The α3(IV)185–206 Peptide from Noncollagenous Domain 1 of Type IV Collagen Interacts with a Novel Binding Site on the β3 Subunit of Integrin αvβ3 and Stimulates Focal Adhesion Kinase and Phosphatidylinositol 3-Kinase Phosphorylation∗

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We have recently identified integrin αvβ3 and the associated CD47/integrin-associated protein (IAP) together with three other proteins as the potential tumor cell receptors for the α3 chain of basement membrane type IV collagen (Shahan, T.A., Ziaie, Z., Pasco, S., Fawzi, A., Bellon, G., Monboisse, J. C., and Kefalides, N. A. (1999) Cancer Res. 59, 4584–4590). Using different cell lines expressing αvβ3, α1β3, and/or CD47 and a liquid phase receptor capture assay, we now provide direct evidence that the synthetic and biologically active α3(IV)185–206 peptide, derived from the α3(IV) chain, interacts with the β3 subunit of integrin αvβ3 independently of CD47. Increased α3(IV) peptide binding was observed on transforming growth factor-β1-stimulated HT-144 cells shown to up-regulate αvβ3 independently of CD47. Also, incubation of HT-144 melanoma cells in suspension induced de novo expression of ligand-induced binding sites epitopes on the β3 subunit similar to those observed following Arg-Gly-Asp-Ser (RGDS) stimulation. However, RGDS did not prevent HT-144 cell attachment and spreading on the α3(IV) peptide, suggesting that the α3(IV) binding domain on the β3 subunit is distinct from the RGD recognition site. α3(IV) peptide binding to HT-144 cells in suspension stimulated time-dependent tyrosine phosphorylation, while the RGDS peptide did not. Two major phosphotyrosine proteins of 120–130 and 85 kDa were immunologically identified as focal adhesion kinase and phosphatidylinositol 3-kinase (PI3-kinase). A direct involvement of PI3-kinase in α3(IV)-dependent β3 integrin signaling could be documented, since pretreatment of HT-144 cells with wortmannin, a PI3-kinase inhibitor, reverted the known inhibitory effect of α3(IV) on HT-144 cell proliferation as well as membrane type 1-matrix metalloproteinase gene expression. These results provide evidence that the α3(IV)185–206 peptide, by directly interacting with the β3 subunit of αvβ3, activates a signaling cascade involving focal adhesion kinase and PI3-kinase.

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Tumor cell invasion and metastasis are complex multistep processes that involve cell attachment to basement membrane or extracellular matrix proteins, degradation of adhesive proteins, and migration through the proteolytically modified tumor cell microenvironment (1, 2). Anchorage of tumor cells to basement membrane proteins is mediated in part by integrins, a large family of heterodimeric cell surface receptors, that function not only as cell adhesion receptors but also as signaling receptors regulating cell growth, cell death, migration, and tissue remodeling (3, 4). Since integrins are devoid of an intrinsic kinase activity, the outside-in signaling process initiated through ligand binding is thought to result in conformational changes of the receptor that are propagated from the ectodomain across the plasma membrane to the integrin cytoplasmic tails, allowing their interaction with intracellular signaling proteins. Integrin signaling leads to the activation of various kinases, such as focal adhesion kinase (FAK),1 phosphatidylinositol 3-kinase (PI3-kinase), mitogen-activated protein kinase (3), as well as integrin-linked kinase (5). Alternatively, integrin signaling can be mediated through transmembrane receptors that are associated with integrins in the cell membrane (6). Integrin-associated protein (IAP) or CD47 is a widely expressed protein that was initially identified through copurification with integrin αvβ3 from human placenta (7). CD47, which is composed of an N-terminal (extracellular) IgG variable domain, followed by five membrane-spanning hydrophobic helices and a cytoplasmic tail, is found in association with β3 and other integrins and has been implicated in multiple β3 integrin-mediated functions, such as enhanced αvβ3-dependent cell spreading or chemotaxis and, in platelets, α1β1-dependent cell spreading and aggregation (8). Both αvβ3 and IAP have been shown to stimulate FAK phosphorylation; however, in contrast to αvβ3, CD47-dependent downstream signaling does not involve PI3-kinase but appears to require a p38 mitogen-activated protein kinase, which can trigger activation of focal adhesion proteins such as Syncytoma and FAK (8, 9).

1 The abbreviations used are: FAK, focal adhesion kinase; CHO, Chinese hamster ovary; LIBS, ligand-induced binding site; mAb, monoclonal antibody; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-matrix metalloproteinase; NC1, noncollagenous domain 1; PI3-kinase, phosphatidylinositol 3-kinase; TGF-β1, transforming growth factor TGF-β1; IAP, integrin-associated protein; AL, anterior lens capsule; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; IMDM, Isceo’s modified Dulbecco’s medium; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
The human metastatic melanoma cell line HT-144 was a gift from Dr. P. Bruguet (Boisnava, Plaisir, France). The Chinese hamster ovary (CHO) cell line CRL9096 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The CHO cell clones expressing human β3 (CHO A13), human αβ3 (CHO A06), or human α2β1 (CHO A10) have been established in our laboratory (22). All cell lines were grown in Iscove’s modified Dulbecco’s medium (IMDM) (BioWhittaker, Verviers, Belgium), supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, and penicillin-streptomycin (100 units/ml).

Immunofluorescence and Flow Cytometry

For flow cytometry analysis, cells were detached from culture plates with EDTA buffer and washed twice with IMDM. The cells (5 × 10^6) were then incubated for 30 min with the primary antibody or with the biotinylated α3(IV)-185–206 peptide, washed with IMDM, and further incubated for 30 min with a FITC-conjugated goat anti-mouse secondary antibody or with FITC-conjugated streptavidin. Cells were then washed and resuspended in phosphate-buffered saline (PBS) (136 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) and subsequently analyzed on an Epics Elite ESP flow cytometer (Coulter Corp., Hialeah, FL). For immunofluorescence microscopy, the labeled cells were fixed by methanol on a glass cover slip, mounted in PBS-glycerol, and examined with a Leica DMRB microscope using a × 63 oil immersion objective.

Immunoprecipitation and Western Blot Analysis

Preparation of Cell Lysates—Cells were detached with EDTA buffer, washed twice in cold PBS, and lysed for 30 min in ice-cold lysis buffer A (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM phenylmethylsulfonyl fluoride). Lysates were cleared by centrifugation at 12,000 × g for 10 min at 4 °C. The protein concentration was determined using Lowry's modified method (23). For the identification of phosphorysine proteins in total cell extracts, HT-144 melanoma cells were resuspended in IMDM and serum-starved for 90 min at 37 °C. The cells (10^6/ml) were then incubated with the α3(IV)-185–206 peptide (20 μg/ml) or the corresponding scrambled peptide for different time points, centrifuged for 2 min at 12,000 × g, and lysed with 2% SDS sample buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and aprotinin). Lysates were immediately heated at 90°C for 5 min prior to protein concentration determination.

Immunoprecipitation—Cell lysate (1 mg of protein) was incubated overnight at 4°C with the anti-α3β1 mAb 29C6 or the anti-CD47 mAb B6H12. Immune complexes were precipitated with protein A-Sepharose beads for 1 h at 4°C. The beads were then washed three times with lysis buffer A, and the precipitates were recovered by boiling the beads in 30 μl of SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4.6% SDS, 20% glycerol, 0.5 mg/ml bromphenol blue). For phosphorysine protein immunoprecipitation, HT-144 melanoma cells were incubated with the α3(IV)-185–206 peptide as described above and lysed with 1% Triton X-100 and 0.1% sodium deoxycholate in lysis buffer B. Cell extracts (1 mg of protein) were incubated overnight at 4°C with either the polyclonal anti-FAK or the anti-PI3-kinase antibody, and the immune complexes were processed as described above.

Western Blot Analysis—Immunoprecipitates or total cell lysates (50 μg of protein) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose Hybrid C membrane (Amersham Pharmacia Biotech) using a semidry transblot apparatus (Amersham Pharmacia Biotech). The membranes were blocked overnight in Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.4, 137 mM NaCl) containing 1% Tween and 5% nonfat dry milk and subsequently incubated for 2 h at room temperature with the anti-β3 mAb 4D10G3 or the anti-CD47 mAb B6H12. Following several washes in TBS-Tween, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG in TBS-Tween containing 5% nonfat dry milk, washed in TBS-Tween, and finally processed using a chemiluminescence kit (SuperSignal; Pierce). For membrane stripping, the membranes were incubated at 50°C in stripping buffer (62.5 mM Tris, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol) and extensively washed. For identification of phosphorysine
**RESULTS**

The α3(IV)185–206 Peptide Binds Specifically to Integrin α3β1 in the Absence of CD47—In an attempt to more precisely identify the α3(IV)185–206 peptide binding site on the α3β1-CD47 complex, different cell types were selected for their expression of either human α3β1 alone, CD47 alone, or the α3β1-CD47 complex, and used as target cells for α3(IV)185–206 peptide binding studies. As shown by flow cytometry analysis (Fig. 1A), erythrocytes expressed CD47 in the absence of α3β1 and HT-144 melanoma cells were positive for both human α3β1 and CD47, whereas CHO transfectants expressing the recom-
binant human αv and β3 integrin subunits were positive for human αvβ3 only. Mock-transfected CHO cells were negative for both human αvβ3 and CD47. The flow cytometry data were further confirmed by immunoprecipitation experiments using anti-αvβ3 or anti-CD47 mAb (Fig. 1B). We next tested the ability of the biotinylated α3(IV)185–206 peptide to bind to these cells using indirect fluorescence microscopy (Fig. 2A) as well as flow cytometry analysis (Fig. 2B). Interestingly, HT-144 melanoma cells and CHO αvβ3 cells, but not erythrocytes or mock-transfected CHO cells, bound the biotinylated α3(IV)185–206 peptide, demonstrating that cells expressing human CD47 alone did not interact with the α3(IV)185–206 peptide, whereas cells expressing human αvβ3 bound the peptide independently of the presence or absence of CD47. In contrast, the corresponding scrambled biotinylated peptide did not bind to any of these cells, underlining the binding specificity of the α3(IV)185–206 peptide.

Recent data from our laboratory have provided evidence that stimulation of HT-144 melanoma cells with the growth factor TGF-β1 up-regulates αvβ3 expression (26). In this study, we have investigated CD47 expression in HT-144 melanoma cells following TGF-β1 stimulation. Interestingly, as shown in Fig. 3, TGF-β1 dissociated the αvβ3-CD47 complex by selectively up-regulating αvβ3 expression. When TGF-β1-treated cells were used for α3(IV)185–206 peptide binding, both αvβ3 expression and biotinylated α3(IV)185–206 peptide binding were increased, whereas CD47 expression did not significantly change, providing further evidence that the α3(IV)185–206 peptide binds to αvβ3 independently of CD47.

Finally, to demonstrate a direct interaction of αvβ3 with the α3(IV)185–206 peptide, a liquid phase receptor capture assay was performed using cell extracts from HT-144 melanoma cells or erythrocytes, as well as mock-transfected or αvβ3-transfected CHO cells. The biotinylated α3(IV)185–206 peptide was added to the different cell lysates and recovered using streptavidin-agarose beads. Captured proteins were then analyzed by SDS-PAGE and Western blotting using an anti-β3 or anti-CD47 mAb. As shown in Fig. 4, no CD47 antigen was recovered when erythrocyte cell extract was incubated with the α3(IV)185–206 peptide. In contrast, αvβ3 was captured from CHO-αvβ3 cells that are negative for CD47, while αvβ3 and CD47 were recovered from HT-144 melanoma cells that coexpress αvβ3 and CD47. The scrambled peptide was unable to retain either αvβ3 or CD47 (data not shown). Taken together, these results provide evidence that the α3(IV)185–206 peptide specifically binds to integrin αvβ3.

The β3 Subunit of Integrin αvβ3 Is Involved in α3(IV)185–206 Peptide Binding—To further localize the α3(IV)185–206 peptide binding site on αvβ3, we used HT-144 melanoma cells and a panel of transfected CHO cell clones previously shown to express either human αvβ3, human αvβ5, or the chimeric receptor αv(hamster)β3(human) for receptor capture experiments. As shown in Fig. 5, for each cell type, the α3(IV)185–206 peptide interacted with the human β3 integrin independently of the associated α subunit. In contrast, the corresponding scrambled peptide did not retain the β3 integrin subunit (data not shown). In summary, these results demonstrate that the α3(IV)185–206 peptide specifically binds to a domain of the β3 integrin subunit.

The α3(IV)185–206 Peptide Contact Site on the β3 Subunit Is Distinct from the RGD Binding Site—Integrin αvβ3 acts as a receptor for a surprisingly large number of proteins, most of which contain the tripeptide recognition sequence RGD. Since the α3(IV)185–206 peptide is devoid of this RGD motif, we
hypothesized that it would not interfere with RGD-dependent cell spreading on immobilized fibrinogen, a specific ligand of \( \alpha_3 \beta_3 \). To test this hypothesis, HT-144 melanoma cells were preincubated with the RGDS peptide (1 \( \mu \)M) or the \( \alpha_3(IV)185-206 \) peptide (25 \( \mu \)M) and seeded on 96-well plates coated with either fibrinogen, the NC1 domain of ALC type IV collagen, or the \( \alpha_3(IV)185-206 \) peptide. As shown in Fig. 6, in the absence of the competing peptide, HT-144 melanoma cells adhered and spread on immobilized fibrinogen as well as the NC1 domain or the \( \alpha_3(IV)185-206 \) peptide. Preincubation of the cells with the RGDS peptide completely inhibited their adhesion to fibrinogen, whereas the same RGDS-treated cells attached and spread normally on the NC1 domain or the \( \alpha_3(IV)185-206 \) peptide. In contrast, preincubation of the cells with the \( \alpha_3(IV)185-206 \) peptide had no effect on cell attachment and spreading on immobilized fibrinogen but inhibited HT-144 adhesion to the NC1 domain or the \( \alpha_3(IV)185-206 \) peptide. The same results were also obtained when CHO \( \alpha_3 \beta_3 \) cells were tested (data not shown). Taken together, these results provide evidence that the \( \beta_3 \) subunit domain involved in \( \alpha_3(IV)185-206 \) peptide binding is distinct from the RGD recognition site.

**De Novo Expression of Ligand-induced Binding Site (LIBS) Epitopes on \( \alpha_3 \beta_3 \) Integrin following \( \alpha_3(IV)185-206 \) Peptide Binding—**In order to determine whether \( \alpha_3(IV)185-206 \) peptide binding to \( \alpha_3 \beta_3 \) was able to initiate outside-in signaling through ligand-induced conformational changes of the ectodomain, we investigated the exposure of ligand-induced neoepitopes on the \( \beta_3 \) subunit, known as LIBS (27, 28). For this purpose, we analyzed the binding of two different anti-LIBS mAbs to HT-144 melanoma cells in the presence or absence of either the \( \alpha_3(IV)185-206 \) peptide or the RGDS peptide, used in this experiment as a positive control. As shown in Fig. 7, pretreatment of HT-144 cells with either 1 \( \mu \)M RGDS or the \( \alpha_3(IV)185-206 \) peptide (20 \( \mu \)g/mL) significantly enhanced the binding of mAbs to HT-144 melanoma cells expressing either \( \alpha_3 \beta_3 \) or \( \alpha_3 \beta_3 \) were incubated with the biotinylated \( \alpha_3(IV)185-206 \) peptide or the RGDS peptide, used in this experiment as a positive control. As shown in Fig. 7, pretreatment of HT-144 cells with either 1 \( \mu \)M RGDS or the \( \alpha_3(IV)185-206 \) peptide (20 \( \mu \)g/mL) significantly enhanced the binding of mAbs to HT-144 melanoma cells expressing either \( \alpha_3 \beta_3 \) or \( \alpha_3(IV)185-206 \) peptide. Peptide-interacting proteins were captured with streptavidin-agarose beads and analyzed using the following antibodies: VNR139 (anti-\( \alpha_3 \)) plus 4D10G3 (anti-\( \beta_3 \)) on blots a and S1.3 (anti-\( \alpha_3 \)) plus 4D10G3 (anti-\( \beta_3 \)) on blots b.

**De Novo Expression of Ligand-induced Binding Site (LIBS) Epitopes on \( \alpha_3 \beta_3 \) Integrin following \( \alpha_3(IV)185-206 \) Peptide Binding—**In order to determine whether \( \alpha_3(IV)185-206 \) peptide binding to \( \alpha_3 \beta_3 \) was able to initiate outside-in signaling through ligand-induced conformational changes of the ectodomain, we investigated the exposure of ligand-induced neoepitopes on the \( \beta_3 \) subunit, known as LIBS (27, 28). For this purpose, we analyzed the binding of two different anti-LIBS mAbs to HT-144 melanoma cells in the presence or absence of either the \( \alpha_3(IV)185-206 \) peptide or the RGDS peptide, used in this experiment as a positive control. As shown in Fig. 7, pretreatment of HT-144 cells with either 1 \( \mu \)M RGDS or the \( \alpha_3(IV)185-206 \) peptide (20 \( \mu \)g/mL) significantly enhanced the binding of mAbs to HT-144 melanoma cells expressing either \( \alpha_3 \beta_3 \) or \( \alpha_3(IV)185-206 \) peptide. Peptide-interacting proteins were captured with streptavidin-agarose beads and analyzed using the following antibodies: VNR139 (anti-\( \alpha_3 \)) plus 4D10G3 (anti-\( \beta_3 \)) on blots a and S1.3 (anti-\( \alpha_3 \)) plus 4D10G3 (anti-\( \beta_3 \)) on blots b.

**De Novo Expression of Ligand-induced Binding Site (LIBS) Epitopes on \( \alpha_3 \beta_3 \) Integrin following \( \alpha_3(IV)185-206 \) Peptide Binding—**In order to determine whether \( \alpha_3(IV)185-206 \) peptide binding to \( \alpha_3 \beta_3 \) was able to initiate outside-in signaling through ligand-induced conformational changes of the ectodomain, we investigated the exposure of ligand-induced neoepitopes on the \( \beta_3 \) subunit, known as LIBS (27, 28). For this purpose, we analyzed the binding of two different anti-LIBS mAbs to HT-144 melanoma cells in the presence or absence of either the \( \alpha_3(IV)185-206 \) peptide or the RGDS peptide, used in this experiment as a positive control. As shown in Fig. 7, pretreatment of HT-144 cells with either 1 \( \mu \)M RGDS or the \( \alpha_3(IV)185-206 \) peptide (20 \( \mu \)g/mL) significantly enhanced the binding of mAbs to HT-144 melanoma cells expressing either \( \alpha_3 \beta_3 \) or \( \alpha_3(IV)185-206 \) peptide. Peptide-interacting proteins were captured with streptavidin-agarose beads and analyzed using the following antibodies: VNR139 (anti-\( \alpha_3 \)) plus 4D10G3 (anti-\( \beta_3 \)) on blots a and S1.3 (anti-\( \alpha_3 \)) plus 4D10G3 (anti-\( \beta_3 \)) on blots b.
in Fig. 8a, incubation of HT-144 cells in suspension with the α3(IV)185–206 peptide induced a time-dependent tyrosine phosphorylation of at least two major proteins of apparent molecular mass of 120 and 85 kDa. An identical result was also obtained when the cells were allowed to attach to the immobilized α3(IV) peptide (data not shown). The phosphorylation time course for the two proteins was similar, with a phosphorylation peak between 20 and 30 min. In contrast, the scrambled peptide was unable to induce a similar phosphorylation profile (Fig. 8b). An absence of tyrosine phosphorylation was also observed with the RGDS peptide (data not shown). The 120- and 85-kDa proteins were further immunologically identified by immunoprecipitation experiments using anti-FAK and anti-PI3-kinase antibodies. Immunoblots of the precipitates were first probed with PY-20 mAb and then stripped and reprobed with an anti-FAK or anti-PI3-kinase mAb. As shown in Fig. 9, stimulation of tyrosine phosphorylation of FAK and PI3-kinase was again time-dependent, with a peak at 15–30 min, providing evidence that FAK and PI3-kinase are involved in the signaling pathway elicited by the binding of the α3(IV)185–206 peptide to the β3 integrin subunit.

Wortmannin Reverts the Inhibitory Effect of the α3(IV)185–206 Peptide on Cell Proliferation and MT1-MMP Gene Expression—We have previously demonstrated an inhibitory effect of both the NC1 domain and the biologically active α3(IV) peptide...
on tumor cell proliferation (18), and tumor cell migration, due to an increase in MT1-MMP expression, the physiological receptor and activator of MMP-2 (17). To test whether PI3-kinase signaling could be involved in these processes, we analyzed the effect of wortmannin, a PI3-kinase inhibitor, on cell proliferation and expression of MT1-MMP. In these experiments, HT-144 or CHO α3β3 cells were seeded onto 96-well plates coated with poly-L-lysine, and preincubated for 1 h with or without wortmannin (0.1 μM) followed by the addition of the α3(IV)185–206 peptide. Proliferation was measured after a 48-h incubation period using the MTT assay. As shown in Fig. 10A, pretreatment of the cells with wortmannin reverted the inhibitory effect induced by the α3(IV)185–206 peptide on HT-144 or CHO α3β3 cell proliferation. Similarly, pretreatment of the cells with wortmannin reverted the inhibitory effect on MT1-MMP gene expression (Fig. 10B). Taken together, these results demonstrate the involvement of PI3-kinase in the α3(IV)185–206 peptide signaling pathway, leading to inhibition of tumor cell proliferation and migration.

**DISCUSSION**

Basement membranes are thin specialized extracellular matrices that are functionally important for embryonic development, maintenance of tissue architecture, tissue remodeling during development and wound healing, and protection of tissues and organs from mechanical stress or exogenous factors (29). A major component of all basement membranes is type IV collagen (10), which promotes cell adhesion and migration (14–16). Tumor cell interactions with type IV collagen have been shown to rely on α3β1 or α5β1 integrins, leading to changes in their invasive properties as well as changes in their expression of various MMPs, such as MMP-1 or MMP-2 (30, 31). We have previously reported that a peptide sequence corresponding to residues 185–203 in the α3(IV) chain of type IV collagen was able to inhibit in vitro tumor cell proliferation and migration through reconstituted basement membranes. The peptide sequence contains a SN5 triplet in position 189–191 that is unique to the α3(IV) collagen chain, and replacement of each of the two serine residues by an alanine abolished its biological activity (17, 18). The cysteine residue at position 185 is also required for the biological activity of the α3(IV) peptide. Engelbreth-Holm-Swarm type IV collagen, which lacks the α3(IV) chain, failed to inhibit tumor cell proliferation and migration. Finally, a tentative affinity chromatography of melanoma cell extract on a α3(IV)179–208 peptide column identified the α3β3-CD47 complex as the tumor cell receptors (20).

**FIG. 9.** Effect of the α3(IV)185–206 synthetic peptide on FAK and PI3-kinase tyrosine phosphorylation in HT-144 melanoma cells. HT-144 melanoma cells in suspension were incubated with the α3(IV)185–206 peptide (20 μg/ml) for different incubation periods. Cell extracts were prepared as described under “Experimental Procedures” and immunoprecipitated with an anti-FAK or anti-PI3-kinase polyclonal antibody. Tyrosine phosphorylation of the precipitated proteins was first assayed by anti-phosphotyrosine immunoblotting. The blots were then stripped and reprobed with an anti-FAK and anti-PI3-kinase mAb.

**FIG. 10.** Effect of wortmannin on the inhibitory activity of the α3(IV)185–206 peptide on tumor cell proliferation and MT1-MMP gene expression. For cell proliferation assays (A), HT-144 melanoma cells (white bars) or CHO α3β3 cells (black bars) were seeded into 96-well plates coated with poly-L-lysine, preincubated for 1 h with (+ W) or without wortmannin (0.1 μM), and incubated for 48 h in the presence or absence of the α3(IV)185–206 peptide. Proliferation was measured as described under “Experimental Procedures.” Differences from control were as follows. NS, not significant; **, p < 0.001. For MT1-MMP gene expression analysis (B), HT 144 melanoma cells were grown on poly-L-lysine-coated dishes in the presence or absence of the α3(IV)185–206 peptide, with or without wortmannin treatment (0.1 μM). MT1-MMP and β-actin mRNAs were evaluated by semiquantitative RT-PCR. C, control; W, wortmannin; α3, α3(IV)185–206 peptide.

In the present study, we have characterized in detail the α3(IV)185–206 peptide binding site on the α3β3-CD47 complex and provide evidence that the peptide specifically interacts with the β subunit of integrin α3β3. Indeed, cells expressing α3β3 interacted with the α3(IV)185–206 peptide, independently of the presence or absence of CD47, whereas cells expressing CD47 in the absence of α3β3 did not. Furthermore, to demonstrate a direct interaction of α3β3 with the α3(IV)185–206 peptide, we performed receptor capture experiments using the biotinylated α3(IV)185–206 peptide and cell extracts of various cell types. When HT-144 melanoma cell extract was used, the α3β3-CD47 complex was recovered, in accordance with our previous data (20). The α3(IV)185–206 peptide also interacted with α3β3 in the absence of CD47, whereas it was unable to capture CD47 in the absence of α3β3. Since we have previously shown that the blocking anti-CD47 mAb B6H12 partially abolished the inhibitory effect of the α3(IV) peptide on tumor cell proliferation (20), a possible explanation for this result could be an antibody-dependent steric hindrance of the αβ3/peptide interaction. Indeed, data by Gresham et al. (32) have provided evidence that the anti-CD47 mAb B6H12 specifically inhibits the enhancement of neutrophil phagocytosis by inhibiting the RGD-dependent integrin/ligand interaction, although affinity-purified CD47 was unable to interact with the RGD sequence. Alternatively, the blocking anti-CD47 mAb could induce conformational changes of α3β3, preventing its interaction with the α3(IV) peptide. This hypothesis is supported by data showing that stimulation of CD47 by its agonist 4N1K, a peptide derived from the COOH-terminal cell binding domain of thrombospondin, spontaneously activates αmβ3, as demonstrated by...
enhanced binding of the conformationally sensitive mAb PAC-1 (9).

Integrin α₃β₃ constitutively interacts with a large variety of RGD-containing adhesive proteins present in the extracellular matrix (33), and this interaction is mediated through the metal ion-dependent adhesion site-like domain of the β₃ subunit (34). By using different cell lines expressing either α₃β₃ or α₁β₃β₃, we provide evidence that the α₃(IV) peptide binds to the β₃ subunit. However, the α₃(IV) peptide binding site is clearly distinct from the RGD recognition site, since inhibition experiments with an RGDS peptide did not inhibit melanoma cell adhesion to immobilized α₃(IV)185–206 peptide, whereas RGDS completely inhibited melanoma and CHO α₃β₃ cell adhesion to immobilized fibrinogen.

Ligand binding to β₃ integrins is known to induce conformational changes of the integrin receptor ectodomain, reflecting the most earliest events in integrin-dependent outside-in signaling. These conformational changes can be monitored with the earliest events in integrin-dependent outside-in signaling of the integrin receptor ectodomain, reflecting the conformational changes of the integrin receptor ectodomain, reflecting the most earliest events in integrin-dependent outside-in signaling. These conformational changes can be monitored with the RGDS peptide epitope on the β₃ integrin subunit. More surprisingly, however, the α₃(IV) peptide, in contrast to the RGDS peptide, was also able to trigger intracellular signaling processes in melanoma cells in suspension, such as tyrosine phosphorylation of FAK and PI3-kinase. There is convincing evidence that a dimeric ligand is necessary to initiate β₃ integrin-dependent intracellular tyrosine phosphorylation, since the monomeric cell recognition peptide RGDS fails to cause intracellular tyrosine phosphorylation (36, 37), despite the fact that RGDS binds to the receptor and induces conformational changes of its ectodomain (27, 38). Bhattacharyya et al. (39) have shown that monomeric vitronectin does not enhance intracellular tyrosine phosphorylation in both pulmonary arterial endothelial cells, whereas multimeric vitronectin elicits time- and concentration-dependent increases in tyrosine phosphorylation of proteins such as FAK, paxillin, Shc, cortactin, or ezrin. Since the α₃(IV) peptide contains a cysteine at position 185 that is essential for its biological activity, one possible explanation is that the α₃(IV) peptide, through its binding to α₃β₃, allows the establishment of disulfide bonds that induce clustering of the α₃β₃ integrin, necessary for outside-in signaling. Such receptor clustering can indeed be observed on the microphotographs of HT-144 and CHO α₃β₃ cells incubated with the biotinylated α₃(IV) peptide.

In a previous report, we have provided evidence that inhibition of tumor cell proliferation by the α₃(IV) peptide relies on elevated levels of cAMP and involves cAMP-dependent protein kinase A (40). Since PI3-kinase has previously been shown to activate protein kinase A in a cAMP-dependent manner (41), we wondered whether PI3-kinase could be involved in the signaling pathway, leading to inhibition of melanoma cell proliferation. The fact that pretreatment of HT-144 and CHO α₃β₃ cells with wortmannin, a PI3-kinase inhibitor, completely reversed the inhibitory effect of the α₃(IV) peptide on cell proliferation, confirms this hypothesis. Our previously published data have also shown that the α₃(IV) peptide inhibits tumor cell migration by decreasing MT1-MMP expression. MT1-MMP is known to activate latent MMP-2 (42), thereby facilitating matrix degradation and cellular invasion. Also, since data by Yu et al. (43) have shown that an increase in intracellular MMP inhibits MT1-MMP expression, we studied the possible involvement of PI3-kinase in this signaling pathway. Pretreatment of HT-144 melanoma cells with wortmannin suppressed the inhibitory effect induced by the α₃(IV) peptide on MT1-MMP gene expression, as demonstrated by semiquantitative RT-PCR analysis, providing evidence that PI3-kinase is involved in the α₃(IV) peptide signaling pathway leading to an inhibition of MT1-MMP gene expression. These results are in good agreement with those of Esparza et al. (44), who reported that inhibition of PI3-kinase by wortmannin strongly increased fibronectin-induced MMP production. Taken together, our results highlight a new α₃β₃-dependent signaling pathway initiated through collagen type IV binding to the integrin β₃ subunit that stimulates FAK and PI3-kinase activation. PI3-kinase, by activating adenylyl cyclase, could thus represent the missing link in the signaling pathway leading to cAMP-dependent inhibition of tumor cell proliferation.

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$\alpha_\beta_3$ Is the Receptor for the $\alpha_3$ Chain of Type IV Collagen