirm and extend the observations that $\alpha v\beta3$ can be retained on an RGD column (56) whereas $\alpha5\beta1$ cannot (11). Furthermore, these data are in agreement with the recent report that αv- and α3- but not α5-containing integrins are bound by a column containing a Fn fragment lacking the synergy region (57). Similarly, it has been reported that $\alpha IIb\beta3$ but not $\alpha v\beta3$ binding to Fn can be inhibited by an 11-amino acid peptide from 3Fn9 that also contains the PHSRN sequence (25). Thus, RGD is sufficient for binding to Fn through $\alpha v\beta3$,

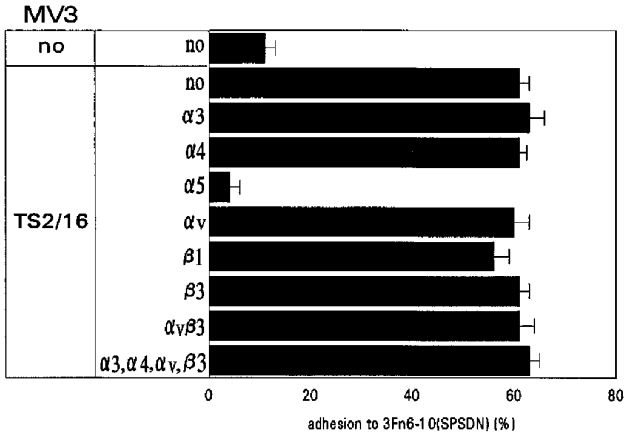

FIG. 7. **Inhibition of stimulated adhesion of MV3 to 3Fn6–10(SPSDN) with integrin mAbs.** MV3 cells were incubated in the absence or in the presence of TS2/16 and allowed to adhere to wells coated with 32 μg/ml 3Fn6–10(SPSDN). Inhibitory mAbs to integrin subunits were added as indicated. Adhesion to BSA was less then 3%. One experiment of three is shown.

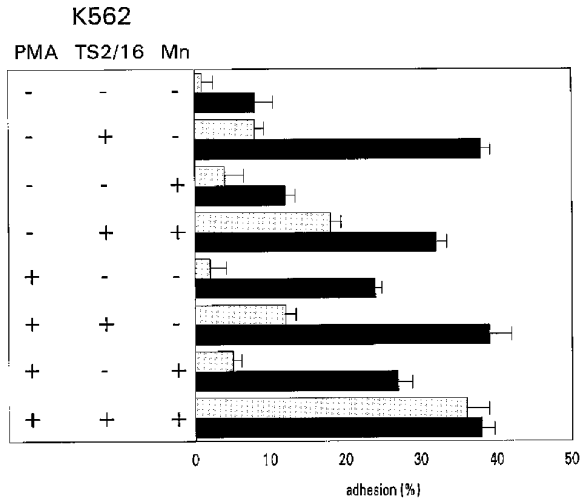

FIG. 8. **Stimulation of adhesion of K562 to recombinant Fn polypeptides.** K562 cells were incubated in the absence or in the presence of various combinations of PMA, TS2/16, and Mn^{2+} as indicated and allowed to adhere to wells coated with 32 μg/ml 3Fn6–10(SPSDN) (*dotted bars*) or 3Fn6–10 (*filled bars*). Adhesion to BSA was less then 4%. One experiment of three is shown.

whereas α5β1 and αIIbβ3 require the synergy region for efficient binding to Fn (24, 25).

Parenthetically, it has been reported that cross-talk between αvβ3 and α5β1 can occur (58, 59). Therefore, the induced adhesion to 3Fn6–10(SPSDN) upon expression of αvβ3 in MV3 cells did not necessarily have to be due to αvβ3-mediated adhesion. Even though Blystone *et al.* (59) show that αvβ3 regulates only α5β1-mediated phagocytosis, in our system αvβ3 might influence α5β1-mediated adhesion. Ligation of αvβ3 with LM609 mAbs might induce a signal that inhibits α5β1. To exclude this possibility, we used C17 anti-β3 for adhesion inhibition assays. The fact that these mAbs inhibit adhesion of β3-transfected MV3 cells to 3Fn6–10(SPSDN) suggests that direct binding through αvβ3 rather than signaling to α5β1 is involved.

The major conclusion from this study is that the requirement for the PHSRN synergy site for α5β1-mediated adhesion to the CCBD depends on the activation state of α5β1. This is based on three findings. First, stimulation of K562 cells that express only α5β1, with Mn^{2+} or stimulatory β1-integrin mAbs, induces adhesion to GRGDSP and 3Fn6–10(SPSDN). Second, in the presence of the combination of PMA, TS2/16, and Mn^{2+}, the mutated and control polypeptide are equally effective in promoting K562 cell adhesion. Third, treatment of MV3 cells with these agents induces adhesion to GRGDSP and enhances adhesion to 3Fn6–10(SPSDN) to the level of adhesion to 3Fn6–10, and this effect is completely blocked by antibodies to α5 but not by mAbs to α3, α4, or αv or the combination.

Even though the αvβ3-negative K562 and MV3 cells express similar levels of α5β1, they differ dramatically in binding to Fn120 kDa through this receptor. The view of cell type-specific regulation of α5β1 affinity proposed by O'Toole *et al.* (60) suggests that the default low affinity state of the integrin as observed in K562 is switched to a high affinity state in MV3. As a result, MV3 but not K562 cells bind strongly to Fn120 kDa. Our finding that K562 cells bind poorly to Fn120 kDa and that 8A2 increases that adhesion two to three times is in line with earlier findings (61). As expected, Mn^{2+} and stimulatory β1 mAbs do not affect the strong adhesion of MV3 to Fn120 kDa. However, our findings demonstrate that these agents do in fact alter the avidity of α5β1 in MV3 cells but that this change can only be observed in the absence of the PHSRN synergy site. One interpretation of these findings is that intracellular factors (induced by PMA for K562 and factors already present in MV3) can increase the affinity of α5β1 to a level that RGD is recognized in the Fn molecule and that additional extracellular events are required for the final activation of α5β1, leading to full adhesion to RGD in Fn. The synergy site could be involved in the last step by locking the RGD site in the α5β1 binding pocket, and in the presence of TS2/16 or Mn^{2+} that last step seems to be no longer required. Our finding that PMA enhances K562 adhesion to the control polypeptide, whereas by itself it has no effect on adhesion to the mutated polypeptide, is in line with this idea. Furthermore, the fact that K562 cells in the presence of TS2/16 bind strongly to the control polypeptide without the need for PMA demonstrates that optimal extracellular stimulation (the synergy site plus stimulatory β1 mAbs) can abbrogate the need for intracellular activation (PMA).

It is of interest that comparable observations have been reported for α4β1 (35). Even though Jurkat and Ramos cells express an active form of α4β1 in the sense that they are capable of binding to the CS1 domain of Fn, they only bind to a peptide containing the EILDV recognition sequence from CS1 in the presence of stimulatory β1 mAbs. The authors suggest that sequences may be present in the NH_2-terminal portion of CS1 that strengthen α4β1 binding to EILDV, although none have yet been identified. Thus, the presence of sites that synergistically enhance binding of integrins to their recognition sequence might be a general mechanism, and activation by stimulatory β1 mAbs and Mn^{2+} may bypass the dependence on such sites. Our report, however, provides the first example of substitution of the function of a well characterized synergy site by agents that activate the integrins involved.

For leukocytes, stimulatory β1 mAbs also increase the affinity of α4β1 for CS1 (35, 62) and for VCAM-1 (29), and they can even induce α4β1 binding to the RGDS sequence (34). MV3 cells adhere to CS1 and tumor necrosis factor α-stimulated endothelial cells in the absence of stimuli (not shown), indicating the expression of active α4β1 on these cells. For MV3 cells in the absence or presence of stimuli, we do not observe any inhibition of binding to the CCBD with HP2/1 anti-α4 mAbs, whereas these mAbs inhibit binding to CS1 (not shown). Thus, the reported recognition of RGD by stimulated α4β1 does not play a role in our assay. This difference may be explained by the fact that Ramos cells, as used by Sanchez-Aparicio *et al.* (34), do not express α5β1. In MV3, the effect of TS2/16 on α4β1

may be masked by the binding to RGD through $\alpha5\beta1$. Alternatively, as previously reported (63), stimulatory $\beta1$ mAbs may selectively activate $\alpha5\beta1$ while leaving $\alpha4\beta1$ unaffected.

A possible interpretation of our findings may be that the PHSRN synergy site binds to the same epitope as recognized by the stimulatory $\beta1$ mAbs. It has been previously suggested that the epitope where these mAbs bind may physically interact with extracellular proteins (55). However, the fact that binding of K562 to 3Fn6–10 can be enhanced in the presence of 8A2 or TS2/16 when PMA is absent indicates that the synergy site and stimulatory mAbs can have additional combined stimulatory effects. Therefore, these data seem to support a model where the synergy site and stimulatory mAbs have different binding sites on $\alpha5\beta1$. This is in agreement with the recent report that the mechanism of binding of integrin $\alpha5\beta1$ to Fn seems to be through binding of the $\alpha5$ subunit to the synergystic regions and of the $\beta1$ subunit to RGD (64).

In conclusion, our data demonstrate that $\alpha5\beta1$ but not $\alpha v\beta3$ requires the PHSRN synergy site for cell adhesion to RGD in the CCBD of Fn but that induction of a high affinity state of $\alpha5\beta1$ with PMA, stimulatory mAbs, and/or Mn^{2+} abrogates this dependence on the PHSRN sequence.

Acknowledgments—We thank Drs. David Cheresh, Barry Coller, Carl Figdor, Eberhard Klein, Nicholas Kovach, Francisco Sánchez-Madrid, and Arnoud Sonnenberg for kindly providing antibodies. We thank Dr. René de Waal-Malefijt for the pBJ1-neo expression vector, Dr. Erkki Ruoslahti for the $\beta3$-cDNA, and Dr. Nancy Hogg for the K562 cell line. We are indebted to Arie Pennings for expert assistance in the flow cytometric cell sorting procedure and to Dr. Carl Figdor for critical reading of the manuscript.

REFERENCES

1. Mosher, D. A. (1989) *Fibronectin*, Academic Press, San Diego
2. Hynes R. O. (1990) *Fibronectins*, Springer Verlag, New York
3. Petersen, T. E., Thogersen, H. C., Skorstengaard, K., Vibe-Pedersen, Sahl, P., Sottrup-Jensen, L., and Magnusson, S. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 137–141
4. McCarthy, J. B., Chelberg, M. K., Mikelson, D. J., and Furcht, L. T. (1988) *Biochemistry* **27,** 1380–1388
5. Humphries, M. J., Komoriya, A., Akiyama, S. K., Olden, K., and Yamada, K. M. (1987) *J. Biol. Chem.* **262,** 6886–6892
6. Kornblihtt, A. R., Umezawa, K., Vibe-Pedersen, K., and Baralle, F. E. (1985) *EMBO J.* **4,** 1755–1759
7. Hynes, R. O. (1987) *Cell* **48,** 549–554
8. Hynes, R. O. (1992) *Cell* **69,** 11–25
9. Elices, M. J., Urry, L. A., and Hemler, M. E. (1991) *J. Cell Biol.* **112,** 169–181
10. Akiyama, S. K., and Yamada, K. M. (1985) *J. Biol. Chem.* **260,** 4492–4500
11. Pytela, R., Pierschbacher, M. D., and Ruoslahti, E. (1985) *Cell* **40,** 191–198
12. Vogel, B. E., Tarone G., Giancotti, F. G., Gailit, J., and Ruoslahti, E. (1990) *J. Biol. Chem.* **265,** 5934–5937
13. Charo, I. F., Nannizzi, L., Smith, J. W., and Cheresh, D. A. (1990) *J. Cell Biol.* **111,** 2795–2800
14. Ginsberg, M. H., Forsyth, J., Lightsey, A., Gediak, J., and Plow, E. F. (1983) *J. Clin. Invest.* **71,** 619–624
15. Gardner, J. M., and Hynes, R. O. (1985) *Cell* **42,** 439–448
16. Busk, M., Pytela, R., and Sheppard, D. (1992) *J. Biol. Chem.* **267,** 5790–5796
17. Pierschbacher, M. D., and Ruoslahti, E. (1984) *Nature* (*Lond.*) **309,** 30–33
18. Yamada, K. M., and Kennedy, D. W. (1984) *J. Cell Biol.* **99,** 29–36
19. Bowditch, R. D., Halloran, C. E., Aota, S., Obara, M., Plow, E. F., Yamada, K. M., and Ginsberg, M. H. (1991) *J. Biol. Chem.* **266,** 23323–23328
20. Obara, M., Kang, M. S., and Yamada, K. M. (1988) *Cell* **53,** 649–657
21. Aota, S., Nagai, T., and Yamada, K. M. (1991) *J. Biol. Chem.* **266,** 15938–15943
22. Kimizuka, F., Ohdate, Y., Kawase, Y., Shimojo, T., Taguchi, Y., Hashino, K., Goto, S., Hashi, H., Kato, I., Sekiguchi, K., and Titani, K. (1991) *J. Biol. Chem.* **266,** 3045–3051
23. Nagai, T., Yamakawa, N., Aota, S., Yamada, S. S., Akiyama, S. K., Olden K., and Yamada, K. M. (1991) *J. Cell Biol.* **114,** 1295–1305
24. Aota, S., Nomizu, M., and Yamada, K. M. (1994) *J. Biol. Chem.* **269,** 24756–24761
25. Bowditch, R. D., Hariharan, M., Tominna, E. F., Smith, J. W., Yamada, K. M., Getzoff, E. D., and Ginsberg, M. H. (1994) *J. Biol. Chem.* **269,** 10856–10863
26. Diamond, M. S., and Springer, T. A. (1994) *Curr. Biol.* **4,** 506–517
27. Neugebauer, K. M., and Reichardt, L. F. (1991) *Nature* (*Lond.*) **350,** 68–71
28. Arroyo, A. G., Sànchez-Mateos, P., Campanero, M. R., Martín-Padura, I., Dejana, E., and Sànchez-Madrid, F. (1992) *J. Cell Biol.* **117,** 659–670
29. Kovach, N. L., Carlos, T. M., Yee, E., and Harlan, J. M. (1992) *J. Cell Biol.* **116,** 499–509
30. Van de Wiel van Kemenade, E., van Kooyk, Y., de Boer, A. J., Huijbens, R. J. F., Weder, P., van de Kasteele, W., Melief, C. J. M., and Figdor, C. G. (1992) *J. Cell Biol.* **117,** 461–470
31. Robinson, M. K., Andrew, D., Rosen, H., Brown, D., Ortlepp, S., Stephens, P., and Butcher, E. C. (1992) *J. Immunol.* **148,** 1080–1085
32. 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) *Cell Regul.* **1,** 883–893
33. Chan, B. M. C., and Hemler, M. E. (1993) *J. Cell Biol.* **120,** 537–543
34. Sanchez-Aparicio, P., Dominguez-Jiménez, C., and Garcia-Pardo, A. (1994) *J. Cell Biol.* **126,** 271–279
35. Wayner, E. A., and Kovach, N. L. (1992) *J. Cell Biol.* **116,** 489–497
36. Pierschbacher, M. D., Hayman, E. G., and Ruoslahti, E. (1981) *Cell* **26,** 259–267
37. Ruoslahti, E., Hayman, E. G., Engvall, E., Cothran, W. C., and Butler, W. T. (1981) *J. Biol. Chem.* **256,** 7277–7281
38. Peeters, J. M., Hazendonk, T. G., Beuvery, E. C., and Tesser, G. I. (1989) *J. Immunol. Methods* **120,** 133–143
39. Brüggen, J., Sorg, C., and Macher, E. (1978) *Cancer Immunol. Immunother.* **5,** 53–68
40. Van Muijen, G. N. P., Jansen, C. F. J., Cornelissen, I. M. H. A., Smeets, D. F. C. M., Beck, J. L. M., and Ruiter, D. J. (1991) *Int. J. Cancer* **48,** 85–91
41. Wayner, E. A., and Carter, W. G. (1987) *J. Cell Biol.* **105,** 1873–1884
42. Sanchez-Madrid, F., De Landazuri, M. O., Morago, G., Cebrian, M., Acevedo, A., and Bernabeu, C. (1986) *Eur. J. Immunol.* **16,** 1343–1349
43. Morimoto, C., Letvin, N. L., Boyd, A. W., Hagan, M., Brown, H. M., Kornacki, M. M., and Schlossman, S. F. (1985) *J. Immunol.* **134,** 3762–3769
44. Kantor, R. R. S., Mattes, M. J., Lloyd, K. O., Old, L. J., and Albino, A. P. (1987) *J. Biol. Chem.* **262,** 15158–15165
45. Tetteroo, P. A. T., Lansdorp, P. M., Leeksma, O. C., and Von Dem Borne, A. E. G. (1983) *Br. J. Haematol.* **55,** 509–521
46. Suzuki, S., Argraves, W. S., Pytela, R., Arai, H., Krusius, T., Pierschbacher, M. D., and Ruoslahti, E. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8614–8618
47. Coller, B. S., Peerschke, E. I., Scudder, L. E., and Sullivan, C. A. (1983) *J. Clin. Invest.* **72,** 325–338
48. Cheresh, D. A., and Harper, J. R. (1987) *J. Biol. Chem.* **262,** 1434–1437
49. Hemler, M. E., Sanchez-Madrid, F., Flotte, T. J., Kremsky, A. M., Burakoff, S. J., Bhan, A. K., Springer, T. A., and Strominger, J. L. (1984) *J. Immunol.* **132,** 3011–3018
50. Danen, E. H. J., Van Muijen, G. N. P., Van de Wiel-van Kemenade, P., Jansen, C. F. J., Ruiter, D. J., and Figdor, C. G. (1993) *Int. J. Cancer* **54,** 315–321
51. Van Kuppevelt, T. H. M. S. M., Languino, L. R., Gailit, J. O., Susuki, S., and Ruoslahti, E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5415–5418
52. Lin, A. Y., Devaux, B., Green, A., Sagerström, C., Elliott, J. F., and Davis, M. M. (1990) *Science* **249,** 677–679
53. Wigler, M., Silverstein, S., Lee, L. S., Pellicer, A., Cheng, Y., and Axel, R. (1977) *Cell* **11,** 223–232
54. Danen, E. H. J., Jansen, C. F. J., Van Kraats, A. A., Cornelissen, I. M. H. A., Ruiter, D. J., and Van Muijen, G. N. P. (1995) *Int. J. Cancer* **61,** 491–496
55. Takada, Y., and Puzon, W. (1992) *J. Biol. Chem.* **268,** 17597–17601
56. Pytela, R., Pierschbacher, M. D., Ginsberg, M. H., Plow, E. F., and Ruoslahti, E. (1986) *Science* **231,** 1559–1562
57. Akiyama, S. K., Aota, S., and Yamada, K. M. (1995) *Cell Adh. Comm.* **3,** 13–25
58. Bauer, J. S., Schreiner, C. L., Giancotti, F. G., Ruoslahti, E., and Juliano, R. L. (1992) *J. Cell Biol.* **116,** 477–487
59. Blystone, S. D., Graham, I. L., Lindberg, F. P., and Brown, E. J. (1994) *J. Cell Biol.* **127,** 1129–1137
60. O'Toole, T. E., Katagiri, Y., Faull, R. J., Peter, K., Tamura, R., Quaranta, V., Loftus, J. C., Shattil, S. J., and Ginsberg, M. H. (1994) *J. Cell Biol.* **124,** 1047–1059
61. Faull, R. J., Kovach, N. L., Harlan, J. M., and Ginsberg, M. H. (1993) *J. Cell Biol.* **121,** 155–162
62. Matsumoto, A., and Hemler, M. E. (1993) *J. Biol. Chem.* **268,** 228–234
63. Faull, R. J., Kovach, N. L., Harlan, J. M., and Ginsberg, M. H. (1994) *J. Exp. Med.* **179,** 1307–1316
64. Obara, M., and Yoshizato, K. (1995) *Exp. Cell Res.* **216,** 273–276

Requirement for the Synergy Site for Cell Adhesion to Fibronectin Depends on the Activation State of Integrin α5β1

Erik H. J. Danen, Shin-ichi Aota, Annemieke A. van Kraats, Kenneth M. Yamada, Dirk J. Ruiter and Goos N. P. van Muijen

J. Biol. Chem. 1995, 270:21612-21618.
doi: 10.1074/jbc.270.37.21612