Structural analysis of the role of the β3 subunit of the αVβ3 integrin in IGF-I signaling

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SUMMARY

The disintegrin echistatin inhibits ligand occupancy of the αVβ3 integrin and reduces Insulin-like growth factor I (IGF-I) stimulated migration, DNA synthesis, and receptor autophosphorylation in smooth muscle cells. This suggests that ligand occupancy of the αVβ3 receptor is required for full activation of the IGF-I receptor.

Transfection of the full-length β3 subunit into CHO cells that have no endogenous β3 and do not migrate in response to IGF-I was sufficient for IGF-I to stimulate migration of these anchorage dependent cells. In contrast, transfection of either of two truncated mutant forms of β3 (terminating at W^{715} or E^{731}) or a mutant with substitutions for Tyr^{747} Tyr^{759} (YY) into either CHO or into porcine smooth muscle cells did not restore the capacity of these cells to migrate across a surface in response to IGF-I. This effect was not due to loss of IGF-I receptor autophosphorylation since the response of the receptor to IGF-I was similar in cells expressing either the full-length or any of the mutant forms of the β3 subunit. Echistatin reduced IGF-I receptor phosphorylation in cells expressing the full-length or the YY mutant forms of β3 subunit, but it had no effect in cells expressing either of two truncated forms of β3. A cell-permeable peptide homologous to the C-terminal region of the β3 subunit (amino acids 747-762) reduced IGF-I stimulated migration and receptor autophosphorylation of non-transfected porcine smooth muscle cells. These results demonstrate that the full-length β3 with intact tyrosines at positions 747 and 759 is required for CHO cells to migrate in response to IGF-I. Furthermore, a region of critical amino acids between residues 742-762 is required for echistatin to induce its regulatory effect on receptor phosphorylation. Since the IGF-I receptor does not bind to αVβ3 the results suggest that specific but distinct regions of the β3 subunit interact with intermediary proteins to facilitate IGF-I stimulated cell migration and echistatin induced inhibition of IGF-I signal transduction.

Key words: Growth factors, Extracellular matrix, Integrin, Disintegrin, Smooth muscle cells

INTRODUCTION

The migration of cells is an important aspect of many biologic events, such as wound healing, tumor cell metastasis and embryonic development (Lauffenberger and Horwitz, 1996). During the formation of atherosclerotic plaques and in restenosis following angioplasty the migration and proliferation of smooth muscle cells are key events (Ross, 1993). Vascular smooth muscle cells have insulin-like growth factor I (IGF-I) receptors and respond to exogenously added IGF-I with increased protein and DNA synthesis (Conover, 1991; Clemmons, 1984) and increased migration (Bornfeldt et al., 1993).

In previous studies we have demonstrated that ligand occupancy of the αVβ3 integrin receptor is required for IGF-I induced migration of vascular smooth muscle cells (SMC) (Jones et al., 1996). In the absence of serum, these cells will migrate in response to IGF-I only if they are plated on vitronectin, an αVβ3 ligand. Blocking ligand occupancy of αVβ3 with either the disintegrin echistatin or the αVβ3 specific monoclonal antibody (LM609) results in marked attenuation of the IGF-I induced increase in migration (Jones et al., 1996). One explanation for these data is that blocking ligand occupancy of an integrin receptor inhibits IGF-I stimulated migration by blocking physical contact with the extracellular matrix and thereby blocking the physical forces required for migration. However, our subsequent studies demonstrated that blocking ligand occupancy of the αVβ3 receptor with echistatin, in cells that were attached to their ECM, also blocked IGF-I stimulated DNA and protein synthesis and inhibited IGF-I induced receptor autophosphorylation. This suggests that ligand occupancy of the αVβ3 receptor was required for full activation of the IGF-I receptor and intracellular signaling by IGF-I (Zheng and Clemmons, 1998).

The β3 subunit of the αVβ3 receptor has been implicated as the major link between the integrins and the cytoskeleton and as an anchor for important components of several transduction systems (Sastry and Horwitz, 1993). Some of these binding interactions and signaling functions have been shown to require specific residues within the cytoplasmic domain of β3, in particular the two tyrosines (Tyr^{747}) (Tyr^{759}).

The common experimental paradigm used to investigate integrin signaling involves detaching adherent cells from tissue culture plates and replating them on surfaces coated with purified ECM proteins. Signaling events are then examined over a short period of time as cells reattach. In many of these studies the cells that are being analyzed have been derived from

solid tissues and therefore the use of this model is not an accurate reflection of the tissue context in which integrins and growth factors normally co-operate to regulate cellular responses. It is therefore important to distinguish between the role of integrin signaling in response to cellular reattachment and the co-operative signaling between integrins and growth factor receptors that occurs in cells that have been stably attached for prolonged periods.

An important step in understanding the mechanistic basis of the interaction between the αVβ3 integrin and the IGF-I receptor is to define the signaling components that are activated by ligand occupancy of αVβ3 and how this signal is transmitted to the IGF-I receptor. In previous studies, we have shown that there is no direct interaction between the IGF-I receptor and αVβ3 (Zheng and Clemmons, 1998). Therefore as a first step to better define the mechanism, we have attempted to identify the regions of the β3 subunit that are required for IGF-I mediated signaling. Using in vitro mutagenesis, we modified the structure of the β3 subunit and in particular the two tyrosines and determined if these changes altered IGF-I stimulated cell migration or the effect of the disintegrin antagonist echistatin on IGF receptor linked signaling using cells that had stably attached to their ECM for a prolonged time period.

MATERIALS AND METHODS

All chemicals were purchased from the Sigma Chemical Company (St Louis, MO) unless otherwise stated. Porcine aortic SMCs (pSMC) were obtained as previously described (Gockerman et al., 1995). CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD). An anti-β3 integrin receptor subunit polyclonal antibody was purchased from Chemicon International (Temecula, Ca). A polyclonal anti-IGF-I receptor antiserum and a monoclonal phosphotyrosine antibody (PY99) were purchased from Santa Cruz (Santa Cruz Ca). IGF-I was a gift from Genentech (South San Franscisco, CA). EZ-link Sulfo NHS SS biotin, Supersignal chemiluminescence substrate and the BCA protein assay kit were purchased from Pierce (Rockford, Il).

Synthesis of cell-permeable synthetic peptides

Peptides were synthesized by step-wise solid phase peptide synthesis method and purified by C18 reverse-phase high performance liquid chromatography by the Peptide Synthesis Facility at the University of North Carolina, Chapel Hill. The cell permeable peptides were designed using the hydrophobic VTVLALGALALVGVG region of the signal peptide sequence of human β3 followed by either the last 16 amino acids of the human β3 cytoplasmic tail, YKEATSTFTNITYRGT (test peptide) or the last 16 amino acids of the human β1 cytoplasmic tail YKSAVTTVVNPKYEGK (control peptide).

Generation of expression vectors

Full-length human integrin β3 receptor subunit cDNA (kindly provided by Dr G. White UNC Chapel Hill) was subcloned into both the pRcRSV and pMEP4 expression vectors (Invitrogen, Carlsbad, CA).

Generation of truncated forms of the β3 receptor

The two truncated forms (termed WK and E) of the β3 receptor were generated using single stranded mutagenesis with mutagenic primers that were complementary to the full-length β3 cDNA, but which also introduced an *Xba*I restriction endonuclease site and a stop codon, resulting in truncation at either amino acid residue 715 (WK) or 731 (E). For the WK mutant, the primer was complementary to nucleotides 2235-2255, except for a base change indicated by * to introduce the *Xba*I site: 5′-GAT GAG GAG TT<u>T CT*A GAT</u> GAG-3′. For the E mutant the primer was complementary to nucleotide 2277-2301, except for base changes indicated by * to introduce the *Xba*I site: 5′-GCG TTC TT<u>T* CT*A GA*</u>A TTT TCG CTT-3′. In both cases the introduced *Xba*I site is underlined. The pRcRSV plasmid was digested at the introduced *Xba*I site using 100 units/ml, and at the *Xba*I site that was present in the polylinker of the pRcRSV vector. The vector and truncated insert were then relegated and the modified DNA was then subcloned into pMEP4.

Generation of Tyr^{747}Phe/Tyr^{759}Phe mutant β3

The polymerase chain reaction was used to mutate the full-length β3 coding sequence at tyrosines 747 and 759 by using a mutagenic primer (5′-TTA AGT GCC CCG GAA CGT GAT ATT GGT GAA GGT AGA CGT GGC CTC TTT AAA CAG TGG GTT GTT GG-3′). The full-length β3 cDNA was used as a template. The N-terminal primer sequence used was 5′-ATG CGA GCG CGC CCG CGG CC-3′. Following sequencing to confirm mutagenesis, the polymerase chain reaction product was subcloned into pRcRSV and pMEP4.

The sequences of the native human β3 cytoplasmic tail and the truncated and mutant receptors that were generated and expressed are shown in Fig. 1.

Transfection of CHO-K1 cells and pSMCs

CHO-K1 cells were transfected with the pRcRSV expression vector containing either the full-length β3 DNA or one of the truncated or mutant forms of the receptor or vector alone (control). G418 resistant CHO-K1 clones were selected in αMEM containing 5% FBS with 800 μg/ml G418 and positive clones were maintained in alpha MEM containing 5% FBS with 400 μg/ml G418 (growth medium). The pSMCs (passage 4) were transfected with the pMEP4 expression vector. In all cases transfections were performed using the poly-l ornithine method as previously described (Imai et al., 1997). Hygromycin resistant pSMCs were selected and maintained in DMEM-H containing 15% FBS and 100 μg/ml hygromycin (growth medium). Both transfected and non-transfected pSMCs were used in subsequent experiments between passage 6 and 20.

Biotinylation of cell surface proteins to detect β3 expression

Transfected cells were grown to subconfluency, rinsed three times

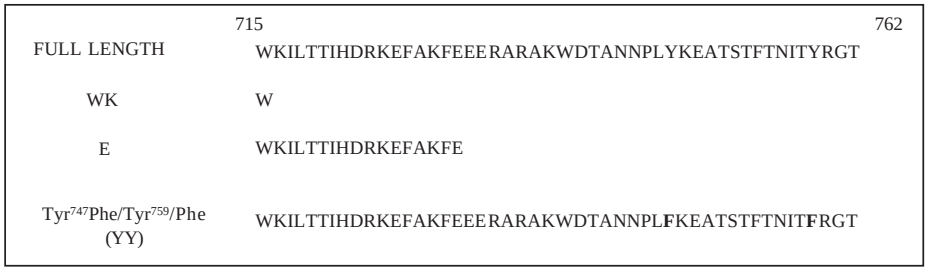

Fig. 1. Diagram showing the sites of the two truncations introduced into the β3 integrin receptor cytoplasmic tail and the position of the two tyrosine residues substituted with phenylalanine. The expression vectors were constructed and CHO-K1 cells and pSMCs were transfected as described in Materials and Methods.

with ice-cold PBS (pH 8.0) then incubated at 4°C for 30 minutes with PBS + 0.5 mg/ml biotin. Cells were then washed three times with PBS + glycine (15 mM) to quench the biotinylation. Cells were then lysed as described below and the protein content of each sample was measured. An equal amount of protein from each cell lysate was immunoprecipitated with a 1:300 dilution of a polyclonal anti human β3 antibody as described below. The immune complexes were then separated by SDS-PAGE, 8% gel, under non-reducing conditions. Biotinylated β3 was visualized by immunoblotting with peroxidase conjugated ExtrAvidin as described below.

Immunoprecipitation for the detection of biotinylated proteins, IGF-I receptor or focal adhesion kinase (FAK) phosphorylation

Cells were cell-surface biotinylated as described above, or incubated overnight in serum-free DMEM (SFM) plus 0.01% BSA and then exposed to 100 ng/ml IGF-I for various lengths of time prior to lysis in RIPA buffer (1% NP40, 0.25% sodium deoxycholate, 1 mM EGTA, 150 mM sodium chloride, 50 mM Tris-HCl, pH 7.5, 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 14,000 ***g*** for 10 minutes. The supernatant was incubated overnight at 4°C with either a 1:300 dilution of an IGF-I receptor antibody or a 1:300 dilution of a monoclonal antiphosphotyrosine antibody (PY99). Immune complexes were then precipitated by adding Protein A-Sepharose and incubating for a further 2 hours at 4°C. The samples were then centrifuged at 14,000 ***g*** for 10 minutes and the pellets washed 4 times with RIPA buffer. The pellet was suspended in 45 μl of Laemmli buffer and boiled for 5 minutes and the proteins separated by SDS-PAGE, 8% gel. To examine the effect of echistatin on IGF-I receptor phosphorylation cells were incubated overnight in SFM plus 0.01% BSA and 10^{-8} M echistatin prior to exposure of cells to IGF-I. To examine the effect of the synthetic peptides, cells were incubated in SFM plus 0.01% BSA overnight then incubated with either the test or control peptide (50 μM) for 6 hours prior to exposing the cells to IGF-I (100 ng/ml).

Western immunoblotting

Following SDS-PAGE proteins were transferred to PVDF membrane. The membranes were blocked in 1% BSA in Tris-buffered saline with 0.1% Tween (TBST) for 2 hours at room temperature. The membranes were incubated with primary antibody (monoclonal anti-phosphotyrosine (PY99) at 1:10,000 dilution, or polyclonal anti-FAK at 1:500 dilution) overnight at 4°C or peroxidase-conjugated ExtrAvidin (1:1,000) for one hour at room temperature. Following incubation with primary antibodies membranes were then washed three times in TBST prior to incubation with appropriate horseradish peroxidase-conjugated secondary antibody. Binding of the peroxidase label was visualized using enhanced chemiluminescence using the manufacturer's instructions (Pierce, Rockford IL).

Cell wounding and migration assay

On plastic

Cells were plated in six-well plates (Falcon Labware, Division of Beckton Dickinson Fairborne NJ) in growth medium. pSMCs were grown to confluency over six days with one medium change and CHOs were grown to confluency in three days. Wounding was performed as previously described (Jones et al., 1993). Briefly, a razor blade was used to scrape an area of cells leaving a denuded area and a sharp visible wound line. The wounded monolayers were then rinsed three times in SFM and then ten 1 mm areas along the wound edge were selected and recorded for each treatment. The wounded monolayers were then incubated with SFM (plus 2% FBS pSMCs only) with or without 100 ng/ml IGF-I for 24-48 hours at 37°C. The cells were then fixed and stained (Diff Quick, Dade Behring, Inc., Newark, DE) and the number of cells migrating into the wound area counted. At least five of the previously selected 1 mm areas at the edge of the wound were counted for each data point.

To test the effect of the synthetic peptides on IGF-I stimulated migration, cells were incubated for 6 hours after wounding with either 50 μM of test or control peptide. The medium was aspirated, and the peptide was then re-added with IGF-I (100 ng/ml) and the incubation continued for 48 hours.

On vitronectin

Six well plates were coated with human vitronectin (purified as previously described) at a concentration of 5 μg/ml in PBS plus 1 mM $CaCl_2$ overnight at 4°C. Subconfluent cells were dislodged and resuspended in SFM + 0.01% BSA and plated onto the coated wells to achieve 80-90% confluency following attachment and spreading overnight at 37°C. Wounding was then performed as described above, but cells were incubated with SFM containing 0.01% BSA and the individual treatments.

Our previous analysis of the wounded monolayer by [^{3}H]thymidine autoradiography demonstrated that the labeling index of pSMCs at the wounded edge was 7±4% at the basal level and 18±7% after treatment with IGF-I. Therefore, less than 10% of cells present in the denuded area at the end of the migration assay are considered to result from cell division rather than cell migration (Jones et al., 1996).

The Mann-Whitney test and Student's *t*-test were used to compare differences between the control and test groups. Band intensities on autoradiographs were measured by scanning densitometry and analyzed using NIH Image, version 1.61.

RESULTS

Expression of human β3 integrin receptor

To determine if the human β3 that was expressed following transfection was localized on the cell surface, cell impermeable biotin was utilized and the biotinylated cell surface proteins were immunoprecipitated with anti-human β3 antiserum. Fig. 2 shows the cell surface expression of human β3 integrin receptor in CHO-K1 cells (A) and pSMC cells (B). CHO-K1

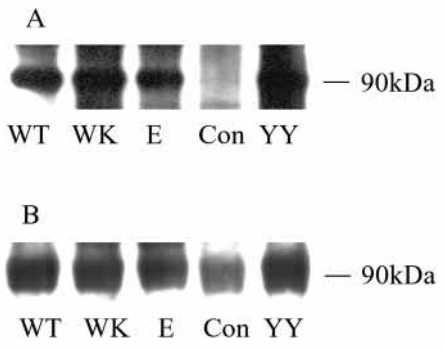

Fig. 2. Constitutive expression of full-length, truncated or mutant forms of the human β3 integrin receptor in CHO-K1 cells (A) or pSMCs (B). CHO-K1 cells and pSMC cells were transfected with the different human β3 integrin receptor constructs and positive clones were selected as described in Materials and Methods. Following cell surface biotinylation, equal amounts of cell lysate were immunoprecipitated with a β3 polyclonal antibody. Biotinylated proteins were then visualized with peroxidase conjugated ExtrAvidin. This figure is representative of the level of β3 expression in the clones used for this study. Lane 1, full-length β3 receptor; lane 2, WK mutant receptor; lane 3, E mutant receptor; lane 4, empty vector (con); lane 5, $Tyr^{747}Phe/Tyr^{759}$/Phe (YY) mutant receptor.

cells transfected with empty vector alone (control) have no detectable β3 integrin receptor by immunoblotting; however, following transfection with expression vectors encoding either the full-length, truncated, or double tyrosine mutant forms of the receptor, a single band at approximately 90 kDa is detectable, corresponding to the expected size of β3 run under non-reducing conditions. In the pSMC transfected with the empty vector alone there is a band at 90 kDa corresponding to endogenous porcine β3; however, following transfection with expression vectors encoding the full-length and the mutant forms of human β3 there is an increase in the level of detectable human β3. Successful transfected expression of human β3 was confirmed by reverse transcriptase-polymerase chain reaction (data not shown).

Integrin β3 expression permits IGF-I stimulated migration in CHO-K1 cells

To establish a role for the β3 subunit of the αVβ3 integrin receptor in IGF-I-stimulated migration, the IGF-I stimulated response of CHO-K1 cells transfected with the full-length human β3 receptor was compared with cells transfected with the empty vector. Fig. 3 shows that CHO-K1 cells transfected with the empty vector alone do not increase their migration in response to IGF-I. However, following transfection with the full-length human β3 receptor, IGF-I stimulated a 79±28% (mean ± s.e.m. *n*=3) increase in migration in cells plated and grown on plastic and a 95±8.5% (mean ± s.e.m. *n*=3) increase if they had been plated on vitronectin. As the β3 subunit is required for high affinity vitronectin binding, the results further confirm that IGF-I stimulated migration of adherent cells is mediated through the αVβ3 receptor.

Since the β3 subunit of the αVβ3 receptor is required for IGF-I stimulated migration we used this model system to further define the region of the subunit required for this response. CHO-K1 cells expressing either of the two truncated forms of the receptor (WK,E) or the receptor with the double tyrosine substitution (YY) showed no increase in migration following incubation with IGF-I (Fig. 3). A lack of response to IGF-I was observed whether the cells were grown on plastic (A) or plated on vitronectin (B). In comparison control pSMCs that express a full-length wild-type porcine β3 subunit migrated normally in response to IGF-I (194±38% increase, mean ± s.e.m. *n*=3). In cells transfected with the full-length human β3 subunit there was a similar response (IGF-I stimulated migration by 194±28% mean ± s.e.m. *n*=3). In contrast, pSMCs expressing either of the truncated forms of the human β3 subunit or the double tyrosine mutant receptor demonstrated no significant increase in migration in the presence of IGF-I (124±24, 136±18, and 107±8%, p, NS, respectively) (mean ± s.e.m. *n*=3) (Table 1).

Table 1. Cell migration of pSMCs expressing the different forms of the β3 integrin receptor compared with control cells transfected with the empty vector alone

Cell type	Cell migration (% control)*	*P*‡
Control	194±38	*P*<0.05
β3 full-length receptor	194±28	*P*<0.05
WK	124±24	
E	136±18	
YY	107±08	

Cells were plated at 80-90% confluency on 6-well plates pre-coated with vitronectin. Following wounding cell migration was determined in the presence or absence of IGF-I (100 ng/ml). For each treatment at least 5 different pre-selected 1 mm wound areas were counted.

*Results shown are calculated from the mean ± s.e.m. from 3 independent experiments. The number of cells migrating in the absence of IGF-I is expressed as 100%. Results shown are expressed as the percentage of cells migrating in the presence of IGF-I compared with the same cell type migrating in the absence of IGF-I.

‡*P*<0.05 when treatment without IGF-I is compared with treatment with IGF-I.

A cell-permeable synthetic peptide homologous to the C-terminal region of β3 reduces IGF-I stimulated migration

In order to further substantiate our data using a non-transfected physiologically relevant cell model we used a technique that

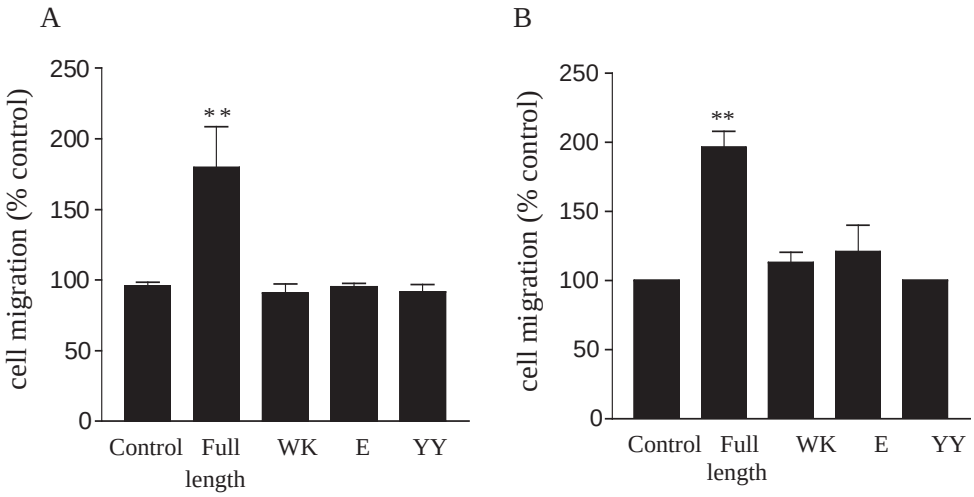

Fig. 3. Cell migration of CHO-K1 cells transfected with the full-length β3 integrin receptor, truncated or mutant forms of the receptor compared with migration of empty vector transfected cells. Cells were grown to confluency on uncoated 6-well plates (A) or plated at 80-90% confluency on 6-well plates pre-coated with vitronectin (B). In both cases cells were then wounded and cell migration was determined in the presence or absence of IGF-I (100 ng/ml). For each treatment at least 5 different 1mm pre-selected wound areas were counted. Results shown are calculated from the mean ± s.e.m. from 3 independent experiments. The number of cells migrating in the absence of IGF-I is expressed as 100%. The results shown are expressed as the percent of cells migrating in the presence of IGF-I compared with the same cell type migrating in the absence of IGF-I. ***P*<0.05 when migration in the absence of IGF-I is compared with migration in the presence of IGF-I.

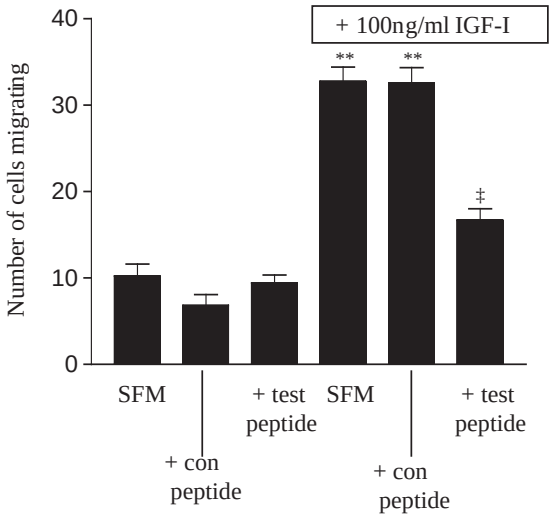

Fig. 4. Cell migration of non-transfected pSMCs incubated with a cell-permeable synthetic peptide homologous to the C-terminal region of the human β3 integrin receptor. Cells were grown to confluency on 6-well plastic dishes then wounded and pre-incubated with 50 μM of the test or control peptide with and without IGF-I (100 ng/ml) as described in Materials and Methods. The number of cells migrating across the wound at 8 pre-selected 1 mm regions of the wound was counted. Results shown are the mean ± s.e.m. from one representative experiment that was repeated four times with similar results. ***P*<0.05 when migration in the presence of IGF-I is compared with migration in the absence of IGF-I. ‡*P*<0.05 when migration in the presence of IGF-I test peptide is compared with migration in the presence of IGF-I alone.

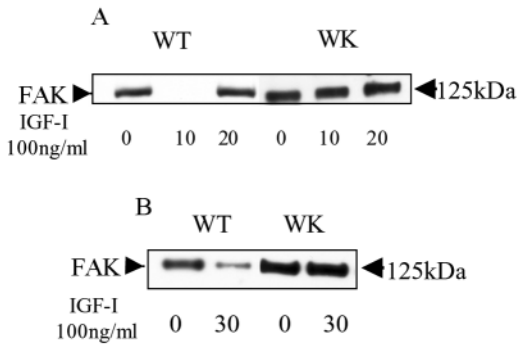

Fig. 5. FAK phosphorylation in cells expressing full-length or truncated (WK) forms of the human β3 integrin receptor with or without exposure to IGF-I. Cells were grown to 80-90% confluency in 10 cm^2 plastic dishes in growth medium and then incubated overnight in SFM plus 0.01% BSA. The cells were then exposed to IGF-I for either 10, 20 or 30 minutes (pSMCs) or 30 minutes (CHOs). Following cell lysis and immunoprecipitation with an antiphosphotyrosine antibody, the amount of FAK in each sample was determined by immunoblotting with an anti FAK antibody.

results in the uptake of a small synthetic peptide sequence homologous to the C-terminal region of the β3 receptor. The addition of an extra 20 amino acids from the signal sequence of β3 results in the uptake of this peptide into the cell cytoplasm where it presumably can compete with the endogenous receptor for signaling molecules. This technique has already been used by other laboratories to begin to identify the functional role of different regions of both β3 (Liu et al., 1996) and β1 integrins (Buttery et al., 1999) for cell attachment or platelet activation.

A 6-hour pre-incubation of non-transfected pSMCs with the synthetic peptide prior to incubation with IGF-I, while having no effect on basal migration resulted in a significant decrease in IGF-I-induced migration (36±5%, mean ± s.e.m. n=4, P<0.05 when migration in the presence of test peptide and IGF-I is compared with migration in the presence of IGF-I alone). The control peptide had no effect on migration in the presence or absence of IGF-I (Fig. 4).

The β3 integrin receptor cytoplasmic tail is not required for FAK phosphorylation but is required for IGF-I stimulated FAK dephosphorylation

The tyrosine phosphorylation, and its associated activation, of FAK have been implicated as a key event in integrin mediated signal transduction. IGF-I has been reported to stimulate both phosphorylation and dephosphorylation of FAK depending upon the cell type and experimental design (Casamassima and Rozengurt, 1998; Baron et al., 1998; Guvakova and Surmacz, 1999). We therefore compared the levels of phosphorylated FAK in confluent monolayer cultures expressing the full-length β3 integrin subunit with the level of phosphorylated FAK in cells expressing the truncated form of β3 (WK). In addition we compared the effect of IGF-I treatment on FAK phosphorylation in the same two cell types.

It can be seen in Fig. 5A that there is no detectable difference in basal levels of FAK phosphorylation between pSMCs expressing the full-length or truncated form of β3. Similar results were obtained when CHO cells expressing the different forms of β3 were compared (Fig. 5B). When pSMCs expressing the full-length β3 were treated with IGF-I a marked decrease in FAK phosphorylation was apparent at 10 minutes, this decrease was transient as levels returned to base line following 20 minutes treatment with IGF-I. In contrast, exposure of stably anchored cells expressing the truncated form of β3 (WK) to IGF-I resulted in no apparent change in the level of FAK phosphorylation. Similar results were obtained with the CHO cells expressing the full-length β3 except the rate of FAK dephosphorylation was slower i.e. no decrease was seen until 30 minutes IGF-I treatment (Fig. 5B). Again no decrease in FAK phosphorylation is apparent in CHO cells expressing the truncated form of β3 (Fig. 5B).

The β3 integrin receptor subunit cytoplasmic tail is not required for IGF-I receptor phosphorylation

Following a 10-minute incubation of pSMC with IGF-I there was no difference in the level of IGF-I receptor phosphorylation between cells expressing the full-length β3 receptor (Fig. 6A) or either of the two truncated of the receptor forms or the receptor with the double tyrosine substitution. The level of phosphorylation after IGF-I stimulation was comparable to that seen in the control cells (Fig. 6A). This finding was further supported by the results with CHO-K1 cells shown in Fig. 6B. There was a marked increase in the level of IGF-I receptor phosphorylation following stimulation with IGF-I in CHO-K1 cells transfected with the empty vector

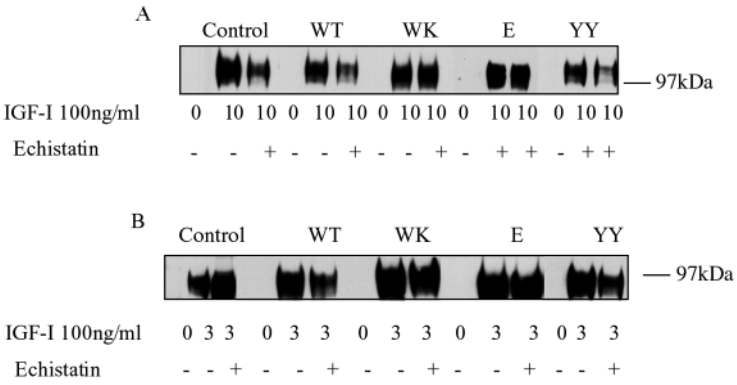

Fig. 6. IGF-I receptor phosphorylation in cells expressing the different forms of the human β3 integrin receptor in the presence or absence of echistatin. Cells were grown to 80-90% confluency in 10cm^2 plastic dishes in growth medium and then incubated overnight in SFM plus 0.01% BSA with or without 10^{-8} M echistatin. The cells were then exposed to IGF-I for either 10 minutes (pSMCs) or 3 minutes (CHOs). Following cell lysis and immunoprecipitation of the IGF-I receptor, the phosphorylation of the IGF-I receptor in each sample was determined by immunoblotting with an anti-phosphotyrosine antibody. (A) pSMCs expressing the different forms of the human β3 integrin receptor. (B) CHO-K1 cells expressing the different forms of the human β3 integrin receptor. This figure is representative of results obtained from 3 separate experiments.

alone, i.e. with no β3 expression. The level of phosphorylation was comparable to that seen in cells expressing the full-length receptor and in the cells expressing both of the truncated forms of the receptor as well as in cells expressing the receptor with the double tyrosine substitution.

The cytoplasmic tail of the β3 integrin receptor subunit is required for the inhibitory effect of echistatin on IGF-I stimulated receptor phosphorylation

Our previous results demonstrating that blocking ligand occupancy of the αVβ3 integrin receptor with the disintegrin echistatin attenuated IGF-I cellular responses suggested a direct relationship between the activation state of the integrin receptor and full activation of the IGF-I signaling. Having demonstrated that the cytoplasmic tail of β3 is not required for IGF-I receptor phosphorylation we next tested whether echistatin could still have an inhibitory effect on the receptor in the absence of the β3 cytoplasmic tail.

Fig. 6A shows that pre-incubation of pSMCs transfected with the empty vector alone or the full-length β3 receptor with echistatin resulted in a significant reduction in the level of IGF-I receptor phosphorylation, a 33±0.75% and 32±0.5% (± s.e.m. n=3, P<0.05) reduction, respectively, when analyzed by scanning densitometry. However, echistatin had no effect on the level of IGF-I receptor phosphorylation in cells expressing either of the two truncated forms of the receptor. In contrast, echistatin still had a significant inhibitory effect on IGF-I receptor phosphorylation in cells expressing the receptor with the double tyrosine substitution, a 29.5±1.25% (± s.e.m. n=3, P<0.05) reduction.

Fig. 6B shows similar results were obtained with the CHO-K1 cells. Echistatin had no inhibitory effect on IGF-I receptor phosphorylation in cells transfected with the empty vector alone consistent with the absence of β3. As with the pSMCs, echistatin had a negative effect on the level of IGF-I receptor phosphorylation in cells expressing the full-length β3 receptor (a 42.7±1.25% (± s.e.m. n=3, P<0.005) reduction). Again, echistatin had no effect in cells expressing either of the two truncated forms of the receptor. As with the pSMCs, the CHO cells expressing the receptor with the double tyrosine substitution were sensitive to the effects of echistatin, resulting in a 23.7±0.65% (± s.e.m. n=3, P<0.005) reduction in phosphorylation when analyzed by scanning densitometry.

A cell-permeable synthetic peptide homologous to the C-terminal region of β3 reduces IGF-I receptor phosphorylation

Although our findings with transfected CHO cells suggested that an intact β3 subunit was not required for IGF-I to stimulate receptor phosphorylation, they do not exclude the possibility that in the presence of full-length β3 both ligand occupancy of β3 and a full complement of β3 protein interactions, as well as the IGF-I receptor occupancy, is required for optimal IGF-I receptor phosphorylation. To investigate that possibility, nontransfected pSMC were pre-incubated with the cell-permeable peptide that had been shown to inhibit IGF-I stimulated migration. This resulted in significant inhibition of IGF-I stimulated receptor phosphorylation. When analyzed by scanning densitometry, the test peptide reduced the level of IGF-I receptor phosphorylation by 20±3.6% (± s.e.m. n=3, P<0.05, when the level of IGF-I receptor phosphorylation in the presence of test peptide is compared with IGF-I receptor phosphorylation in the absence of peptide) whereas the control peptide had no effect. A representative experiment is shown in Fig. 7.

DISCUSSION

In previous studies that utilized stably attached cells, we have demonstrated a requirement for ligand occupancy of the αVβ3 integrin receptor for optimal IGF-I receptor-mediated signaling

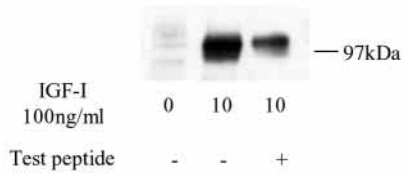

Fig. 7. IGF-I receptor phosphorylation of untransfected pSMCs following pre-preincubation with the synthetic peptide homologous to the C-terminal of the human β3 integrin receptor. Cells were grown to 80-90% confluency in 10 cm^2 plastic dishes in growth medium and then incubated overnight in SFM plus 0.01% BSA. Cells were then incubated for a further 6 hours with 50 μM of the test or control peptide. Cells were then exposed to IGF-I (100 ng/ml) for 10 minutes. Following cell lysis and immunoprecipitation of the IGF-I receptor, phosphorylation of the IGF-I receptor in each sample was determined by immunoblotting.

(Jones et al., 1996; Zheng and Clemmons, 1998). In this study we have analyzed this interaction by determining in stably attached cells the role of cytoplasmic tail of the β3 subunit in modulating both IGF-I-stimulated migration and IGF-I receptor phosphorylation. Using two independent experimental approaches we have shown that the C-terminal region of the β3 cytoplasmic tail is required for both IGF-I stimulated migration and the inhibitory effect of echistatin on IGF-I receptor phosphorylation. A crucial role for the C-terminal region of β3 and more specifically for Tyr^{747}, Tyr^{759} in IGF-I stimulated migration is supported by other studies of β3 integrin function. Phosphorylation of Tyr^{747} has been demonstrated following platelet aggregation and the signaling proteins SHC and Grb2, have been shown to associate with phosphorylated β3 peptides (Law et al., 1996). Law et al. have shown that mutation of these tyrosines results in defective αIIbβ3 function in mice (Law et al., 1999). The ^{744}NPXY sequence of β3 was shown to be essential for melanoma cell migration on vitronectin but in that study that key structural requirement was the presence of a tyrosine residue and not its phosphorylated state (Filardo et al., 1995). A blocking peptide comparable to that used in this study was shown to block adhesion of melanoma cells to vitronectin and mutation of Tyr^{747} and Tyr^{759} resulted in loss of the inhibitory function of this peptide (Liu et al., 1996). Although we have not identified the specific protein(s) that interact with the C-terminal region of β3 to modulate the cell migration response, our results suggest that there is an interaction between such a protein and either one of the downstream (post receptor) signaling elements in a pathway that is activated by the IGF-I receptor or some intracellular protein that is induced by IGF-I.

The disentegrin echistatin, has been shown to be a potent inhibitor of the IGF-I receptor phosphorylation response (Zheng and Clemmons, 1998) and in these studies it was unable to exert an inhibitory effect on receptor phosphorylation in the absence of a β3 cytoplasmamic tail. This result would predict that cells expressing truncated β3 would have an attenuated IGF-I receptor phosphorylation response. In contrast, the level of IGF-I receptor phosphorylation was equivalent in cells expressing different forms of the receptor. However, if β3 signaling to the IGF-I receptor had no effect on IGF-I stimulated receptor phosphorylation, then it would be predicted that the cell permeable β3 peptides would also have no effect. Since they were effective we propose an alternative explanation that is based on the fact that integrin function can be altered by different intracellular signals. These signals regulate integrin conformation, ligand affinity, and activation of signal transduction. The αVβ3 integrin has been shown to be present in a constitively high or low affinity state and the low affinity state can be induced by intracellular suppressors (Wary et al., 1998). We propose that under basal conditions wherein there is no ligand occupancy, the β3 integrin functions through intermediary proteins to suppress IGF-I receptor phosphorylation (Fig. 8). When β3 is occupied by an appropriate ligand such as vitronectin, this suppression is lifted allowing IGF-I mediated receptor activation. In this model, truncation or absence of the β3 receptor does not result in the loss of the ability of IGF-I to reduce receptor phosphorylation but rather also functions to lift suppression resulting in no change in the ability of IGF-I to activate the receptor when compared the full-length β3 that is ligand occupied. Exposure of cells to echistatin prevents the lifting of this suppression by ligands therefore resulting in a reduction in IGF-I receptor phosphorylation. Since in our cells expressing truncated β3 there was no suppression at baseline, it is predictable that echistatin would have no effect. Also echistatin may have additional functional activities. Echistatin binding to αVβ3 has been shown to induce extensive conformational changes (Cheresh and Spiro, 1987). It is possible that a conformational change in β3 may be an additional mechanism by which the interaction between β3 and β3-linked signaling molecules is perturbed. The β3 synthetic peptides which have been shown in other studies to compete with signaling molecules for binding to β3 (Liu et al., 1996) clearly disrupted the receptor phosphorylation response to IGF-I suggesting that they effected this change by altering the binding of intermediary signaling molecules to β3 thus perturbing the mechanism by which ligand occupancy of β3 acts to lift suppression. The elucidation of the components of this signaling mechanism will be an important goal in future studies.

The tyrosine phosphorylation of FAK is considered a key event in integrin mediated signal transduction. FAK phosphorylation is an initial event that follows cellular adhesion to ECM proteins in a number of different cell types (Burridge et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992). Integrin-engagement triggered phosphorylation of FAK creates binding sites for SH2 domains of other signaling molecules, thus providing a link between integrin engagement and downstream signaling events (Cobb et al., 1994; Schaller et al., 1994; Calab et al., 1995). Cells from

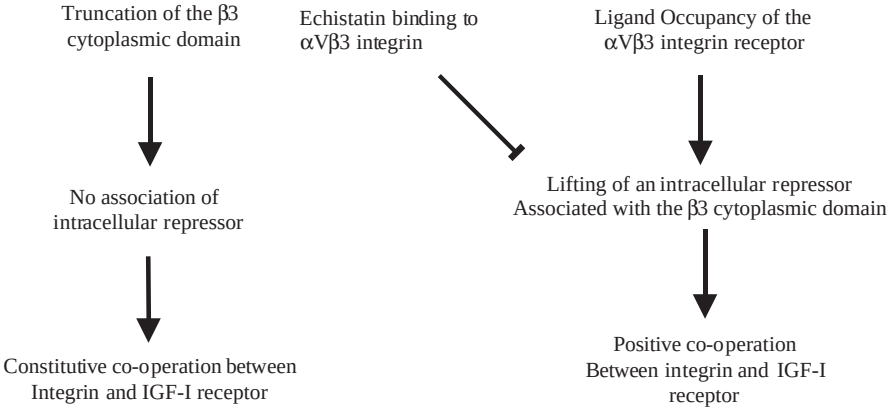

Fig. 8. Ligand occupancy of the αVβ3 integrin lifts the intracellular repression associated with the β3 integrin resulting in postive co-operation between the integrin and IGF-I receptor. In the presence of echistatin the repression can not be lifted and there is no positive co-operation between the two receptors. Truncation of the β3 cytoplasmic tail prevents association of the intracellular repressor resulting in constitutive co-operation between the two receptors.

FAK$^{-/-}$ mice have reduced migration compared with their wild-type counterparts (Ilic et al, 1995). In contrast to other growth factors, IGF-I has been shown to both promote both FAK phosphorylation and dephosphorylation depending upon the cell type and experimental design (Casamissma and Rozengurt, 1998; Baron et al., 1998; Guvakova and Surmacz, 1999) Our results using stably attached cells show that in contrast to recently attached cells, stimulation of FAK phosphorylation is not required for IGF-I to stimulate cell migration. Additionally, although integrin beta subunit cytoplasmic domains are important for the activation of attachment-mediated FAK phosphorylation, in stably attached cells the truncation of the β3 cytoplasmic tail had no effect on the basal level of FAK phosphorylation.

In contrast, IGF-I did induce a rapid and transient decrease in FAK phosphorylation in cells expressing the full-length β3, but it had no effect on the cells expressing the truncated form of the β3. Attenuation of FAK dephosphorylation in response to IGF-I was shown to be associated with reduced IGF-I stimulated migration in MCF-7 breast cancer cells expressing a catalytically inactive SHP-2 mutant (Manes et al., 1999). Reduced spreading and migration of fibroblasts from SHP-2$^{-/-}$ mice has also been reported (Yu et al., 1998). Since hyperphosphorylation of FAK is also observed in these mice, it was hypothesized that the SHP-2 phosphatase could mediate FAK dephosphorylation and turnover required for motility and spreading. The role of the β3 cytoplasmic tail in the FAK dephosphorylation response to IGF-I is as yet unclear, however, since SHP-2 is a cytosolic phosphatase and FAK localizes to focal adhesions following integrin engagement, one hypothesis is that truncation of the β3 cytoplasmic tail prevents the IGF-I stimulated recruitment of SHP-2 from the cytosol to the focal adhesions and the lack of FAK turnover prevents localized cell detachment that may be required for migration.

In summary, our studies have begun to uncover the complexity of the integrin/IGF-I receptor interaction by demonstrating that, in stably anchored cells, different components of the cytoplasmic tail of β3 are required for different aspects of IGF-I signaling. The identification of the exact regions of this integrin receptor that affect the different aspects of IGF-I signaling offers the potential to specifically modify selected IGF-I mediated effects without disrupting others.

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