We investigated the influence of the activation state of integrin α5β1 on its dependence on the PHSRN synergy site for binding to RGD in fibronectin. K562 and MV3 cells lacked αvβ3 expression and adhered to fibronectin through α5β1. Mel57 cells adhered through αvβ3 and α5β1. A recombinant fibronectin polypeptide, containing five type III repeats from the central cell binding polypeptide, expression of αvβ3 in MV3 induced strong adhesion of K562 and MV3 to GRGDSP. In the presence of TS2/16 or Mn2+, α5β1-mediated adhesion of K562 and MV3 to GRGDSP. In the presence of a combination of phorbol 12-myristate 13-acetate, Mn2+, or TS2/16 induced weak K562 binding to the mutated polypeptide, and in the presence of the RGD sequence (24). This sequence is also present in an integrin adhesiveness can be stimulated by phorbol esters and other more physiologically relevant agonists (8, 26). In addition, antibodies have been described to integrin αIIbβ3 (19) and α5β1 (20-23). For α5β1 binding to Fn, the synergistic regions 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 αIIbβ3 (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 β1 (27-30), β2 (31), and β3 (32) subunits, which induce a high affinity state of the integrins. Studies with stimulatory β1 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 α5β1 and αvβ3-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 artificial 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 TF7 phage promoter 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. 2A). The amino-terminal sequence of 3Fn6 starts immediately after an initiation codon for methionine. To create...
TABLE 1  
Fibronectin-binding integrins on K562, MV3, and Mel57 cells  

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substitution mutants, two complementary oligonucleotides with appropriate sequences were synthesized, annealed, and then cloned between the BamHI and EcoRI sites of 3Fn9. This yielded a mutated polypeptide 3Fn6-10(SPSDN) where the PHSRN sequence from 3Fn9 was substituted by SPSDN (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, pLyS). 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 were pooled and dialyzed for purity by SDS-polyacrylamide gel electrophoresis, pooled, dialyzed against phosphate-buffered saline without Ca2+ or Mg2+ 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 erythroblastic 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-a3 (41), purchased from Telios Pharmaceuticals Inc. (San Diego); HP2/1 anti-a4 (42), provided by Dr. Francisco Sanchez-Madrid; NKI-Saml anti-a5 (30), provided by Dr. Carl Figdor; 4B4 anti-aβ1 (43), purchased from Life Technologies, Inc.; Eberhard Klein; C17 anti-aβ3 (45), provided by Dr. Arnoud Sonnenberg; 10E5 anti-allb (47), provided by Dr. Barry Coller; and LM142 anti-aν. The stimulatory anti-integrin aβ3 mAbs were purchased from Telios Pharmaceuticals Inc. (San Diego); HP2/1 anti-a4 (45), purchased from Life Technologies, Inc.). After 48 h, stably transfected cells were selected by selection method (53), using the calcium phosphate transfection system (Life Technologies, Inc.).

RESULTS

K562, MV3, and Mel57 Differentially Adhere to the CCBD—We investigated adhesion of K562 human erythroblastic 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 α5β1 (Table I). MV3 and Mel57 expressed αβ1, α4β1, and α5β1. In addition, Mel57 but not MV3 expressed αβ3. MV3 and Mel57 expressed other αβ integrins including αβ5 (54) and possibly αβ1 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 α5 or β1. Even though MV3 expressed several Fn-binding integrins, adhesion was fully blocked by mAbs to α5, whereas mAbs to α3 or α4 or polyclonal anti-αβ had no effect. Adhesion of Mel57 was inhibited approximately 35% by mAbs to α5 or β1 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(PSDNS) (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 αβ3 on MV3 induces adhesion to 3Fn6–10(PSDNS).** A, MV3 cells were untransfected (dotted line), transfected with pBJ1neo alone (thin line), or transfected with pBJ1neo including integrin β3 cDNA followed by sorting with LM609 anti-αβ3 mAbs (thick line). Shown is the relative fluorescence after incubation with LM609 and a fluorescein-isothiocyanate-labeled second antibody. B, MV3neo or MV3-β3 cells were allowed to adhere to wells coated with increasing concentrations of 3Fn6–10(PSDNS) (dotted line) or 3Fn6–10 (line) as indicated. Filled bars represent remaining adhesion to wells coated with 32 μg/ml 3Fn6–10(PSDNS) 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 β3 or αvβ3 or by polyclonal αv. The combination of mAbs to α5 and αvβ3 completely blocked adhesion of Mel57.

Thus, K562 adheres weakly to the CCBD through α5β1, MV3 binds strongly through α5β1, and Mel57 binds strongly through α5β1 and αvβ3.

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 3Fn6–5Fn10 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 αvβ3, we transfected MV3 cells with β3 cDNA, resulting in αvβ3 surface expression (Fig. 4A), and used these cells in adhesion assays. Expression of αvβ3 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-β3 mAbs (Fig. 4B). A similar level of inhibition was found with LM609 anti-αvβ3 (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 α5β1 versus αvβ3.

Stimulation of α5β1-mediated RGD Binding with β1 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 α5β1, suggested that binding of α5β1 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 β1 mAbs, with Mn2+, 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. 5A). A control β1-integrin mAb A2B2 had no effect (not shown). The strong binding of Mel57 was not enhanced by 8A2 or TS2/16. Mn2+ 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 α5β1 or to the recruitment of other integrins. Induced adhesion of K562 to GRGDSP in the presence of 8A2 (Fig. 5B) or TS2/16 (not shown) was blocked by mAbs to α5 and not by any of the other mAbs. In addition, even though 8A2 and TS2/16 may activate α5β1, αvβ3, and possibly αvβ1 on MV3 cells, induced adhesion of MV3 to GRGDSP was completely blocked by mAbs to α5, whereas mAbs to α3, α4, and αv had no effect (Fig. 5B). The fact that the 4B4 anti-β1 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 β1 subunit (55).

Thus, the strength of α5β1 binding to RGD can be increased by Mn2+ and by activating β1 antibodies.

Requirement for the PHSRN Synergy Site Depends on the Activation State of α5β1—As stimulatory β1 mAbs and Mn2+ induced α5β1-mediated adhesion to GRGDSP, we hypothesized that the activation state of α5β1 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 Mn2+ and allowed them to adhere to the mutated and control Fn polypeptides. TS2/16 and, to a lesser extent, Mn2+ 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 Mn2+, 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 Mn2+ 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 α5β1 on K562 cells was not maximally activated by these agents. To exclude possibility 1, we used mAbs to α3, α4, α5, αv, β1, β3, αvβ3, or the combination of these mAbs in the absence of anti-α5 for inhibition of TS2/16-stimulated adhesion of MV3 cells to the mutated polypep-
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(PSDNS) (dotted line) or 3Fn6-10 (line) as indicated. Adhesion to BSA was less than 4%. One experiment of three is shown.

tide. Stimulated adhesion was blocked by the anti-a5 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 a5β1 and not to recruitment of other integrins.

To investigate possibility 2, we incubated K562 cells with PMA, TS2/16, or Mn2+ 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 Mn2+, 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 Mn2+, the control and the mutated polypeptide were equally effective in promoting adhesion of K562 cells. This adhesion was blocked by a5 mAbs (not shown).

From these results, we conclude that requirement of the PHSRN synergy site for a5β1-mediated adhesion to RGD in the COBD depends on the activation state of a5β1.

**DISCUSSION**

In line with earlier reports, we find that αvβ3 does not require the PHSRN site. We base this conclusion on 2 observations. First, Mel57 cells express αvβ3 and adhere equally well to all molecules tested containing RGD, i.e. GRGDSP, the mutated polypeptide lacking the synergy site 3Fn6-10(PSDNS), the control polypeptide 3Fn6-10, and Fn120 kDa. Second, the αvβ3 negative MV3 cells do not adhere to RGD-containing ligands that lack the PHSRN site, and transfection with β3 cDNA resulting in αvβ3 surface expression leads to binding of these cells to GRGDSP and 3Fn6-10(PSDNS).

These findings confirm and extend the observations that αvβ3 can be retained on an RGD column (56) whereas α5β1 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 αIIbβ3 but not αvβ3 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 αvβ3,
Modulated Requirement for Fn Synergy Adhesion Site

One experiment of three is shown.

cated and allowed to adhere to wells coated with 32 μg/ml 3Fn6-

Three findings. First, stimulation of K562 cells that express

Inhibition assays. The fact that these mAbs inhibit adhesion of

To exclude this possibility, we used C17 anti-β3 for adhesion in­

ates only a5β1-mediated phagocytosis, in our system avβ3 might influence a5β1-mediated adhesion. Ligation of avβ3 with LM609 mAbs might induce a signal that inhibits a5β1. To
eclude this possibility, we used C17 anti-β3 for adhesion inhi­

bation. The major conclusion from this study is that the requirement

Parenthetically, it has been reported that cross-talk between

whereas a5β1 and αIIIβ3 require the synergy region for ef­

cient binding to Fn (24, 25).

Parenetically, it has been reported that cross-talk between

avβ3 and a5β1 can occur (58, 59). Therefore, the induced ad­

hesion to 3Fn6–10(SPSDN) upon expression of avβ3 in MV3
cells did not necessarily have to be due to avβ3-mediated ad­

hesion. Even though Blystone et al. (59) show that avβ3 regu­
lates only a5β1-mediated phagocytosis, in our system avβ3 might influence a5β1-mediated adhesion. Ligation of avβ3

with LM609 mAbs might induce a signal that inhibits a5β1. To

exclud this possibility, we used C17 anti-β3 for adhesion in­
hbition assays. The fact that these mAbs inhibit adhesion of

β3-transfected MV3 cells to 3Fn6–10(SPSDN) suggests that
direct binding through avβ3 rather than signaling to a5β1 is

involved.

The major conclusion from this study is that the requirement

for the PHSRN synergy site for a5β1-mediated adhesion to the

CCBD depends on the activation state of a5β1. This is based on

three findings. First, stimulation of K562 cells that express

only a5β1, with Mn²⁺ or stimulatory β1-integrin mAbs, in­
duces adhesion to GRGDSP and 3Fn6–10(SPSDN). Second, in

the presence of the combination of PMA, TS2/16, and Mn²⁺, the

mutated and control polypeptide are equally effective in pro­
moting K562 cell adhesion. Third, treatment of MV3 cells with

these agents induces adhesion to GRGDSP and enhances ad­
hesion to 3Fn6–10(SPSDN) to the level of adhesion to 3Fn6–

10, and this effect is completely blocked by antibodies to a5 but

not by mAbs to α3, α4, or αv or the combination.

Even though the avβ3-negative K562 and MV3 cells express

similar levels of a5β1, they differ dramatically in binding to

Fn120 kDa through this receptor. The view of cell type-specific

regulation of a5β1 affinity proposed by O'Toole et al. (60) sug­
gests 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, Mn²⁺ and stimulatory β1 mAbs
do not affect the strong adhesion of MV3 to Fn120 kDa. How­

ever, our findings demonstrate that these agents do in fact

alter the avidity of a5β1 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 a5β1 to a level that RGD is recog­
nized in the Fn molecule and that additional extracellular

events are required for the final activation of a5β1, 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 a5β1 binding

pocket, and in the presence of TS2/16 or Mn²⁺ 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 extracel­

lular stimulation (the synergy site plus stimulatory β1 mAbs)
can abrogate 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 NH₂-terminal portion

of CS1 that strengthen a4β1 binding to EILDV, although none

have yet been identified. Thus, the presence of sites that syn­

ergistically enhance binding of integrins to their recognition

sequence might be a general mechanism, and activation by

stimulatory β1 mAbs and Mn²⁺ 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 affini­

ity 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), indicat­
ing 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

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 than 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 Mn²⁺ 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 than 4%. One experiment of three is shown.
may be masked by the binding to RGD through α5β1. Alternatively, as previously reported (63), stimulatory β1 mAbs may selectively activate α5β1 while leaving α4β1 unaffected.

A possible interpretation of our findings may be that the PHSRN synergy site binds to the same epitope as recognized by the stimulatory β1 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 TS216 when PMA is absent indicates that the synergy site and stimulatory mAbs have different binding effects. Therefore, these data seem to support a model where the synergy site and stimulatory mAbs can have additional combined stimulatory effects. In conclusion, our data demonstrate that α5β1 but not α4β1 requires the PHSRN synergy site for cell adhesion to RGD in the CCBD of Fn but that induction of a high affinity state of α5β1 with PMA, stimulatory mAbs, and/or Mn2+ abrogates this dependence on the PHSRN sequence.

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REFERENCES