# Serine 785 phosphorylation of the β1 cytoplasmic domain modulates β1A-integrin-dependent functions

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## SUMMARY

**The integrin β1 cytoplasmic domain plays a key role in a variety of integrin-mediated events including adhesion, migration and signaling. A number of studies suggest that phosphorylation may modify the functional state of the cytoplasmic domain, but these studies frequently only examine the effect of substituting amino acid mimics that cannot be phosphorylated. We now demonstrate, using site directed mutagenesis, that substituting either an unphosphorylated (S to M) or a phosphorylated (S to D) mimic in place of serine can modify integrin function. Specifically, we show that expressing a residue that mimics a dephosphorylated form of the protein promotes cell spreading and directed cell migration, whereas a residue mimicking a phosphorylated form of the protein promotes attachment but inhibits cell spreading or migration. The significance of these observations is strengthened by the fact that the β1 mutations display the same properties in both a fibroblast cell line (GD25) and a teratocarcinoma cell line (F9). The results indicate that changes in the phosphorylation state of S785 modulates β1 integrin function.**

Key words: Phosphorylation, β1 Integrin, Serine, GD25, F9

## INTRODUCTION

Integrins are cell surface glycoproteins composed of an α/β heterodimer that mediate cell-cell and cell-extracellular matrix (ECM) interactions. The pairing of particular α and β subunits confers specificity for a given ECM ligand. Integrin subunits have an extensive extracellular domain, a transmembrane domain and a relatively short cytoplasmic domain that can interact with the actin-based cytoskeleton in specialized structures called focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996; Hynes, 1992). The β1 subunit is a versatile member of the integrin family that can interact with at least ten different α subunits. Alternative RNA processing of the β1 subunit results in functionally different subunits with distinct cytoplasmic domains (Balzac et al., 1993; Belkin et al., 1997; Belkin et al., 1996; Cooper et al., 1991; DiPersio et al., 1995; Languino and Ruoslahti, 1992). The cytoplasmic domain of β1A (referred to here as β1) is the most common isoform and interacts with numerous proteins of the focal adhesion complex, including α actinin (Otey et al., 1990; Otey et al., 1993) and talin (Burn et al., 1988), as well as numerous signaling proteins, including FAK, Src, ILK, ICAP and adaptor proteins (Chang et al., 1997; Dedhar and Hannigan, 1996; Keely et al., 1998; LaFlamme et al., 1997; Yamada, 1997). The β1D isoform, for example, is found in cardiac and skeletal muscle, and forms more stable interactions with the cytoskeleton, leading to a more adherent, less motile, phenotype (Belkin et al., 1996).

Two regions of the β1 cytoplasmic domain appear to be key in mediating interactions with these focal adhesion proteins. The first is a membrane proximal region that has been implicated in FAK and paxillin associations and in stabilizing α/β interactions, presumably by creating a salt bridge between the aspartate residue (D759) and a conserved arginine in the α cytoplasmic tail. The second region contains two highly conserved NPXY domains that are present in all of the β subunits except β4 (Filardo et al., 1995; O'Toole et al., 1995; Reszka et al., 1992). This second region has been implicated in associations with a variety of proteins including α-actinin, FAK, talin and paxillin.

Many protein-protein interactions are modulated by reversible phosphorylation. The β1 integrin cytoplasmic domain contains five potential phosphorylation sites (Y783, Y795, S785, T788, T789) within a sequence of 16 amino acids (780-795) that includes the two highly conserved NPXY motifs. Several observations suggest that these phosphorylation sites are important for regulating the function of β1 integrins. First, tyrosine-phosphorylated β1 in Rous Sarcoma virus-transformed fibroblast cells localizes to podosomes, whereas the unphosphorylated form is found in focal adhesions (Johansson et al., 1994). Focal adhesion targeting is also inhibited by site directed substitutions at S785, Y783 and Y795, when these residues are mutated to mimic specific phosphorylation states (Barreuther and Grabel, 1996; Reszka et al., 1992). Conservative Y to F mutations at Y783 and Y795 result in wild-type levels of adhesion to ECM substrates and these proteins localize to focal adhesion sites but fail to mediate directed cell motility effectively (Sakai et al., 1998). Conservative T to A mutations at threonine residues 788 and 789 produce a β1 that cannot support fibronectin fibril formation or attachment to fibronectin substrates (Wennerberg et al., 1998). In addition, these T to A mutations result in an inactive, non-ligand-binding form of the extracellular domain, suggesting that

phosphorylation at these threonines may regulate inside-out signaling (Wennerberg et al., 1998).

Most of these studies are limited in that they only investigate the functional outcomes of conservative mutations from a residue that can be phosphorylated to one that cannot be phosphorylated. This approach will not detect a loss of function owing to phosphorylation. In other words, if an unphosphorylated residue supports β1 function and phosphorylation at this site does not, it would be missed. Therefore, in our investigations of the functional significance of S785, we included a site-directed substitution of an amino acid mimicking a constitutively phosphorylated form of the protein, which allows us more fully to reveal the functional significance of phosphorylation at this site.

As F9 cells differentiate into parietal endoderm in the presence of cyclic AMP plus retinoic acid, focal adhesions form and there is a concomitant dephosphorylation of the β1 cytoplasmic domain. Levels of β1 phosphorylation in parietal endoderm cells are increased twofold by treatment with the phosphatase inhibitor okadaic acid (Mulrooney et al., 2000). Phosphoamino acid analysis revealed that phosphorylation is on a serine residue and that there is only one serine residue, at amino acid 785 (Dahl and Grabel, 1989). These results led us to hypothesize that the dephosphorylation of serine 785 was required for focal adhesion localization. We tested this by expressing chicken β1 with site-directed substitutions in F9 cells and determining the subcellular localization of the integrin using an antibody specific for the chicken form of the protein (Barreuther and Grabel, 1996). When transfected F9 cells expressed chicken β1 with either a serine at position 785 (wildtype) or a methionine at this site, the integrin localized to focal adhesion sites. By contrast, β1 containing an aspartate at this site, which mimics a constitutively phosphorylated residue, failed to localize to the focal adhesion sites (Barreuther and Grabel, 1996). In this study, we were restricted from more completely examining the role of S785 in β1 function by the presence of wild-type endogenous β1. We now report two approaches that allow us to examine the role of S785 in multiple β1-supported functions. First, we have used a mouse-specific β1-function-blocking antibody to examine a number of β1-dependent behaviors in F9 cell lines expressing various forms of chicken β1. Second, we have expressed these mutant chicken β1 constructs in the β1-deficient mouse fibroblast cell line GD25. The data suggest that phosphorylation at S785 promotes adhesion but inhibits cell spreading and directed cell migration in both F9 and GD25 cells.

## MATERIALS AND METHODS

### Cell lines and reagents

Wild-type F9 teratocarcinoma cells were cultured as previously described (Dahl and Grabel, 1989). Briefly, F9 stem cells were induced to differentiate into parietal endoderm by supplementing the medium with 0.1 μM retinoic acid plus 0.25 mM dibutyrl cAMP (Sigma) for 5 days. The GD25-WTβ1 and the GD25 β1 null fibroblast cell lines were a generous gift from Reinhard Fassler. GD25-S785A was a generous gift from Deane Mosher. The mouse anti-chicken β1 monoclonal antibody (mAb) W1B10 was obtained from Sigma and rabbit anti-vinculin was obtained from Telios. The rat anti-mouse α6 mAb GOH3 was obtained from Immunotech. The mouse specific β1 antibody, CD29 HA2/5, was obtained from Research Diagnostics. Anti-mouse IgG FITC and anti-rabbit IgG rhodamine conjugates were obtained from Cappel. Strepavidin-horseradish peroxidase (HRP) was obtained from Amersham. Mouse anti-α-fodrin was obtained from ICN. Goat anti-mouse IgG-HRP was obtained from Transduction Laboratories and laminin-1 was obtained from Sigma.

### Transfection of GD25 with chicken β1

Three RSV-neo constructs encoding the sequence of chicken β1, (WT, S790M, S790D) were obtained from A. Horwitz. The β1 sequence was excised with *Eco*RI and *Xho*I and ligated into the same sites of the multiple cloning region of pCDNA3.1zeo (Invitrogen). The constructs were linearized and electroporated as previously described (Wennerberg et al., 1996) into GD25 fibroblast cells. Cells were selected for two weeks in the presence of 500 μg $ml^{-1}$ Zeocin (Invitrogen).

### FACS analysis

Zeocin resistant clones were removed from the dishes using calcium and magnesium free Hepes-Hanks Buffer (CMF-HH) supplemented with 0.02% EDTA, collected and resuspended in 500 μl CMF-HH containing 2% bovine serum albumin (BSA) (blocking buffer; BB). 20 μg of W1B10 (chicken β1 specific antibody) was added and incubated for 30 minutes at 37°C. Cells were centrifuged, washed in BB three times, resuspended in BB containing FITC-conjugated goat anti-mouse IgG (1:100) (Cappel Laboratories) and incubated for 30 minutes at 37°C. Cells were washed twice in BB and three times in CMF-HH, and resuspended in CMF-HH. Cells were then analyzed and sorted in a Becton-Dickson FACScan (Yale Flow Cytometry Center). Resulting cell populations were maintained in culture medium supplemented with 500 μg $ml^{-1}$ Zeocin.

### Immunoprecipitation

GD25 transfected and untransfected cells were washed in cold phosphate buffered saline (PBS) and the cell surface proteins labeled with 0.25 mg $ml^{-1}$ Sulfo-NHS-biotin (Pierce) for 40 minutes at room temperature. Cells were then washed once with serum free DMEM and three times with PBS. They were then lysed in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 1 mM $CaCl_2$, 0.5% sodium deoxycholate, 1% NP-40 and 2 mM PMSF in the presence of a protease inhibitor cocktail (10 μg $ml^{-1}$ leupeptin, 10 μg $ml^{-1}$ pepstatin, 10 μg $ml^{-1}$ aprotinin, 10 μg $ml^{-1}$ benzamidine) for 1 hour on ice. Lysates were precleared by centrifugation at 13,000g for 15 minutes. Samples were then incubated with 10 μg of anti-chicken β1 antibody (W1B10) or 10 μg of anti-mouse α6 antibody (GOH3) overnight at 4°C. The samples were then incubated with 50 μl protein G beads for 1 hour at 4°C with constant agitation. The bound material was then washed three times with precipitation buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1% NP-40) and once with 1 M Tris-buffered saline pH 7.4 (TBS). The unbound fraction (resulting supernatant) was reserved for use as a loading control. Samples were solubilized in 2× Laemmli buffer, boiled for 5 minutes and the proteins resolved by SDS -PAGE. Proteins resolved by SDS-PAGE were electrophoretically transferred to Immobilon-P (Millipore) and the membranes blocked for 1 hour at room temperature with TBS containing 0.03% Tween-20 (TBST) and 3% dry milk. The blots were incubated for 1 hour at room temperature with HRP-conjugated strepavidin diluted in TBST (1:1000). For the loading control, blots were incubated with mouse anti-α-fodrin (spectrin), using manufacturers specifications, for one hour at room temperature followed by five washes with TBST and then a 1 hour incubation with anti-mouse IgG-HRP (1:10,000) at room temperature. Blots were then processed by enhanced chemiluminescence, and visualized on Kodak XAR film.

### Immunofluorescence

GD25 transfected cells were fixed for 10 minutes in 3.7%

formaldehyde and then washed twice with PBS and permeabilized with 0.5% Triton-X100. Cells were then washed twice in PBS and blocked for 30 minutes at 37°C in PBS supplemented with 2% BSA. Cells were then washed with PBS/BSA and incubated for 1 hour at 37°C with 20 μg ml$^{-1}$ W1B10 and 20 μg ml$^{-1}$ rabbit anti-vinculin. Cells were then washed five times in PBS for 5 minutes. Cells were then incubated with FITC-conjugated goat anti-mouse IgG (1:100) or rhodamine-conjugated goat anti rabbit IgG (1:100) for 1 hour at room temperature followed by five 5-minute washes with PBS. Cells were washed, mounted and examined using a Nikon fluorescence microscope and images captured using a SPOT digital camera and Adobe Photoshop software.

### Cell attachment assay

96-well tissue culture plates were coated with 30 μg ml$^{-1}$ laminin overnight at room temperature. Control wells were coated with 2% BSA. The plates were then washed with PBS and blocked for 2 hours with 2% BSA at 37°C. Plates were then washed and then cells were added and allowed to attach for 1 hour as previously described (Stephens et al., 1993). After washing the wells the extent of attachment was determined by colorimetric detection at 595 nm using Bradford Assay Reagent (Pierce). For those experiments involving F9 cells, endogenous β1 function was inhibited by incubating the cells with the function-blocking antibody CD29 HA2/5 (Research Diagnostics) for 1 hour prior to seeding. The level of attachment of GD25 transfected cells was determined by subtracting the residual attachment to BSA from attachment to laminin. All samples were analyzed in quadruplicate.

### Cell spreading assay

24-well tissue culture plates were coated with 30 μg ml$^{-1}$ laminin-1 overnight at room temperature. Equal quantities of cells were seeded and the cells allowed to attach and spread for 4-6 hours. Cells were then washed with PBS and photographed. For the F9 spreading assays, cells were allowed to attach for 1 hour before 20 μg ml$^{-1}$ CD29 HA2/5 was added to the media. All samples were analyzed in triplicate.

### GD25 cell migration

Cell migration assays were performed as previously described (Sakai et al., 1998). Briefly, assays were performed in modified Boyden chambers containing Nucleopore polycarbonate membranes (5 μm pore size, Costar). Filters were coated overnight in 30 μg ml$^{-1}$ laminin. The filters were rinsed with PBS supplemented with 2% BSA and placed into the chambers. 500 μl PBS containing 2% BSA and 100 μM epidermal growth factor (EGF) (Upstate Biotechnology) was added to the chamber. Cells were suspended in PBS with 2% BSA and added to the upper chamber and then incubated overnight. The cells on the upper side of the filter were removed with a cotton swab to ensure that only those cells that had passed through the membrane were counted, and the filters were then washed with PBS and stained with Crystal Violet. Cells on the underside of the filter were then counted. All samples were analyzed in quadruplicate.

### F9 cell migration and parietal endoderm outgrowth assays

F9 parietal endoderm outgrowth assays were performed as previously described (Jiang and Grabel, 1995). Briefly, F9 cells were kept in suspension culture in the presence of 0.1 μM retinoic acid until mature visceral endoderm was present on the outer edge (9-11 days). Mature embryoid bodies were then plated on laminin-coated 33 mm tissue culture dishes and allowed to attach for 16 hours. After attachment, 20 μg ml$^{-1}$ CD29 HA2/5 was added to the cultures and the outgrowth photographed after 5 hours and 20 hours. The migration distance of the outgrowth for each embryoid body was measured in ten locations and the average migration distance used as one data point. A minimum of ten embryoid bodies from each cell line were used to quantify migration of parietal endoderm.

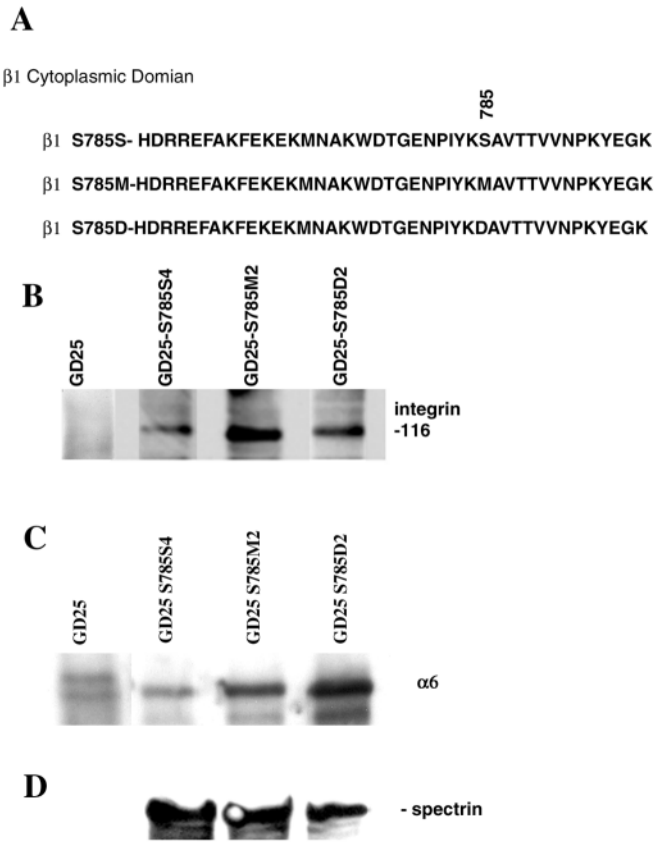


**Fig. 1.** Expression of chicken β1 in GD25 cells. Three constructs expressing chicken β1 with site directed substitutions at S785 of the cytoplasmic domain (A) were transfected into GD25 cells. Biotin surface labeled cells from clones expressing chicken S785S, S785M or S785D β1 were immunoprecipitated using anti-chicken-β1 antibody (W1B10) and the precipitates analyzed by SDS-PAGE followed by western blotting probed with streptavidin (B). Biotin surface labeled cells from clones expressing chicken S785S, S785M or S785D β1 were immunoprecipitated using anti-mouse-α6 antibody (GOH3) and the precipitates analyzed by SDS-PAGE followed by western blotting probed with streptavidin (C). As a loading control, supernatant fractions (see Methods) were analyzed for spectrin content by western analysis (D).

## RESULTS

### Generation of GD25 cell lines expressing chicken β1

To determine the functional significance of the phosphorylation state of serine 785 of the β1 integrin cytoplasmic domain, we transfected the β1 null fibroblast cell line GD25 with constructs encoding chicken β1 containing site directed substitutions (Fig. 1A). The constructs encode wild-type chicken β1 either with a serine (S785S) or a methionine (S785M) residue at position 785, mimicking a dephosphorylated residue, or with an aspartate at this site, mimicking a constitutively phosphorylated form of the protein (S785D) (Reszka et al., 1992). The successful transfection and expression of chicken β1 integrin was analyzed by flow cytometry (data not shown). To establish mutant cell lines, transfected cells were selected to have comparable mean fluorescent intensities after subtracting out the background fluorescent intensity from untransfected GD25 (mean fluorescent intensities for each cell

line: GD25-S785S4, 23.96; GD25-S785M2, 23.10; GD25-S785D2, 28.05). These data suggest that the total β1 expression levels in each cell line are relatively equal. To verify that each cell line was a homogenous population of β1 expressing cells, we calculated the proportion of cells expressing chicken β1 using indirect immunofluorescence for each of the transfected GD25 cell lines. These data suggest that 90-95% of transfected cells express the chicken β1 isoform (data not shown). We analyzed two cell lines expressing chicken β1 for each of our mutant constructs and observed no differences in β1 expression levels or behavior in the various assays between cell lines expressing the same construct. The subsequent results therefore show data obtained from one cell line for each amino acid substitution.

Cell lines were surface biotinylated and immunoprecipitated with either the anti-chicken-β1 antibody W1B10 or the anti-α6 antibody GOH3, followed by western-blot analysis using streptavidin-HRP. Each clone expresses chicken β1 at the cell surface (Fig. 1B). Densitometry tracings suggest that the expression of chicken β1 for each cell line differs by a factor of less than two (data not shown). Each clone expresses a relatively equivalent level of α6 (Fig. 1C), suggesting that each cell line expresses an α subunit capable of heterodimerizing with the chicken β1. To confirm that each lane was equally loaded for total protein, supernatant fractions (see Methods) from each precipitation were analyzed for the cytoplasmic protein spectrin. This analysis showed roughly equivalent levels of spectrin for each cell line (Fig. 1D). The cell lines transfected with chicken β1 presented in this study are called GD25-S785S4, GD25-S785M2 and GD25-S785D2. The F9 cell lines used in this study (F9-S785S, F9-S785M, and F9-S785D) have been previously described (Barreuther and Grabel, 1996).

## Role of S785 in cell attachment of F9 and GD25 cell lines

To address the functional role of the different chicken β1 proteins in F9 stem cells, the contribution of the endogenous mouse β1 was inhibited by the addition of CD29-HA2/5, a function-blocking antibody specific for mouse β1. In each experiment, the ability to attach to laminin was determined for cells expressing the different forms of the chicken β1 protein. (The role of the β1 subunit was assayed using laminin substrates, because attachment to laminin relies heavily if not totally upon the β1-containing integrin α6β1 (Jiang and Grabel, 1995; Wennerberg et al., 1996).) All F9 cell lines attached well to laminin in the absence of antibody (Fig. 2). Increasing concentrations of the CD29 HA2/5 antibody progressively inhibited attachment of untransfected F9 cells to laminin because the antibody selectively blocks the endogenous mouse β1. By contrast, F9-S785S cells demonstrate residual attachment in the presence of the antibody owing to expression of the chicken protein. F9-S785D cells can also attach to laminin in the presence of the antibody but F9-S785M cells cannot. Attachment of F9-S785S cells to laminin can be completely inhibited if the cells are incubated with both the mouse-specific CD29 HA2/5 and the chicken-β1-specific W1B10 (data not shown). This supports our conclusion that residual attachment observed in the presence of the antibody is due to the chicken β1. These experiments were repeated with F9-derived parietal endoderm from all cell lines and the data are consistent with those presented for stem cells in Fig. 2A (data not shown). These data suggest that mutations mimicking the dephosphorylated form of chicken β1 interfere with attachment to laminin.

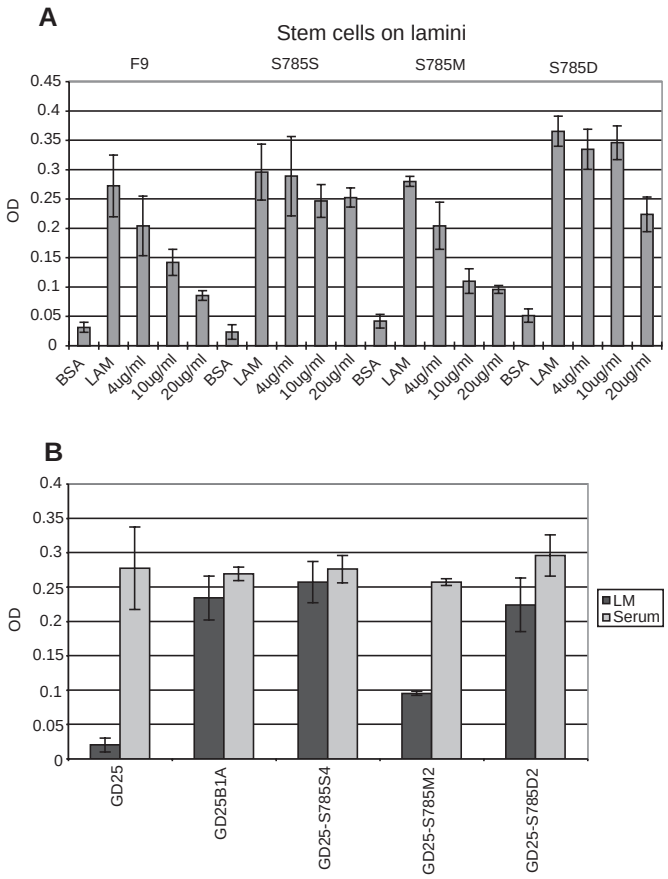


**Fig. 2.** Role of S785 in cell attachment. Attachment to laminin (LAM) was examined for F9 and GD25 cell lines expressing chicken β1. Untransfected F9 cells attachment is inhibited by increasing concentrations of CD29 HA2/5, a mouse-specific function-blocking β1 antibody. Both the F9-S785S and F9-S785D cell lines continue to attach in the presence of the antibody, whereas the F9-S785M cell line does not (A). Untransfected and transfected GD25 cells attach equally well to serum coated substrates (B). By contrast, only GD25β1A, GD25-S785S4 and GD25-S785D2 attach well to laminin (LM) (B). Error bars represent ±s.e. of quadruplicate experiments.

It has been previously shown that the parental GD25 cell line can attach to fibronectin but not to laminin (Sakai et al., 1998; Wennerberg et al., 1998). Here, we compared the ability of untransfected and transfected GD25 cell lines (GD25-S785S4, GD25-S785M2 and GD25-S785D2) to attach to serum- or laminin-coated substrates; serum-coated substrates are rich in fibronectin and vitronectin. In addition, we also examined the attachment behavior of GD25 cells transfected with wild-type mouse β1 (GD25-β1wt). To rule out nonspecific cell adhesion, the attachment attributable to 2% BSA alone was subtracted from attachment levels observed on serum and laminin substrates. The attachment level observed on serum-coated substrates was similar for all cell lines (Fig. 2B). However, consistent with the previous reports, the untransfected GD25 cells attach poorly to laminin. By contrast, both the GD25-

S785S and GD25-S785D cell lines, as well as the GD25-β1wt cell line, are able to attach to laminin. As described for F9-S785M cells, GD25-S785M2 cells attach poorly to laminin. The results presented here for the F9 and GD25 cell lines show that expression of S785S or S785D promotes cell attachment to laminin, whereas expression of a S785M does not.

### Role of serine 785 in cell spreading

For cells to spread on an ECM substrate, the integrin β subunit must bind the ECM as well as engage the actin-based cytoskeleton. To facilitate an examination of spreading and not attachment behavior, F9 cells were allowed to attach for 1 hour prior to addition of the antibody. In the absence of the antibody, all the F9 cells spread on laminin. We inhibited cell spreading of untransfected F9 cells with the addition of the CD29 HA2/5 antibody (Fig. 3A). Both the F9-S785S and F9-S785M cell lines had residual spreading activity in the presence of the antibody. Although expression of an aspartate at 785 allowed cells to attach (Fig. 2), these cells failed to spread and retained a rounded morphology in the presence of CD29HA2/5, as observed for the untransfected F9 cells (Fig. 3A). Spreading of F9-S785 cells could be completely inhibited by the addition of both CD29 HA2/5 and W1B10 antibodies (Fig. 3B). Similar results were obtained when these experiments were repeated using F9 parietal endoderm (data not shown).

As previously observed, untransfected GD25 cells fail to spread on laminin (Sakai et al., 1998) and retain a rounded morphology (Fig. 3C). As observed for the F9 cells, GD25 cells that express either S785S or S785M were able to spread on this substrate. In addition, we examined the ability of GD25-β1wt and GD25-S785A, a cell line that expresses a mutant form of mouse β1, to spread on laminin. Both of these cell lines spread on laminin. The extent of spreading for these two cell lines, which express mouse β1, appears to be greater than that observed in the corresponding cell lines expressing chicken β1 (Fig. 3C). This may be due to higher expression levels of the mouse protein in these cells. By contrast, GD25-S785D cells failed to spread on laminin. GD25-S785D cells were still unable to spread as effectively as wild-type or GD25-S785M cells after longer incubation times of up to 16 hours (data not shown). We repeated these experiments on fibronectin substrates. At earlier time points (1-3 hours), GD25-S785D cells fail to spread or spread more slowly than the other transfected cell lines. However, after longer incubation, the extents of spreading for untransfected GD25 cells and transfected GD25 cells, including GD25-S785D, are indistinguishable. The eventual cell spreading observed is presumably due to the action of non-β1-containing integrin heterodimers and/or alternative fibronectin receptors, because the GD25 parental line, which does not express β1, is able to spread on fibronectin, as previously observed (Fassler et al., 1995). These experiments suggest that β1 containing an aspartate residue at position 785 is unable to mediate the necessary interactions with the cytoskeleton involved in cell spreading.

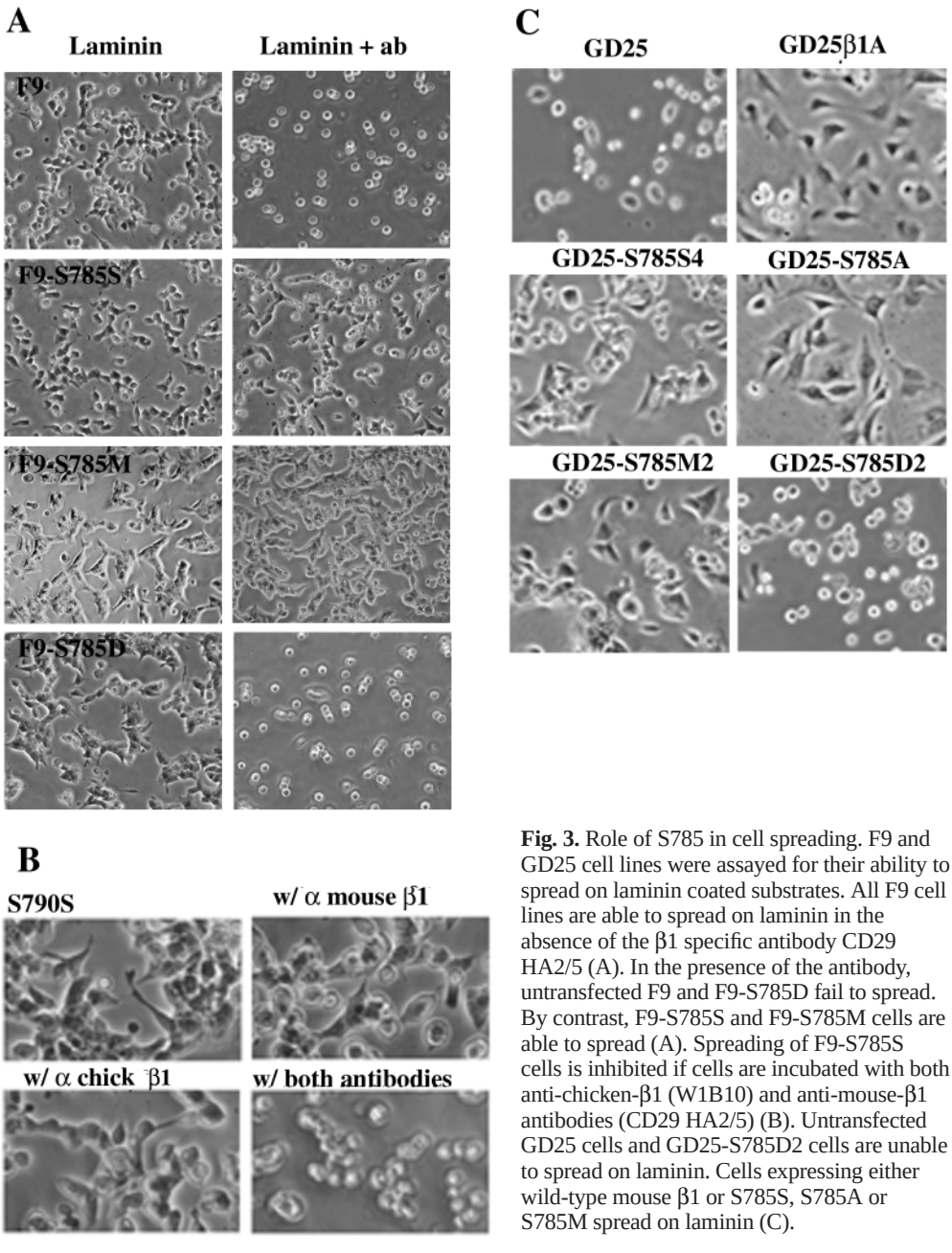


**Fig. 3.** Role of S785 in cell spreading. F9 and GD25 cell lines were assayed for their ability to spread on laminin coated substrates. All F9 cell lines are able to spread on laminin in the absence of the β1 specific antibody CD29 HA2/5 (A). In the presence of the antibody, untransfected F9 and F9-S785D fail to spread. By contrast, F9-S785S and F9-S785M cells are able to spread (A). Spreading of F9-S785S cells is inhibited if cells are incubated with both anti-chicken-β1 (W1B10) and anti-mouse-β1 antibodies (CD29 HA2/5) (B). Untransfected GD25 cells and GD25-S785D2 cells are unable to spread on laminin. Cells expressing either wild-type mouse β1 or S785S, S785A or S785M spread on laminin (C).

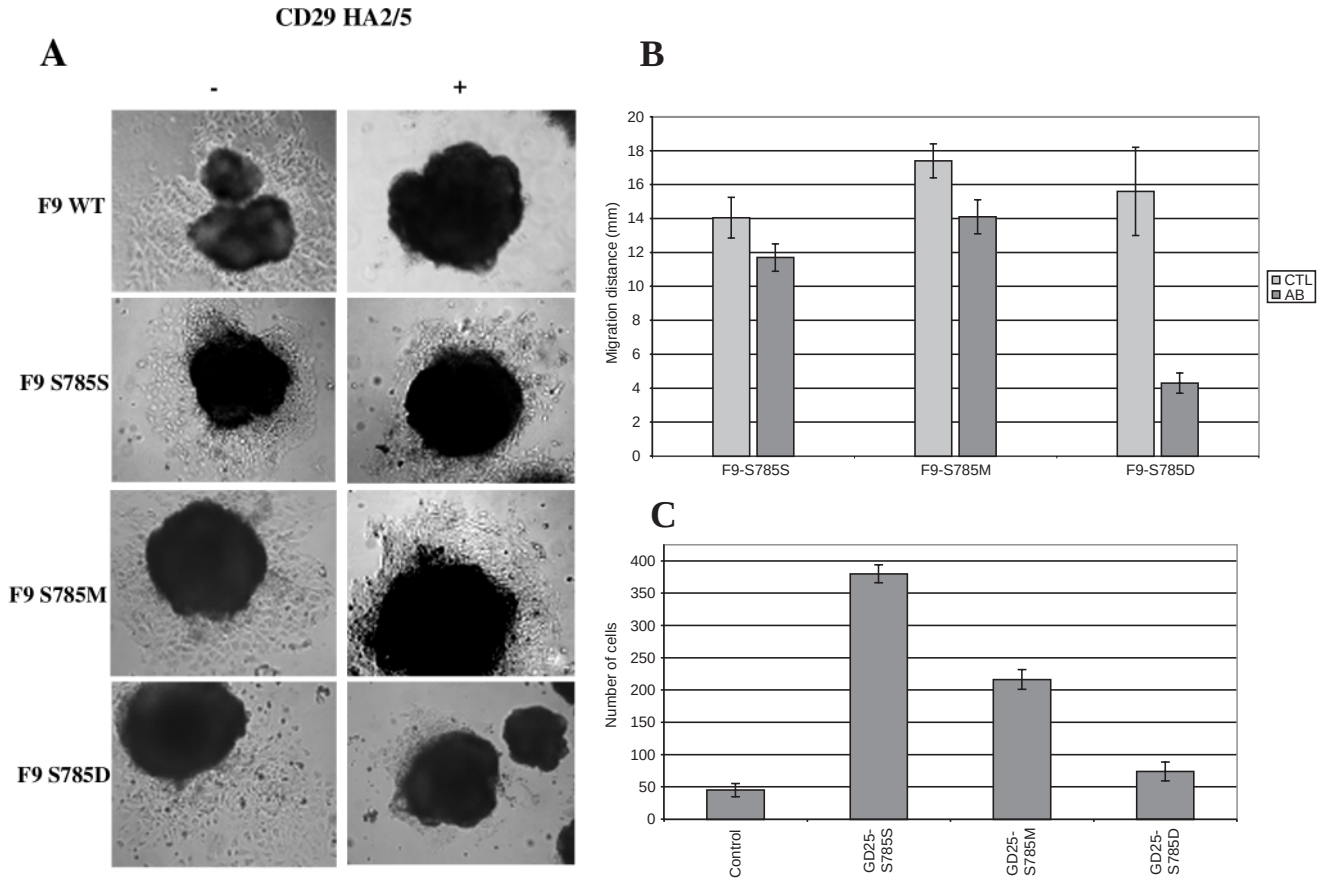


**Fig. 4.** Role of S785 in directed cell migration. F9 outgrowth and GD25 cell migration was examined on laminin coated substrates. Parietal endoderm outgrowth assays were used to measure the role of S785 in F9 cell migration (A). All cell lines produce extensive outgrowth when plated on laminin. F9-S785S and F9-S785M produce moderate to extensive outgrowth in the presence of the antibody CD29 HA2/5. Untransfected and F9-S785D cell outgrowth is inhibited. (B) Extent of outgrowth, for cells treated (AB) or untreated (CTL) with the β1 function-blocking antibody CD29 HA2/5, was quantified by measuring the distance from the edge of the embryoid body to the outermost edge of outgrowth at ten points for ten embryoid bodies. Error bars represent ±s.e. of triplicate experiments. (C) Boyden chamber assays were used to measure migration of GD25 cell lines. Error bars represent ±s.e. of quadruplicate experiments. An intermediate level of migration is seen with the GD25-S785M2 and significantly reduced migration with GD25-S785D2.

### Role of serine phosphorylation in F9 cell migration

To determine the role of serine phosphorylation in F9 cell migration, we used parietal endoderm outgrowth assays. Previous work in our laboratory has shown that migration on laminin is mediated by the α6β1 integrin (Jiang and Grabel, 1995). When mature embryoid bodies are plated on laminin in the presence of an antibody directed against α6β1 (GOH3), the extent of outgrowth is inhibited (Jiang and Grabel, 1995). To determine the effects of expressing β1 with different residues at position 785, we performed these outgrowth assays on laminin substrates in the presence of the β1 function-blocking antibody, CD29 HA2/5, using the transfected cell lines. Note that all the F9 cell lines produce outgrowth in the absence of the function blocking antibody (Fig. 4A). Embryoid bodies derived from untransfected controls as well as F9-S785D failed to produce substantial parietal endoderm outgrowth in the presence of the antibody. However, embryoid bodies from both F9-S785S and F9-S785M cells produced extensive outgrowth. We calculated the average distance of outgrowth migration at ten points for ten embryoid bodies from each cell line (see Methods) (Fig. 4B). In the presence of the antibody, embryoid bodies derived from both the F9-S785S and F9-S785M display somewhat decreased migration distances than their untreated controls, although the difference is not statistically significant. By contrast, the migration of parietal endoderm from F9-S785D derived embryoid bodies is inhibited by 66% in the presence of the antibody. In further support for a role for β1 phosphorylation in parietal endoderm migration, we have shown that parietal endoderm from untransfected F9 embryoid bodies show a marked decrease in migration distance when cultured in the presence of okadaic acid, a treatment that promotes a twofold increase in β1 phosphorylation levels (Barreuther and Grabel, 1996) (data not shown).

### Role of serine phosphorylation in GD25 cell migration

GD25-S785D cells are able to attach to but not to spread on laminin, which suggests an inability of β1 to associate with the cytoskeleton. Thus, we reasoned that these cells might be deficient in cell migration. We therefore assayed the ability of the GD25 transfected cells to migrate using a modified Boyden chamber assay. Untransfected GD25 cells or one of the

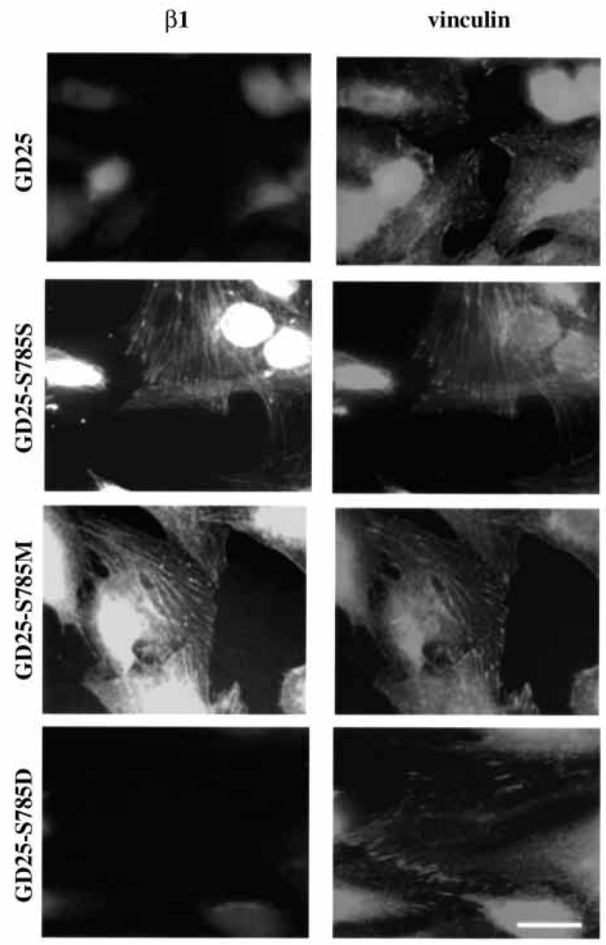


**Fig. 5.** Localization of β1 in GD25 cells expressing site directed substitutions at S785. Cells were plated on fibronectin and double labeled with antibodies directed against vinculin and chicken β1. There is no β1 in untransfected GD25 cells. β1 does not localize to focal adhesion sites in GD25-S785D2 cells. By contrast, β1 in both GD25-S785S4 and GD25-S785M2 cells colocalizes with vinculin to these structures.

transfected GD25 cell lines were placed in the upper well of the chamber, separated by a laminin-coated nucleopore filter from the lower chamber, which contained EGF to direct the migration. The ability of the different cell lines to migrate was determined by counting those cells that had passed through the filter after 16 hours. The untransfected GD25 cells migrate poorly through the filter (Fig. 4C), whereas the GD25-S785S cells had relatively high levels of migration. By contrast, GD25-S785D cell migration was markedly reduced, with levels similar to untransfected controls. GD25-S785M cells were able to migrate through the filter, although to a lesser extent than wild-type transfectants. These results, along with the data from the F9 outgrowth assays, suggest that expressing an aspartate in place of the serine at position 785 inhibits cell migration.

### Localization of chicken β1 with site substituted mutations

We have previously shown that β1 from F9-S785D cells failed to localize to focal adhesion sites, whereas β1 containing a serine or methionine residue at this site was able to localize to the focal adhesion (Barreuther and Grabel, 1996). We have extended those studies to determine whether these amino acid substitutions have the same effect on β1 localization in GD25 cells. We examined the localization of the chicken β1 protein in the transfected GD25 cell lines using indirect immunofluorescence (Fig. 5). To facilitate focal adhesion formation by all the cell lines, cells were plated on fibronectin because untransfected GD25 cells can assemble these structures on fibronectin using other integrins, such as αVβ3 (Fassler et al., 1995; Wennerberg et al., 1996). The presence of β1 containing focal adhesions was assayed by double labeling for vinculin and β1. As previously reported for the transfected F9 cell lines, we show that the chicken β1 with an aspartate substitution fails to localize to the focal adhesion, whereas wildtype or methionine-substituted β1 colocalizes with vinculin to focal adhesions in these cells. Taken together with our previous F9 work, these data suggest dephosphorylation at S785 is important for the placement of β1 in focal adhesion sites.

## DISCUSSION

The cytoplasmic domain of the β1 integrin is involved in many important functions. Specific regions within the domain are necessary for ligand binding, targeting of the integrin and cytoplasmic proteins to focal adhesions, protein-protein interactions and for integrin mediated signaling (Burridge and Chrzanowska-Wodnicka, 1996; Dedhar and Hannigan, 1996; LaFlamme et al., 1997; Schwartz et al., 1995). Many studies have focused on the key role of the 16 amino acid stretch that includes both NPXY domains and the five phosphorylatable residues. Most of these studies have introduced conservative mutations, resulting in subunits that behave similarly to the wildtype for most integrin functions examined. In this report, we demonstrate, using site directed mutagenesis, that expressing both an unphosphorylated (S to M) and a phosphorylated (S to D) mimic in place of serine can modify integrin function. Specifically, we show that expressing a residue that mimics a dephosphorylated form of the protein promotes cell spreading and directed cell migration, whereas a residue mimicking a phosphorylated form of the protein promotes attachment but inhibits cell spreading or migration.

The results from our attachment and spreading assays were unexpected, in that distinct phenotypes resulted from the same mutation. Expression of an aspartate at 785, mimicking a phosphorylated residue, results in attachment levels similar to or better than wild-type S785 in both the F9 and GD25 cell lines, whereas the same mutation was unable to support spreading or migration. By contrast, expressing a methionine at this site results in reduced attachment levels but can support spreading and migration. This result is less surprising when taken in the context of previous work, which suggests that integrin-mediated attachment and spreading are two separable behaviors that can be distinguished by whether or not the integrin links to the cytoskeleton (Tawil et al., 1993). Integrins may mediate attachment without subsequent cytoskeletal linkage. This is exemplified by the fact that α and β isoforms can have identical extracellular domains, which facilitate attachment, but alternate cytoplasmic domains, which may or may not effectively associate with the actin-based

cytoskeleton. For example, after attachment the β1A isoform is found predominantly in focal adhesions, whereas the β1C isoform mediates attachment but does not localize to an adhesion site, suggesting an inability to connect to the cytoskeleton (Fornaro and Languino, 1997). The α6A and α6B isoforms both promote attachment to laminin but only the α6A isoform makes stable interactions with the cytoskeleton and can promote cell migration (Jiang and Grabel, 1995; Shaw et al., 1995). Our data suggest that phosphorylation, in addition to isoform switching, can regulate the ability of the integrin subunit to interact with the cytoskeleton.

The reduced attachment levels observed for the cells expressing chicken β1 with S785M may be attributable to a conformational change in the integrin subunit, resulting in reduced ligand binding. Integrins with similar mutations introduced at the two threonine residues (T788, 789A) display reduced attachment but can promote focal adhesion formation and FAK activation, two processes requiring cytoskeletal interactions. It was proposed that this mutation leads to a shift in conformation of the extracellular domain to a non-ligand-binding form of the protein (Wennerberg et al., 1998).

The ability of a cell to spread or migrate is dependent on the association of the integrin cytoplasmic domain with the actin-based cytoskeleton. It has been suggested that migrating cells must make and subsequently break contacts between the ECM and the plasma membrane (Sheetz et al., 1998). At the leading edge of the cell, extensions or lamellipodia elongate on the substrate and integrins make contacts with the underlying ECM (Regen and Horwitz, 1992; Schmidt et al., 1993). A contractile force is then generated from both ends of the cell towards the nucleus as these integrins engage the ECM, as a result of their linkage to the cytoskeleton (Felsenfeld et al., 1996). Receptor release at the rear of the cell causes forward movement (Palecek et al., 1998; Palecek et al., 1996). The appearance of shed whole integrin molecules in the tracks of migrating cells suggests that this release occurs at the integrin-cytoskeleton link (Schmidt et al., 1993). We recently proposed a model for migration in which phosphorylation modulates the integrin-cytoskeleton interactions. In a dephosphorylated form, the integrin interacts with the actin-based cytoskeleton and focal adhesion proteins to create a focal adhesion site. We further proposed that phosphorylation at S785 results in the breaking of this integrin-cytoskeleton link. Migration is promoted, in part, by the dynamic regulation of the phosphorylation state of S785, which regulates the integrin actin connection.

In support of our model, we now show that expression of S785D in both F9 and GD25 cell lines inhibits migration, whereas the expression of S785M results in intermediate level of migration in the GD25 cell line. The intermediate levels of migration observed for a substitution of phenylalanine, mimicking a dephosphorylated residue, for either of the two tyrosine (Y783, Y795) residues (Sakai et al., 1998) is consistent with our results. If the ability of a cell to migrate is modulated by subsequent changes in the phosphorylation state of the integrin, one might expect inhibitory effects from a methionine substitution, a mutation that could enhance or strengthen integrin-cytoskeleton interactions. In our studies with the transfected cell line GD25-S785M2, we show an intermediate level of migration, between those observed for GD25-S785S4 and GD25-S785D2. This form of the integrin can make the necessary contacts with the cytoskeleton and spread, but the breaking of the integrin-cytoskeleton link might be inhibited. If this is the case, how do GD25-S785M2 cells migrate? The fact that they can suggests that alternate mechanisms exist for disengaging the integrin from the cytoskeleton. It has been proposed that proteolytic cleavage of integrin extracellular domains may be involved in migration (Alfandari et al., 1997). Alternatively, the integrin-cytoskeleton interaction might be broken by the removal of an actin-binding protein.

Additional support for an alternate mechanism for disengaging the integrin from the cytoskeleton comes from our observation that expression of S785M in F9 cells results in levels of migration similar to wild-type cells. The difference between the ability of GD25-S785M2 and F9-S785M cells to migrate may be attributable to the differences between the two assays. Although both assays measure directed migration, the Boyden chamber assays are more complex, in that the cells must migrate through a filter towards a chemotactic agent. By contrast, parietal endoderm outgrowth assays only measure the ability of the cells to migrate away from the embryoid body. The distinction between these assays is supported by work investigating the chemotactic versus chemokinetic responses by Y to F mutations in the cytoplasmic domain of β1. In these experiments, the Y to F mutation was more inhibitory in chemotactic assays than in chemokinetic assays (Sakai et al., 1998).

Our data supporting a role for S785 phosphorylation suggest that the regulation of phosphorylation at this site is important for β1-dependent functions. The F9 system provides an excellent model for examining the significance of phosphorylation of the β1 integrin subunit, because phosphorylation of S785 is developmentally regulated (Dahl and Grabel, 1989). In an effort to determine the kinase and phosphatase that regulate this phosphorylation event, we have identified a candidate kinase (ILK) and phosphatase (PP2A), both of which colocalize and coimmunoprecipitate with β1 in F9 cells (Mulrooney et al., 2000).

How might phosphorylation modulate β1 function? One possibility is that phosphorylation causes a conformational change of the cytoplasmic tail, which results in altered protein-protein interactions. Previous work designed to elucidate the structure of the β3 cytoplasmic domain suggests that the two NPXY domains result in β reverse turns, forming a hairpin loop (Haas and Plow, 1997). The sequence homology between the cytoplasmic domains of β1 and β3 suggests that they are structurally similar. The serine, two tyrosine and two threonine residues are located within this loop formed by the two NPXY domains. The introduction of a phosphate group on one of these residues could change the secondary structure of the cytoplasmic tail. This change could, in turn, expose or hide interaction domains necessary for cytoskeletal linkage or integrin activation. Interestingly, these NPXY motifs correspond with the binding sites for many focal adhesion and signaling proteins (Dedhar and Hannigan, 1996; LaFlamme et al., 1997). Support for this notion comes from investigations of a naturally occurring serine-to-proline mutation in the β3 tail. This proline substitution, known to introduce dramatic changes in conformation, results in inhibited platelet activation (Chen et al., 1992). The results presented here are consistent with this model in that the distinct phenotypes that are attributable to altered phosphorylation states appear to be related to whether or not the integrin can engage the cytoskeleton.

## REFERENCES


**Alfandari, D., Wolfsberg, T. G., White, J. M. and DeSimone, D. W.** (1997). ADAM 13: a novel ADAM expressed in somitic mesoderm and neural crest cells during *Xenopus laevis* development. *Dev. Biol*. **182**, 314-330.

**Balzac, F., Belkin, A. M., Koteliansky, V. E., Balabanov, Y. V., Altruda, F., Silengo, L. and Tarone, G.** (1993). Expression and functional analysis of a cytoplasmic domain variant of the beta 1 integrin subunit. *J. Cell Biol*. **121**, 171-178.

**Barreuther, M. F. and Grabel, L. B.** (1996). The role of phosphorylation in modulating beta 1 integrin localization. *Exp. Cell Res*. **222**, 10-15.

**Belkin, A. M., Zhidkova, N. I., Balzac, F., Altruda, F., Tomatis, D., Maier, A., Tarone, G., Koteliansky, V. E. and Burridge, K.** (1996). Beta 1D integrin displaces the beta 1A isoform in striated muscles: localization at junctional structures and signaling potential in nonmuscle cells. *J. Cell Biol.* **132**, 211-226.

**Belkin, A. M., Retta, S. F., Pletjushkina, O. Y., Balzac, F., Silengo, L., Fassler, R., Koteliansky, V. E., Burridge, K. and Tarone, G.** (1997). Muscle beta1D integrin reinforces the cytoskeleton-matrix link: modulation of integrin adhesive function by alternative splicing. *J. Cell Biol.* **139**, 1583-1595.

**Burn, P., Kupfer, A. and Singer, S. J.** (1988). Dynamic membrane-cytoskeletal interactions: specific association of integrin and talin arises in vivo after phorbol ester treatment of peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA* **85**, 497-501.

**Burridge, K. and Chrzanowska-Wodnicka, M.** (1996). Focal adhesions, contractility, and signaling. *Annu. Rev. Cell. Dev. Biol*. **12**, 463-518.

**Chang, D. D., Wong, C., Smith, H. and Liu, J.** (1997). ICAP-1, a novel beta1 integrin cytoplasmic domain-associated protein, binds to a conserved and functionally important NPXY sequence motif of beta1 integrin. *J. Cell Biol.* **138**, 1149-1157.

**Chen, Y. P., Djaffar, I., Pidard, D., Steiner, B., Cieutat, A. M., Caen, J. P. and Rosa, J. P.** (1992). Ser-752→Pro mutation in the cytoplasmic domain of integrin beta 3 subunit and defective activation of platelet integrin alpha IIb beta 3 (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. *Proc. Natl. Acad. Sci. USA* **89**, 10169-10173.

**Cooper, H. M., Tamura, R. N. and Quaranta, V.** (1991). The major laminin receptor of mouse embryonic stem cells is a novel isoform of the alpha 6 beta 1 integrin. *J. Cell Biol.* **115**, 843-850.

**Dahl, S. C. and Grabel, L. B.** (1989). Integrin phosphorylation is modulated during the differentiation of F-9 teratocarcinoma stem cells. *J. Cell Biol.* **108**, 183-190.

**Dedhar, S. and Hannigan, G. E.** (1996). Integrin cytoplasmic interactions and bidirectional transmembrane signalling. *Curr. Opin. Cell. Biol*. **8**, 657-669.

**DiPersio, C. M., Shah, S. and Hynes, R. O.** (1995). Alpha 3A beta 1 integrin localizes to focal contacts in response to diverse extracellular matrix proteins. *J. Cell Sci.* **108**, 2321-2336.

**Fassler, R., Pfaff, M., Murphy, J., Noegel, A. A., Johansson, S., Timpl, R. and Albrecht, R.** (1995). Lack of beta 1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. *J. Cell Biol.* **128**, 979-988.

**Felsenfeld, D. P., Choquet, D. and Sheetz, M. P.** (1996). Ligand binding regulates the directed movement of beta1 integrins on fibroblasts. *Nature* **383**, 438-440.

**Filardo, E. J., Brooks, P. C., Deming, S. L., Damsky, C. and Cheresh, D. A.** (1995). Requirement of the NPXY motif in the integrin beta 3 subunit cytoplasmic tail for melanoma cell migration in vitro and in vivo. *J. Cell Biol.* **130**, 441-450.

**Fornaro, M. and Languino, L. R.** (1997). Alternatively spliced variants: a new view of the integrin cytoplasmic domain. *Matrix Biol.* **16**, 185-193.

**Haas, T. A. and Plow, E. F.** (1997). Development of a structural model for the cytoplasmic domain of an integrin. *Protein Eng.* **10**, 1395-1405.

**Hynes, R. O.** (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

**Jiang, R. and Grabel, L. B.** (1995). Function and differential regulation of the alpha 6 integrin isoforms during parietal endoderm differentiation. *Exp. Cell Res.* **217**, 195-204.

**Johansson, M. W., Larsson, E., Luning, B., Pasquale, E. B. and Ruoslahti, E.** (1994). Altered localization and cytoplasmic domain-binding properties of tyrosine-phosphorylated beta 1 integrin. *J. Cell Biol.* **126**, 1299-1309.

**Keely, P., Parise, L. and Juliano, R.** (1998). Integrins and GTPases in tumour cell growth, motility and invasion. *Trends Cell Biol.* **8**, 101-106.

**LaFlamme, S. E., Homan, S. M., Bodeau, A. L. and Mastrangelo, A. M.** (1997). Integrin cytoplasmic domains as connectors to the cell's signal transduction apparatus. *Matrix Biol.* **16**, 153-163.

**Languino, L. R. and Ruoslahti, E.** (1992). An alternative form of the integrin beta 1 subunit with a variant cytoplasmic domain. *J. Biol. Chem*. **267**, 7116-7120.

**Mulrooney, J., Foley, K., Vineberg, S., Barreuther, M. and Grabel, L.** (2000). Phosphorylation of the beta1 integrin cytoplasmic domain: toward an understanding of function and mechanism. *Exp. Cell Res*. **258**, 332-341.

**Otey, C. A., Pavalko, F. M. and Burridge, K.** (1990). An interaction between alpha-actinin and the beta 1 integrin subunit in vitro. *J. Cell Biol*. **111**, 721-729.

**Otey, C. A., Vasquez, G. B., Burridge, K. and Erickson, B. W.** (1993). Mapping of the alpha-actinin binding site within the beta 1 integrin cytoplasmic domain. *J. Biol. Chem*. **268**, 21193-21197.

**O'Toole, T. E., Ylanne, J. and Culley, B. M.** (1995). Regulation of integrin affinity states through an NPXY motif in the beta subunit cytoplasmic domain. *J. Biol. Chem*. **270**, 8553-8558.

**Palecek, S. P., Schmidt, C. E., Lauffenburger, D. A. and Horwitz, A. F.** (1996). Integrin dynamics on the tail region of migrating fibroblasts. *J. Cell Sci.* **109**, 941-952.

**Palecek, S., Huttenlocher, A., Horwitz, A. and Lauffenburger, D.** (1998). Physical and biochemical regulation of integrin release during rear detachment of migrating cells. *J. Cell Sci.* **111**, 929-940.

**Regen, C. M. and Horwitz, A. F.** (1992). Dynamics of beta 1 integrin-mediated adhesive contacts in motile fibroblasts. *J. Cell Biol.* **119**, 1347-1359.

**Reszka, A. A., Hayashi, Y. and Horwitz, A. F.** (1992). Identification of amino acid sequences in the integrin beta 1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* **117**, 1321-1330.

**Sakai, T., Zhang, Q., Fassler, R. and Mosher, D. F.** (1998). Modulation of beta1A integrin functions by tyrosine residues in the beta1 cytoplasmic domain. *J. Cell Biol.* **141**, 527-538.

**Schmidt, C. E., Horwitz, A. F., Lauffenburger, D. A. and Sheetz, M. P.** (1993). Integrin-cytoskeletal interactions in migrating fibroblasts are dynamic, asymmetric, and regulated. *J. Cell Biol.* **123**, 977-991.

**Schwartz, M. A., Schaller, M. D. and Ginsberg, M. H.** (1995). Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell. Dev. Biol*. **11**, 549-599.

**Shaw, L. M., Turner, C. E. and Mercurio, A. M.** (1995). The alpha 6A beta 1 and alpha 6B beta 1 integrin variants signal differences in the tyrosine phosphorylation of paxillin and other proteins. *J. Biol. Chem*. **270**, 23648-23652.

**Sheetz, M. P., Felsenfeld, D. P. and Galbraith, C. G.** (1998). Cell migration: regulation of force on extracellular matrix-integrin complexes. *Trends Cell Biol.* **8**, 51-54.

**Stephens, L. E., Sonne, J. E., Fitzgerald, M. L. and Damsky, C. H.** (1993). Targeted deletion of beta 1 integrins in F9 embryonal carcinoma cells affects morphological differentiation but not tissue-specific gene expression. *J. Cell Biol.* **123**, 1607-1620.

**Tawil, N., Wilson, P. and Carbonetto, S.** (1993). Integrins in point contacts mediate cell spreading: factors that regulate integrin accumulation in point contacts vs. focal contacts. *J. Cell Biol.* **120**, 261-271.

**Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S. and Fassler, R.** (1996). Beta 1 integrin-dependent and -independent polymerization of fibronectin. *J. Cell Biol.* **132**, 227-238.

**Wennerberg, K., Fassler, R., Warmegard, B. and Johansson, S.** (1998). Mutational analysis of the potential phosphorylation sites in the cytoplasmic domain of integrin beta1A. *J. Cell Sci.* **111**, 1117-11126.

**Yamada, K. M.** (1997). Integrin signaling. *Matrix Biol.* **16**, 137-141.