Modulation of Integrin Antagonist Signaling by Ligand Binding of the Heparin–Binding Domain of Vitronectin to the αVβ3 Integrin

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ABSTRACT

The interaction between the arginine glycine and aspartic acid motif (RGD) of integrin ligands such as vitronectin and the integrin receptor αVβ3 in mediating cell attachment has been well described. Similarly, the ability of disintegrins, small RGD containing peptides, to inhibit cell attachment and other cellular processes has also been studied extensively. Recently, we characterized a second site of interaction between vitronectin and its integrin partner. We determined that amino acids within the heparin-binding domain of vitronectin bind to a cysteine loop (C-loop) region of β3 and that this interaction is required for the positive effects of αVβ3 ligand occupancy on IGF-I signaling in smooth muscle cells. In this study we examine the signaling events activated following ligand binding of disintegrins to the αVβ3 and the ability of these signals to be regulated by binding of the heparin-binding domain of vitronectin. We demonstrate that disintegrin ligand binding activates a series of events including the sequential activation of the tyrosine kinases c-Src and Syk. This leads to the activation of calpain and the cleavage of the β3 cytoplasmic tail. Addition of vitronectin or a peptide homologous to the heparin-binding domain inhibited activation of this pathway. Our results suggest that the signaling events that occur following ligand binding to the αVβ3 integrin reflects a balance between the effects mediated through the RGD binding site interaction and the effects mediated by the heparin binding site interaction and that for intact vitronectin the effect of the heparin-binding domain predominates. J. Cell. Biochem. 105: 437–446, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: ECHISTATIN; INTEGRIN; VITRONECTIN

INTRODUCTION

Integrins are heterodimeric proteins consisting of one α- and one β-subunit. Sixteen α- and 8 β-subunits have been identified that can form more than 20 functional combinations (reviewed by [Aplin et al., 1998; Aplin et al., 1999]). Association of integrin receptors and their ligands is required for cells to stably attach and spread to the extracellular matrix. Furthermore, ligand binding of integrins to their ligands is required for most cell types to respond to the growth and survival factors [Aplin et al., 1998; Aplin et al., 1999]. Smooth muscle cells (SMC), proliferating endothelial cells, osteoclasts and certain types of malignant cells have been shown to express the highest levels of the αVβ3 integrin. We have been particularly interested in the role of ligand binding to the αVβ3 integrin in the regulation of the SMC response to the growth factor insulin like growth factor-I (IGF-I) [Jones et al., 1996; Maile et al., 2001; Zheng and Clemmons, 1998]. We have shown that the ability of IGF-I to stimulate migration and proliferation of SMC is dependent upon αVβ3 ligand occupancy [Jones et al., 1996; Maile et al., 2001; Zheng and Clemmons, 1998]. When SMC are deprived of a source of vitronectin (Vn) or if Vn binding to αVβ3 is blocked by the addition of an anti-αVβ3 antibody then they do not respond to IGF-I. Conversely, the addition of Vn enhances both proliferation and migration of these cells in response to IGF-I. Previous studies have shown that cellular attachment via the αVβ3 integrin requires an interaction between the arginine glycine and aspartic acid motif (RGD) in its ligands such as Vn and fibronectin and amino acids tyrosine 122 to methionine 124 of the β3 integrin subunit [Xiong et al., 2001]. Disruption of this interaction with RGD containing disintegrin antagonists causes cell detachment and ultimately

apoptosis [Blobel and White, 1992; Niewiarowski et al., 1994]. We have shown in SMC that are stably attached and spread that addition of disintegrin echistatin, at doses that are low that do not result in cell detachment, inhibits the response of SMC to IGF-I [Jones et al., 1996; Zheng and Clemmons, 1998]. More recently, we identified and characterized a second region of Vn that was required for the enhancing effects of αVβ3 ligand occupancy on the response of SMC to IGF-I [Maile et al., 2006a,b]. We determined that the heparin-binding domain (HBD) of Vn (amino acids 365–381), interacted with a region of the β3 extracellular domain referred to as the cysteine loop region (C-loop; between amino acids 177 and 184) and that this interaction was required for these enhancing effects. The crystal structure of αVβ3 shows that the C-loop region is spatially distinct from the critical residues required for RGD-binding domain (amino acids 122 and 124) [Xiong et al., 2001]. The aim of this study was to determine the signaling events that mediate the inhibitory effects of RGD antagonist binding to αVβ3, whether these effects could be modulated by binding of the HBD region of Vn to β3 and the net effect of simultaneous ligand occupancy of both sites.

MATERIALS AND METHODS

Polyvinyl difluoride membranes (Immobilon P) were purchased from Millipore Corporation (Bedford, MA). Autoradiographic film was obtained from Pierce (Pierce/ThermoFisher, Rockford, IL). Fetal bovine serum (FBS), Dulbecco's modified medium, penicillin and streptomycin were purchased from Life Technologies, (Grand Island, NY). The calpain 1 polyclonal antibody and the monoclonal phosphotyrosine antibody (PY99) were purchased from Santa Cruz (Santa Cruz, CA). The anti-FAK [pY^{397}] and the anti-Src kinase [pY^{418}] phosphospecific polyclonal antibodies were purchased from Biosource International Inc (Camarillo, CA). The anti-Syk and anti-FAK polyclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The antibody against the αV subunit of αVβ3 was purchased from Chemicon International (Temecula, CA). The polyclonal antibody against β3 was prepared by injecting rabbits with two peptides, one containing amino acids 60–85 and the other 653–680 of porcine β3 sequence that had been conjugated to KLH. The β3 antibody specific for the cytoplasmic tail of β3 prepared by injecting rabbits with a peptide containing amino acids 742–762 of β3 (c-β3). The calpain inhibitor E64d, the Syk inhibitor, piceatannol and the Src family kinase inhibitor PP2 were purchased from Calbiochem-Novabiochem, (San Diego, CA). All other reagents were purchased from Sigma Chemical Company (St Louis, MO) unless otherwise stated.

Porcine aortic SMC were isolated as previously described [Gockerman et al., 1995] and maintained in Dulbecco's modified medium supplemented with glucose (4.5 g/l), penicillin (100 units/ml), streptomycin (100 μg/ml) (DMEM-H) and 10% FBS in 10 cm tissue culture plates (Falcon Laboratory, Franklin Lakes, NJ). The cells were used between passage 5 and 16.

GENERATION OF RECOMBINANT WILD TYPE (Wt) AND MUTANT FORMS OF Vn
RT-PCR was used to generate the WtVn cDNA from mRNA prepared from porcine SMC [Yoneda et al., 1996]. Wild-type Vitronectin (WtVn), a form of Vn in which the HBD had been deleted (VnΔHBD), that is deletion of amino acids 342–358 (which contains the amino acid sequence QPKMTKSARRSGKRYRS) and a RGD^{66}E mutant Vn were generated, purified and characterized as we have described previously [Maile et al., 2006b].

A synthetic peptide corresponding to the HBD (amino acids 365–381) of human Vn [Suzuki et al., 1984] (LAKKQRFRHRNRKGYRS) was synthesized, purified and characterized as we have described previously.

CELL TREATMENTS
SMC were plated at a density of 5×10^5 in 10 cm dishes (Falcon # 3003) then grown to 90% confluency (approximately 5×10^6 cells). SMC were incubated in serum free medium supplemented with 0.02% bovine serum albumin (SFM) with or without echistatin (10^{-8}M), synthetic αVβ3 antagonists (10^{-6}M), Vn HBD peptide (10 μg/ml) or intact Vn (2 μg/ml). Where indicated, SMC were preincubated with inhibitors (PP2, 4 μM, piceatannol, 30 μM, E64d, 20 μM or calpastatin, 50 μM) or appropriately diluted vehicle (PP2 and piceatannol = 0.08% DMSO, E64d and calpastatin = water) for 1 h prior to the addition of echistatin.

CELL LYSIS
Following treatment SMC were lysed in ice-cold lysis buffer: 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1 mM EGTA plus 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin. The lysates were clarified by centrifugation at 14,000g for 10 min. To visualize proteins equal amounts of lysate were removed and the proteins were separated by SDS-PAGE and the proteins visualized by western immunoblotting with appropriate antibody as described below.

IMMUNOPRECIPITATION
Equal amounts of cell lysate were incubated overnight at 4°C with the appropriate antibody (PY99, β3, Src kinase, FAK using a 1:500 dilution). Immune complexes were then precipitated by adding protein A sepharose and incubating for a further 2 h at 4°C. The samples were then centrifuged at 14,000g for 10 min and the pellets washed four times with lysis buffer. The pellets were resuspended in 45 μl of reducing Laemmli buffer (0.2M final concentration DTT), boiled for 5 min and the proteins separated by SDS-PAGE, 8% gel.

WESTERN IMMUNOBLOTTING
Following SDS-PAGE the proteins were transferred to Immobilon P membranes. The membranes were blocked in 1% BSA in Tris-buffered saline with 0.1% Tween (TBST) for 2 h at room temperature then incubated with the appropriate primary antibody (β3, c-β3, Calpain 1, PY99, FAK, FAK[pY^{397}], Syk or Src kinase and Src kinase [pY^{418}], using a 1:500 dilution) overnight at 4°C and washed three times in TBST. Binding of the peroxidase labeled secondary antibody was visualized using enhanced chemiluminescence following the manufacturer's instructions (Pierce, Rockford, IL) and the immune complexes were detected by exposure to autoradiographic film.

Chemiluminescent images obtained were scanned using a DuoScan T1200 (AGFA Brussels, Belgium) and band intensities of the scanned images were analyzed using NIH Image, version 1.61. The Student's t-test was used to compare differences between treatments. The results that are shown are representative of at least three separate experiments.

RESULTS

ECHISTATIN TREATMENT ALTERS TYROSINE PHOSPHORYLATION OF THE ß3 SUBUNIT OF αVβ3

Since echistatin binds directly to αVβ3 in SMC we first determined whether we could identify a direct effect of echistatin on integrin activation [Jones et al., 1996]. β3 phosphorylation is a marker of the activation state of αVβ3 [Ling et al., 2003; Maile et al., 2001]. Consistent with our previous reports we detected basal β3 phosphorylation in quiescent SMC (Fig. 1A; top panel) [Ling et al., 2003]. Following echistatin treatment, there was a slight increase in ß3 phosphorylation after 1 h that was still apparent after 4 h (a 2.0 ± 0.1-fold increase compared with cells not treated with echistatin; mean $\pm$ SEM, n = 3, $P < 0.05$). However, after a 5-h exposure to echistatin β3 phosphorylation was undetectable (Fig. 1A; top panel).

ECHISTATIN TREATMENT TRIGGERS CLEAVAGE OF THE β3 SUBUNIT OF αVβ3

Immunoblotting of cell lysates from the same experiment revealed however, that there was a significant decrease in the amount of β3 that could be detected after 7-h treatment with echistatin compared with cells incubated in SFM alone (a $79 \pm 10\%$ decrease; mean $\pm$ SEM, n = 3, $P < 0.05$). In addition the β3 that remained appeared to have increased electrophoretic mobility on the SDS gel as the β3

immunoreactive band appeared to have a lower molecular weight compared with the untreated control. The increase in mobility suggested a decrease in the molecular weight of intact β3 protein following 5 and 7 h of treatment.

Since it has been reported that the cytoplasmic tail of β3 is susceptible to proteolytic cleavage [Du et al., 1995; Pfaff et al., 1999] we hypothesized that the changes in β3 detection following echistatin treatment may be due to the proteolytic cleavage. To assess this we examined ß3 protein levels using an antibody specific for the C-terminal region of ß3 [Du et al., 1995]. Treatment with echistatin for 5 and 7 h resulted in a significant, $82.75 \pm 5\%$ decrease (mean $\pm$ SEM, n = 3; $P < 0.05$), in the level of β3 protein detectable with the antibody specific for the cytoplasmic domain (c-β3), compared with control cultures (Fig. 1C).

To compare the effects of the RGD antagonist with both intact Vn and a peptide corresponding to the HBD region of Vn, SMC were also incubated for 7 h either intact Vn or the Vn HBD peptide. Both Vn and the Vn HBD peptides stimulated significant increases (a 4.8 ± 1.3-fold increase compared with untreated cells; mean $\pm$ SEM, n = 3, $P < 0.05$) in β3 phosphorylation consistent with our previous studies [Maile et al., 2006b]. There was no significant difference in total β3 protein levels from cells incubated for 7 h in the presence or absence of Vn or the Vn HDB peptide and the molecular weight estimate of β3 indicated that it remained intact (Fig. 1B).

EFFECT OF AN αVβ3–SPECIFIC SYNTHETIC ANALOGUE

Although our previous studies had suggested that echistatin binds primarily to αVβ3 on smooth muscle cells [Jones et al., 1996] it can bind to other αV integrins so we determined if cleavage could be stimulated by a synthetic analogue that binds specifically to αVß3 [Clemmons et al., 1999]. We have reported previously that
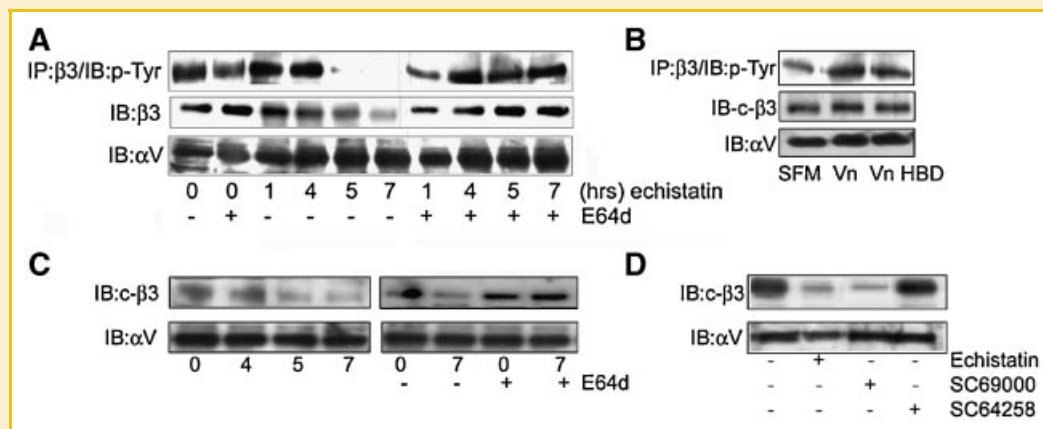

Fig. 1. Echistatin treatment triggers the cleavage of the ß3–subunit of αVß3 SMC were incubated for various times as indicated with echistatin (10^{-8}M), vitronectin (Vn; 2 μg/ml) Vn HBD (10 μg/ml), SC69000 or SC64258 (10^{-6}M). In some cases SMC were preincubated with E64d (20 μM) for 1 h prior to the addition of the other compounds. A: Following lysis and immunoprecipitation with an anti–β3 antibody, phosphorylation of β3 was determined by immunoblotting with an anti–phosphotyrosine antibody (p-Tyr). Equal amounts of total protein from cell lysates were analyzed and the amount of β3 was determined by immunoblotting with an anti–β3 antibody. To control for loading the amount of αV protein in each sample is also shown. B: Aliquots of cell lysates from the same experiment as described in panel A were immunoblotted with an antibody specific for the cytoplasmic tail of β3 (c–β3). To control for loading the amount of αV protein in each sample is also shown. C: Following lysis and immunoprecipitation with an anti–β3 antibody, phosphorylation of β3 was determined by immunoblotting with an anti–phosphotyrosine antibody (p-Tyr). Equal amounts of total protein from cell lysates were analyzed and the amount of β3 was determined by immunoblotting an antibody specific for the cytoplasmic tail of β3 (c–β3). To control for loading the amount of αV protein in each sample is also shown. D: SMC were treated as indicated for 7 h. Following cell lysis β3 was visualized using an antibody specific for the cytoplasmic tail of β3 (c–β3). To control for loading the amount of αV protein in each sample is also shown. The data in these figures are representative of the results from three similar experiments.

a synthetic αVβ3 antagonist referred to as (SC69000) which blocks I^{125} kistrin binding to αVβ3 on SMC to an extent that is comparable to echistatin [Clemmons et al., 1999], also inhibited IGF-I stimulated cell migration and stimulated IGF-IR dephosphorylation. Exposure to SC69000 (10^{-6}M) for 7 h reduced the levels of β3 protein detected with the cytoplasmic tail specific antibody by 84 ± 12% compared with untreated cells (mean ± SEM, n = 3, $P < 0.05$), a level that was comparable following treatment with echistatin (Fig. 1D). In contrast a control compound, (SC64258), which had been shown to have no significant effect on I^{125} kistrin binding to αVβ3 [Clemmons et al., 1999], had no significant effect on β3 cleavage (Fig. 1D).

CALPAIN ACTIVATION IS REQUIRED FOR ECHISTATIN INDUCED CLEAVAGE OF β3

Activation of the protease calpain has been shown to result in cleavage of the β3 cytoplasmic tail and inhibition of this protease with E64d blocks cleavage [Du et al., 1995]. We therefore examined the effect of E64d on the echistatin-induced decrease in β3 phosphorylation and cleavage of β3. Preincubation of cells with E64d prior to incubation with echistatin completely restored β3 phosphorylation levels to that seen in the absence of echistatin treatment (Fig. 1A; top panel). Similarly, E64d pretreatment inhibited the echistatin-induced cleavage of β3 (Fig. 1A; middle panel) since β3 protein levels in the presence of E64d were 108 ± 1.5% of control levels (mean ± SEM, n = 3). This observation was confirmed using the antibody to β3 that specifically recognizes the cytoplasmic tail (Fig. 1C) (β3 protein levels were 105 ± 5% of control levels (mean ± SEM, n = 3). In these experiments there was no significant effect of E64d on β3 protein levels or phosphorylation in the absence of echistatin treatment. Taken together with the results in Figure 1A this suggests that the decrease in β3 phosphorylation following echistatin treatment is most likely due to the loss of the β3 cytoplasmic tail following proteolysis of β3.

ECHISTATIN ACTIVATES CALPAIN

We next determined whether echistatin could directly activate calpain. A size shift (from 80 kDa to a 74/76 kDa doublet) in calpain has been shown to predict its activation as a result of autoproteolytic cleavage [Saito et al., 1994]. No calpain 2 was detected (data not shown), however, this may be due to a lack of antibody species cross reactivity since the antibody that was used was directed against human calpain and the smooth muscle cells used in this study were of porcine origin. In contrast, calpain 1 was clearly detected in the lysates (Fig. 2). Following a 4-h incubation with echistatin there was a decrease in the amount of intact (inactive) calpain and an increase in the amount of the smaller molecular weight fragment of calpain (active). Analysis of data from three separate experiments revealed a 80 ± 4% decrease (mean ± SEM, n = 3) in intact calpain 1 and a corresponding, 84 ± 6% increase (mean ± SEM) in fragment following 7-h treatment with echistatin (Fig. 2). This change was significantly greater compared to cells treated in SFM alone ($P < 0.05$). Since autoproteolytic activity is a marker of calpain activation this result strongly suggests that echistatin binding to the cell surface triggers and increase in calpain 1 activation. The time course of activation shows that significant calpain activation has
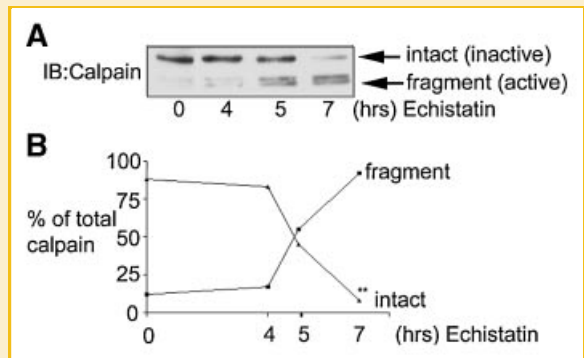

Fig. 2. Activation of calpain following incubation with echistatin. A: SMC in SFM were treated with echistatin (10^{-8}M) for various times as indicated. Following cell lysis calpain 1 levels were determined by immunoblotting with a specific antibody. The arrows indicate the positions of intact calpain and the autoproteolytic calpain fragment. The data in this figure are representative of the results from three similar experiments. B: The decrease in intact calpain 1 protein levels and the associated increase in fragment as determined by scanning densitometric analysis of western immunoblots from three independent experiments is shown. $^*P < 0.05$ when the level of intact calpain 1 after 7-h treatment is compared with 7-h treatment in SFM alone.

occurred after 5 and 7 h incubation with echistatin; which is also the time of maximal β3 change in β3 cleavage.

ECHISTATIN INCREASES THE PHOSPHORYLATION OF INTRACELLULAR PROTEINS

In order to investigate the pathway by which echistatin triggers activation of calpain and thereby cleavage of β3 we examined signaling events that occur between 30 min and 5 h following echistatin exposure. We first examined the total amount of phosphorylated proteins in the SMC before and after echistatin treatment. Treatment of SMC with echistatin stimulated an increase in two specific phosphoprotein bands in the range of 100–120 kDa and 70–80 kDa (Fig. 3 bottom panel). The increase in phosphorylation of the protein(s) with a molecular weight estimate of 120 kDa was first detectable after 1-h treatment with echistatin and the level of phosphorylation continued to increase with up to 4-h treatment (a 2 ± 0.2-fold increase compared with untreated cells; mean ± SEM, n = 3, $P < 0.05$) after which it remained unchanged. There was a small increase in the phosphorylation of the 70–80 kDa band after 1-h incubation with echistatin and the level of phosphorylation increased further after 4-h treatment then it appeared to remain unchanged thereafter (a 1.8 ± 0.3-fold increase compared with untreated cells; mean ± SEM, n = 3, $P < 0.05$). Control blots of cell lysates demonstrated that there was no change in the level of αV protein (Fig. 3 top panel) and that the level of β3 (as measured using the antibody specific for the cytoplasmic tail of β3) decreases over the same time interval (Fig. 3 middle panel). This suggests that the changes in levels of these two phosphorylated proteins are not due to loading differences or changes in the level of total cellular protein.

ECHISTATIN TREATMENT RESULTS IN THE ACTIVATION OF FAK

It has been reported that activation of integrin receptors leads to the phosphorylation and therefore autoactivation of focal adhesion
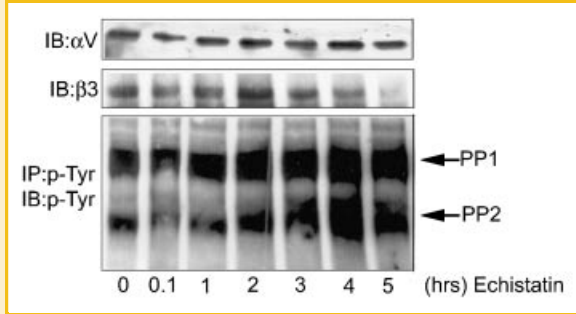
Fig. 3. Echistatin stimulation of protein tyrosine phosphorylation. A: SMC were incubated in SFM with or without the addition of echistatin (10^{-8}M) for various lengths of time as indicated. Cells were then lysed and immuno-precipitation performed with an anti–phosphotyrosine antibody (p-Tyr). This was followed by immunoblotting with the same anti–phosphotyrosine anti-body. The level of αV as determined by immunoblotting of aliquots of cell lysates from the same experiment is shown in the upper panel and the level of β3 as determined by immunoblotting with the antibody specific for the C-terminal of β3 is shown in the middle panel (c-β3). The data in this figure are representative of the results from three similar experiments.

kinase (FAK) a 125-kDa protein [Kornberg et al., 1992]. It has also been reported that binding of another αVβ3 disintegrin, contortrostatin, to mammary carcinoma cells also increased FAK phosphorylation [Ritter et al., 2000]. Since the increase in the 120-kDa phosphoprotein band could be due to an increase in FAK phosphorylation we determined whether an increase in FAK phosphorylation occurred in response to echistatin. We examined the phosphorylation status of tyrosine 397 of FAK specifically as this is the site of autophosphorylation and therefore represents the activated form of FAK. Echistatin treatment resulted in a significant increase in the level of autophosphorylated, FAK (Fig. 4A). This increase was initially detected following 1-h incu-bation with echistatin. A further increase was detected following
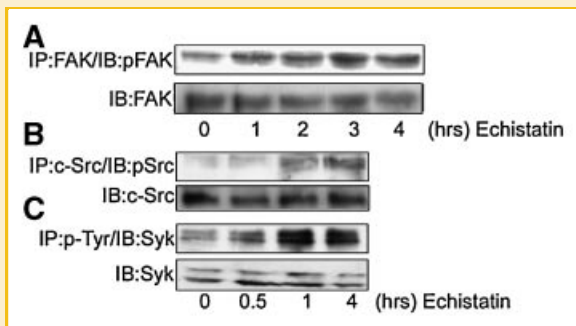

Fig. 4. Echistatin stimulation of FAK, c-Src and Syk phosphorylation. SMC were incubated in SFM with or without the addition of echistatin (10^{-8}M) for various lengths of time as indicated. Cells were then lysed and immuno-precipitated with (A) an anti–FAK antibody followed by immunoblotting with an antibody specific for the phosphorylated form of FAK [pY^{397}] (pFAK), (B) an anti-c-Src antibody followed by immunoblotting with an antibody specific for the active form of c-Src [pY^{418}] (pSrc) and (C) an anti–phosphotyrosine antibody (p-Tyr) and immunoblotting with an anti–Syk antibody. Equal amounts of lysates were also immunoblotted directly with appropriate anti-body to control for differences in protein. The data in these figures are representative of the results from three similar experiments.

3–4 h incubation (a 4.06 ± 0.12-fold increase (mean $\pm$ SEM, n $=$ 3), $P < 0.05$ compared with SFM alone).

## ACTIVATION OF Src AND Syk TYROSINE KINASES FOLLOWS ACTIVATION OF FAK BY ECHISTATIN

The phosphorylation and activation of FAK following integrin activation has been shown to lead to the recruitment and phos-phorylation of c-Src kinase [Schaller et al., 1994]. The association of Src with FAK results in further FAK phosphorylation leading to full catalytic activation. Therefore, we next examined the effect of echistatin on c-Src activity using phosphorylation of tyrosine 418 as a marker of its activation. A 1-h treatment with echistatin stimulated a 3.96 ± 0.3-fold increase in Src kinase phosphorylation (mean $\pm$ SEM, n $=$ 3, $P < 0.05$ compared with SFM alone; Fig. 4B). This increased further following a 4-h incubation (a 7.7-fold $\pm$ 0.6 increase compared with cells treated in SFM alone; mean $\pm$ SEM, n $=$ 3, $P < 0.05$ compared with SFM alone).

Activation of the tyrosine kinase Syk has been shown to be downstream of Src kinase activation following integrin ligation [Kurosaki et al., 1994]. We therefore examined whether the phosphorylation of Syk contributed to the phosphorylated protein band detected at 70–80 kDa following echistatin treatment. After 4-h treatment with echistatin there was a 2.0 ± 0.1-fold increase in Syk phosphorylation (mean $\pm$ SEM, n $=$ 3, $P < 0.05$ compared with SFM alone; Fig. 4B).

EFFECT OF CALPAIN AND c-Src KINASE INHIBITORS ON FAK AND Syk ACTIVATION

To determine whether activation of Src kinase was required for echistatin induced activation of Fak and Syk, cells were preincubated with the Src kinase inhibitor PP2 and the effect of echistatin induced phosphorylation of FAK and Syk was compared to cells that were not preincubated with PP2. In the presence of PP2 the echistatin induced increase in FAK phosphorylation was reduced by $50 \pm 5\%$ (mean $\pm$ SEM, n $=$ 3, $P < 0.05$ compared with echistatin alone). The presence of PP2 had no effect on the phosphorylation of FAK in the absence of echistatin since FAK phosphorylation in the presence of PP2 was $96 \pm 2\%$ (mean $\pm$ SEM, n $=$ ns) of the level of FAK phosphorylation in the absence of echistatin treatment (Fig. 5A). E64d had no significant effect on the phosphorylation of FAK either basally (FAK phosphorylation in the presence of E64d was $110 \pm 15\%$ of the level in the absence of E64d treatment (mean $\pm$ SEM, n $=$ 3, P, ns)) or in the presence of echistatin (FAK phosphorylation in the presence of echistatin and E64d was $120 \pm 12\%$ of the level of FAK phosphorylation following treatment with echistatin alone, (mean $\pm$ SEM, n $=$ 3, $P =$ ns (Fig. 5A)).

The presence of PP2 had no effect on Syk phosphorylation in the absence of echistatin since Syk phosphorylation in the presence of PP2 was $102 \pm 3\%$ (mean $\pm$ SEM, n $=$ 3, $P =$ ns) of the level of Syk phosphorylation in untreated cells. Pretreatment of cells with PP2 prior to the addition of echistatin completely inhibited the increase in Syk phosphorylation [Syk phosphorylation in the presence of echistatin plus PP2 was reduced by $57 \pm 6\%$ [mean $\pm$ SEM, n $=$ 3, $P =$ ns (Fig. 5B)]. This suggests that activation of Src kinase is upstream of the activation of Syk and that the activation of Src kinase contributes to some but not all of the phosphorylation of
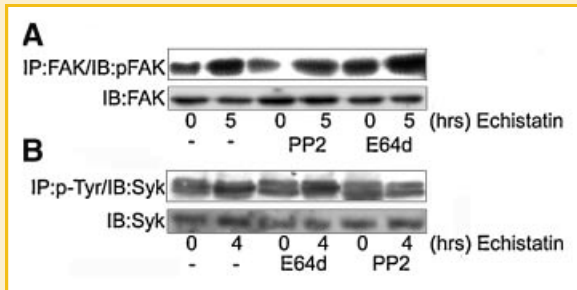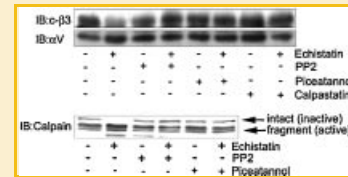
Fig. 5. Inhibition of c–Src inhibits FAK and Syk activation by echistatin. A: SMC were pretreated with PP2 (4 μM) or E64d (20 μM) where indicated prior to treatment with or without echistatin (10^{-8}M) for 5 h. Cells were then lysed and immunoprecipitation was performed with an anti–FAK antibody. This was followed by immunoblotting with an antibody specific for the phosphorylated form of FAK [pY^{397}] (pFAK). Equal volumes of cell lysate were immunoblotted directly with an anti–FAK antibody. B: SMC were pretreated with PP2 (4 μM) or E64d (20 μM) where indicated prior to treatment with or without echistatin (10^{-8}M) for 4 h. Cells were then lysed and immunoprecipitation was performed with an anti–phosphotyrosine antibody. This was followed by immunoblotting with an antibody specific Syk. Equal volumes of cell lysate were immunoblotted directly with an anti Syk antibody. The results shown are from the same gel but discontinuous lanes and the data are representative of the results from three similar experiments.

Fig. 6. Inhibition of ß3 cleavage and calpain activation by inhibitors of Src and Syk kinase. A: SMC in SFM were pretreated with PP2 (4 μM), piceatannol (30 μM) or calpastatin (50 μM) for 1 h prior to a 7–h incubation with echistatin (10^{-8}M). Following cell lysis β3 protein levels in each sample were determined by immunoblotting with an antibody specific for the cytoplasmic tail of β3 (c–β3). Data derived from scanning densitometric analysis expressed as arbitrary scanning units: Lane 1: 31,546, Lane 2: 9433, Lane 3: 20755, Lane 4: 30576, Lane 5: 30832, Lane 6: 28851, Lane 7: 33672, Lane 8: 34078. To control for total protein levels the amount of αV in each sample is also shown. The data in this figure are representative of the results from three similar experiments. B: SMC in SFM were pretreated with PP2 (4 μM) or piceatannol (30 μM) for 1 h prior to a 7–h incubation with echistatin (10^{-8}M). Following cell lysis pre and post autolytic calpain protein levels were visualized in each sample by immunoblotting with an anti–calpain 1 antibody. The arrows denote the position of intact calpain and its autolytic fragments. To control for total protein levels the amount of αV in each sample is also shown. The data in this figure are representative of the results from three similar experiments.

FAK. E64d had no significant effect on the phosphorylation status of Syk either basally or in the presence of echistatin Syk phosphorylation in the presence of echistatin plus E64d was 97 ± 4% of the level of Syk phosphorylation following treatment with echistatin alone [mean ± SEM n = 3, P = ns (Fig. 5B)] suggesting that the activation of calpain is downstream of the activation of Src and Syk kinase activity (Fig. 5 A,B).

INHIBITION OF Syk INHIBITS ECHISTATIN INDUCED ß3 CLEAVAGE

In order to determine whether the activation of signaling components in this pathway were involved in the echistatin induced activation of calpain and cleavage of β3 we examined the effects of PP2 and the Syk kinase inhibitor, piceatannol, on echistatin induced activation of calpain 1 and on cleavage of β3. The 82.75 ± 5% decrease in β3 protein levels (as determined by immunoblotting with the antibody that recognizes the cytoplasmic tail of β3) following a 7-h incubation with echistatin was effectively blocked in the presence of PP2, piceatannol and calpastatin (with β3 protein levels being 92 ± 5, 95 ± 4.7 and 91 ± 8% of controls respectively; mean ± SEM n = 3; Fig. 6A). Each inhibitor had no significant effect on β3 protein levels in the absence of echistatin treatment.

The decrease in intact calpain 1 levels and increase in the lower molecular weight forms of calpain that are detected following echistatin treatment was inhibited by the Src family kinase inhibitor, PP2 and the Syk inhibitor piceatannol, while neither inhibitor had any effect on calpain activation in the absence of echistatin treatment (Fig. 6B). While we observed a certain degree of experiment-to-experiment variation in the basal level of calpain activity, echistatin treatment consistently resulted in conversion to the active fragment comprising > 90% of the total detectable calpain. This change was consistent and the increase was significant compared with the cells incubated in the absence of echistatin

($P < 0.05$). Regardless of the basal level of calpain activity the Src and Syk inhibitors consistently inhibited the formation of the active fragment and the level of intact calpain was comparable to control cultures (Fig. 6B).

Taken together the results shown are consistent with the conclusion that the activation of Src and Syk is upstream of the activation of calpain and is required for calpain activation and subsequent cleavage of β3.

THE Vn HBD NEGATES THE ABILITY OF SC69000 TO ACTIVATE Syk

Intact Vn binds to αVβ3 through both its RGD and HBD sequences. RGD peptides antagonize IGF-I signaling whereas the Vn HBD enhances IGF-I stimulated signaling in SMC [Maile et al., 2006b]. Furthermore, intact Vn enhances IGF-I signaling even though it binds αVβ3 through its RGD and HBD sequences [Maile et al., 2006b]. Since the effect of the Vn HBD peptide is comparable to the effect of intact Vn these findings raise the question as to whether binding of the Vn HBD region modulates the apparent inhibitory effect that is induced by ligand binding to the RGD binding site, on IGF-I mediated signaling. Having characterized the signaling events that are activated following incubation with echistatin we were able to address this question. We first compared Syk activation by SC69000 in the presence or absence of either the VnHBD peptide or intact Vn. SC69000 induced a 2.4 ± 0.3 (mean SEM, n = 3 P < 0.05) fold increase in Syk phosphorylation which was completely inhibited in the presence of either intact Vn or the Vn HBD peptide (Syk phosphorylation was increased in the presence of Vn or the Vn
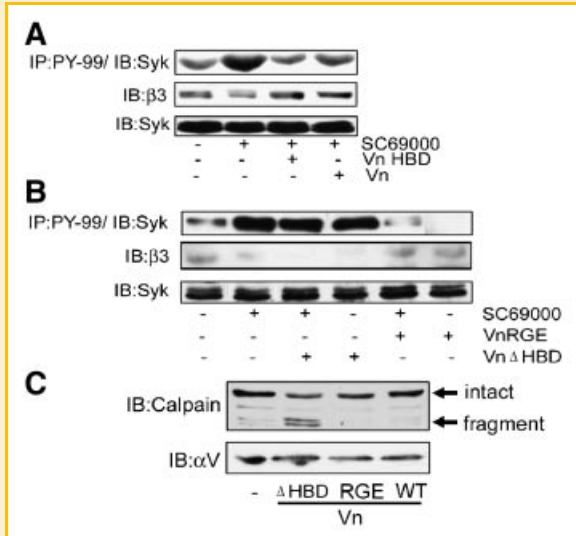
Fig. 7. Vn and the VnHBD inhibit the effects of RGD antagonism. SMC were incubated for with SC69000 (10^{-6}M), wild–type vitronectin (Vn; 2 μg/ml), Vn HBD (10μg/ml), VnRGE or VnΔ HBD (2 μg/ml) as indicated. A, B: Cells were then lysed and immunoprecipitation performed with an anti–phosphotyrosine antibody. This was followed by immunoblotting with an antibody specific Syk (top panel). The level of intact β3, as determined by immunoblotting with the antibody specific for the C–terminal of β3 (c–3) is shown in the middle panel. Equal amounts of cell lysate were immunoblotted directly with an anti–Syk antibody (bottom panel). The results shown are from the same gel but discontinuous lanes and the data are representative of results obtained from three independent experiments. C: Following cell lysis pre and post autolytic calpain protein levels were visualized in each sample by immunoblotting with an anti–calpain 1 antibody. The arrows denote the position of intact calpain and its autolytic fragments. To control for total protein levels the amount of αV in each sample is also shown.

HBD 1.2 ± 0.4-fold compared with untreated cells, p, ns) (Fig. 7A top panel). We next examined β3 cleavage and consistent with the role of Syk in activating the signaling pathway that leads to β3 cleavage following RGD site antagonist binding, the 3 ± 0.12-fold decrease in intact β3 protein that was stimulated by SC69000 was blocked in the presence of intact Vn or the VnHBD peptide (Fig. 7A, middle panel).

To determine the relative roles of the VnHBD and RGD sequences within the context of the whole Vn molecule we compared the effect of adding a Vn mutant in which the HBD had been deleted (VnΔHBD) with a Vn mutant in which the RGD sequence had been changed to RGE (VnRGE), on SC69000 mediated signaling. Vn RGE alone had no effect on Syk activation. Syk activation in the presence Vn RGE was increased 1.1 ± 0.1 compared with untreated cells (p, ns). The addition of Vn RGE however, completely inhibited the effect of SC69000 on Syk activation. In contrast to the 3.7 ± 0.4-fold increase in Syk activation in the presence of SC69000, Syk activation in the presence of Vn RGE was not significantly different from untreated cells (1.1 ± 0.1-fold increase compared with untreated cells, $P =$ ns). The inhibition of Syk activation was reflected in the complete inhibition of the $74 \pm 4\%$ decrease in intact β3 protein since β3 protein in the presence of Vn RGE was $110 \pm 9\%$ of untreated cells (mean ± SEM, n = 3, $P < 0.05$). In contrast addition of the VnΔHBD deletion mutant activated Syk to a similar extent as SC69000 (a $3.5 \pm .2$-fold increase in Syk activation compared with

untreated cells, (mean ± SEM, n = 3, $P < 0.05$) and exposure to VnΔHBD was associated with a $92 \pm 8\%$ decrease in β3 protein levels (mean ± SEM, n = 3, $P < 0.05$). Furthermore, VnΔHBD was unable to inhibit the effect of SC69000 in stimulating Syk activation and it had no effect on SC69000 stimulated β3 cleavage (Fig. 7B). Syk activation was increased by 3.4 ± 4-fold in the presence of SC69000 and VnΔHBD compared with untreated cells (mean ± SEM, n = 3, $P < 0.05$). Similarly β3 protein levels were reduced by $76 \pm 6\%$ in the presence of SC69000 and Vn ΔHBD compared with untreated cells.

Since activation of calpain by echistatin is critical for its effects on β3 cleavage we wished to determine the effect of the RGD sequence within intact Vn and the modulatory role of the HBD sequence on calpain activation. When SMC were treated with intact Vn or VnRGE calpain activation was not significantly changed compared with untreated cells. In contrast when SMC were incubated with VnΔHBD a $72 \pm 9\%$ decrease in intact calpain and a corresponding 84% increase in calpain fragment, (mean ± SEM, n = 3, $P < 0.05$) could be detected indicative of calpain activation (Fig. 7B).

DISCUSSION

Echistatin was first described as an antagonist of integrin function acting to block fibrinogen binding to platelets thereby inhibiting platelet aggregation [Gan et al., 1988]. Our results show that in addition to a passive antagonist function echistatin also actively stimulates signaling events through its binding to the αVβ3 integrin without causing detachment of stably attached SMC. This is consistent with a previous report in which it was observed that echistatin triggered an apoptotic response in breast cancer cells prior to initiating detachment [Brassard et al., 1999]. Signaling initiated by disintegrin binding specifically to αVβ3 has also been reported for the disintegrin contortrostatin [Ritter et al., 2001]. In that report contortrostatin was shown to stimulate hyperphosphorylation of FAK and it disrupted the actin cytoskeleton and causing disassembly of focal adhesions without affecting cell adhesion [Ritter et al., 2001]. Echistatin binding to αVβ3 has been shown to induce conformational changes that result in increased ligand-induced binding sites [Marcinkiewicz et al., 1997]. These conformational changes lead to a further increase in binding affinity for echistatin. Ligand binding of ECM proteins to the integrin receptors also induces conformational changes that contribute to the activation of intracellular signaling pathways, a process that has been termed "outside in signaling" [Aplin et al., 1999]. Therefore, it is likely that the conformational changes induced by echistatin binding are sufficient to initiate outside in signaling. In this study we demonstrate that echistatin binding to αVβ3 leads to outside in signaling as reflected in the sequential activation of FAK, Src and Syk leading to calpain activation and ultimately β3 cleavage. FAK phosphorylation has been shown to be stimulated following integrin agonist binding and to be required for focal adhesion stability. It is also essential for cell migration in many systems [Gilmore and Romer, 1996; Sieg et al., 1999]. Our data and that of Ritter et al. discussed above [Ritter et al., 2001] demonstrate that integrin antagonists can also trigger the phosphorylation of FAK yet they

function to inhibit integrin mediated effects such as cell migration. These responses are somewhat inconsistent with the previous described role for FAK. However, while phosphorylation of FAK is essential for its role in regulating cellular responses other studies have also suggested its rate of dephosphorylation is equally important [Sieg et al., 1999;Gilmore and Romer, 1996]. For example, it has is been shown that in cells expressing a mutant form of SHP-2, which is catalytically inactive, IGF-I cannot stimulate cell migration and this response is associated with a reduction in IGF-I stimulated FAK dephosphorylation [Manes et al., 1999]. This is consistent with our own observations using cells that express mutant forms of β3, that are not activated by ligand occupancy in which inhibition of IGF-I stimulated cell migration is associated with inhibition of IGF-I stimulated FAK dephosphorylation [Maile et al., 2001]. Additionally expression of the mutant forms of β3 is associated with a lack of SHP-2 recruitment to β3 and its subsequent transfer to SHPS-1 [Maile and Clemmons, 2002]. If, as these studies would suggest, SHP-2 recruitment to β3 is necessary for the dephosphorylation of FAK then echistatin induced cleavage of the cytoplasmic tail of β3 due to calpain activation would be predicted to result in an inability of IGF-I to stimulate FAK dephosphorylation as a result of the spatial and temporal dysregulation of SHP-2 transfer. This suggests that the activation state of the integrin is not only a regulator of FAK phosphorylation but that it also regulates FAK dephosphorylation via its ability to regulate the distribution of SHP-2.

Calpain 1 a member of the calpain family of calcium dependent cysteine proteases is a ubiquitous protease that has been shown to be localized to focal adhesions of various types of adherent cells [Glading et al., 2002; Perrin and Huttenlocher, 2002]. Studies have shown that activation of calpain results in the limited proteolysis of various structural and signaling molecules that are co-localized in focal adhesions [Cooray et al., 1996; Fox et al., 1985]. As such calpain has been implicated in remodeling of cytoskeletal and membrane attachments. However, dysregulation of calpain activity results in tissue damage in response to events such as myocardial infarction, stroke, and brain trauma [Goll et al., 2003]. Previous studies have reported that calpain activation is an event that is downstream of integrin activation [Fox et al., 1985]. Furthermore, it was shown that calpain is necessary for the formation of integrin clusters, which accumulate prior to the formation of focal adhesion complexes [Bialkowska et al., 2000]. In addition those clusters were shown to contain calpain cleaved β3. Other studies have shown that β3 is a target for proteolytic cleavage by calpain and more specifically that the cytoplasmic tail of ß3 has been shown to have a number of calpain cleavage sites; the two major sites flank the two tyrosine phosphorylation motifs [Du et al., 1995; Pfaff et al., 1999]. Studies have shown that the cleavage of ß3 is involved in clot retraction since calpain activation was shown to result in marked reduction in platelet-mediated clot retraction while inhibition of calpain prevented this effect [Schoenwaelder et al., 1997]. The effects of calpain were associated with cleavage of Src and talin and the associated disruption of integrin – cytoskeletal attachment is likely to contribute to the effects of calpain on clot retraction. Additionally, the calpain-mediated cleavage of ß3 has been shown to be an early event in detachment-triggered apoptosis in endothelial cells [Meredith et al., 1998]. These previous studies suggest that calpain is an important regulator of cellular events that are regulated by integrin engagement. Our results demonstrate that activation of the αVβ3 integrin following disintegrin binding can lead to a level of calpain activation that can result in significant β3 cleavage. Since we have determined previously that recruitment of signaling molecules such as DOK-1 to β3 is required for IGF-I mediated signaling [Ling et al., 2005] these observations provide a mechanism by which echistatin treatment inhibits signaling events that are stimulated by IGF-I. To our knowledge this is the first report to describe the consequences of calpain mediated cleavage of an integrin cytoplasmic domain on growth factor stimulated signaling.

The obvious question from our studies is why does echistatin binding to αVβ3 and activation of FAK lead to calpain activation and β3 cleavage whereas αVβ3 ligand occupancy with Vn or osteopontin does not result in these changes? We determined previously that the enhancing effect of Vn on IGF-I signaling was dependent upon binding of its HBD but not its RGD region to αVβ3 since addition of a VnΔHBD mutant could not enhance IGF-I mediated signaling whereas the Vn RGE mutant was fully active [Maile et al., 2006b]. In this study we extend those observations to show that a peptide containing the HBD of Vn can inhibit the signaling events that are initiated by RGD antagonists. This effect was not limited to a Vn HBD peptide but was also apparent when the Vn RGE mutant, which also binds to the region of β3 that binds the HBD peptide [Maile et al., 2006a,b], was analyzed. Specifically, this mutant negated the effects of SC69000 on Syk activation and β3 cleavage. Furthermore, while incubation of SMC with wild-type Vn or the Vn RGE mutant had no effect on calpain activation, treatment of SMC with the VnΔHBD mutant caused a significant increase in calpain activation similar to that seen following treatment of cells with the RGD containing antagonists. These results suggest that the effect of αVβ3 ligand occupancy is a balance between signaling events that are triggered by Vn binding to both its RGD and HBD sequences. The results support the conclusion that under usual conditions the net effect of the intact Vn molecule is to inhibit Syk and thus calpain activation as well as β3 cleavage even though it binds to the RGD-binding site. In contrast a mutant form of Vn that did not have the HBD domain could not counteract the effect of the RGD antagonist on β3 cleavage. This clearly demonstrates that the HBD domain of Vn contains the sequence that confers this inhibitory property and that when intact Vn binds to αVβ3 this inhibitory effect on Syk/calpain activation predominates.

The suggested role of αVβ3 in angiogenesis, atherosclerosis, and metastasis has lead to widespread interest in the use of αVβ3 inhibitors as treatments for these conditions. The results from these studies extend our understanding of the complexity of αVβ3 signaling and suggest that using RGD antagonists that activate this specific series of intracellular signaling events may be a less desirable approach as compared to blocking the signaling effects that occur following binding of the Vn HBD region [Maile et al., 2006a,b].

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