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Selective modulation of type 1 insulin-like growth factor receptor signaling and functions by β_1 integrins

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We show here that β_1 integrins selectively modulate insulin-like growth factor type I receptor (IGF-IR) signaling in response to IGF stimulation. The β_{1A} integrin forms a complex with the IGF-IR and insulin receptor substrate-1 (IRS-1); this complex does not promote IGF-I mediated cell adhesion to laminin (LN), although it does support IGF-mediated cell proliferation. In contrast, β_{1C}, an integrin cytoplasmic variant, increases cell adhesion to LN in response to IGF-I and its down-regulation by a ribozyme prevents IGF-mediated adhesion to LN. Moreover, β_{1C} completely prevents IGF-mediated cell proliferation and tumor growth by inhibiting IGF-IR auto-phosphorylation in response to IGF-I stimulation. Evidence is provided that the β_1 cytodomain plays an important role in mediating β_1 integrin association with either IRS-1 or Grb2-associated binder1 (Gab1)/SH2-containing protein-tyrosine phosphate 2 (Shp2), downstream effectors of IGF-IR: specifically, β_{1A} associates with IRS-1 and β_{1C} with Gab1/Shp2. This study unravels a novel mechanism mediated by the integrin cytoplasmic domain that differentially regulates cell adhesion to LN and cell proliferation in response to IGF.

Introduction

Neoplastic prostate growth and metastasis are regulated by interactions between cells and the surrounding ECM proteins (Parise et al., 2000). Members of the laminin (LN) family are the first ECM proteins synthesized during embryonic development (Ekblom et al., 2003). LN-1, composed of $\alpha_1\beta_1\gamma_1$ subunits, is an important component of the epithelial basement membranes, and contributes to epithelial cell adhesion and polarization (Malinda and Kleinman, 1996). LN-1 plays an important role in regulating the functions of normal and malignant human prostate epithelial cells (Bello-DeOcampo et al., 2001). LN interactions with several cells are mediated by integrins (Mercurio, 1995). Integrins are heterodimeric receptors consisting of an α and β subunit mediating cell–cell and cell–ECM interactions (Hynes, 2002). Members of the β_1 subfamily and the $\alpha_6\beta_4$ integrin have been reported to function as LN receptors and to play a role in tumor growth (Juliano, 1993; Mercurio, 1995; Colognato and Yurchenco, 2000).

The integrin cytoplasmic domains modulate several signal transduction pathways (Fornaro and Languino, 1997). The cytoplasmic domain of β_1 exists in five different spliced forms and is known in its most widely expressed form, i.e., β_{1A}, to modulate cell proliferation and migration as well as receptor localization (Fornaro and Languino, 1997). The β_{1C} integrin, an alternatively spliced variant of the β_1 subfamily that contains a unique 48–amino acid sequence in its cytoplasmic domain inhibits normal and cancer cell proliferation (Fornaro and Languino, 1997). This cytoplasmic variant is expressed in nonproliferative, differentiated epithelium and is selectively down-regulated in prostate and breast carcinoma (Fornaro and Languino, 1997; Manzotti et al., 2000).

Insulin-like growth factors (IGFs) and the IGF type I receptor (IGF-IR) are important modulators of growth and differentiation, play a crucial role in the establishment and maintenance of the transformed phenotype (Baserga, 2000),

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Abbreviations used in this paper: β-gal, β-galactosidase; FN, fibronectin; Gab1, Grb2-associated binder1; IGF, insulin-like growth factor; IGF-IR, IGF type 1 receptor; IRS-1, insulin receptor substrate-1; LN, laminin; PI 3-kinase, phosphatidylinositol 3-kinase; RZ, ribozyme; Shp2, SH2-containing protein-tyrosine phosphatase 2; SRB, Sulforhodamine B; tet, tetracycline.

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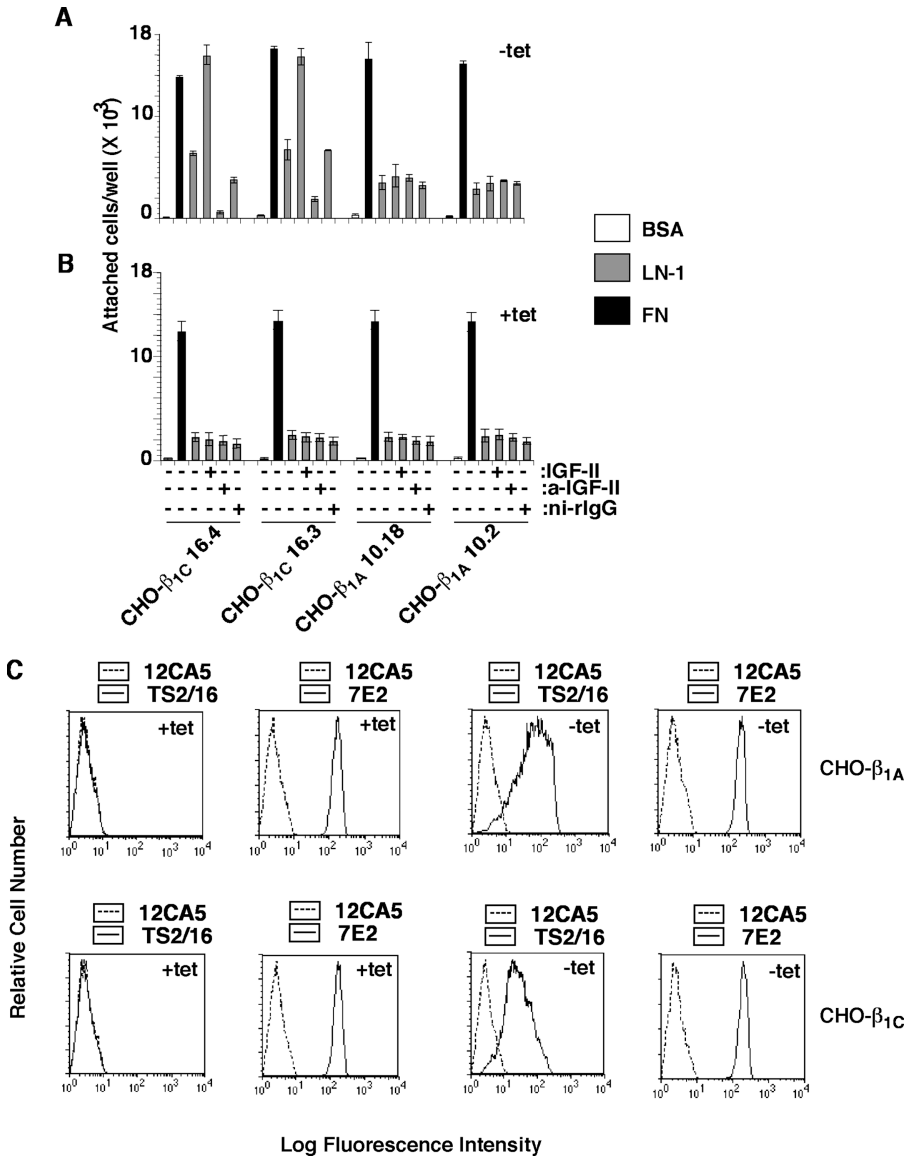

Figure 1. **IGF stimulates adhesion to LN-1 of β_{1C}-expressing cells.** β_{1C}-CHO (clones 16.3 and 16.4) and β_{1A}-CHO (clones 10.18 and 10.2) clones were cultured in the absence (A) or in the presence (B) of tet for 48 h. Cells were labeled using ^{51}Cr-sodium chromate for 1 h at 37°C. ^{51}Cr-labeled cells were incubated in the presence or in the absence of purified rabbit Ab to IGF-II or ni-rIgG for 1 h on ice. Cells were plated on BSA, LN-1, or FN at 37°C for 2 h in the presence or in the absence of IGF-II. Attached cells were washed, lysed, and the amount of ^{51}Cr associated with the attached cells was measured by liquid scintillation counting. Data are expressed as mean ± SEM. (C) Representative β_{1C}-CHO and β_{1A}-CHO clones were cultured in the absence or in the presence of tet for 48 h and analyzed by FACS® using a β_1 integrin Ab TS2/16 specific to human β_1 or 7E2 specific to hamster β_1 or, as negative control, 12CA5.

and are potential targets for anticancer treatment (Surmacz, 2003). The IGF axis appears to contribute to prostate cancer progression, but the mechanisms responsible for this process have not been studied (Reiss et al., 1998). IGF-I and IGF-II bind to IGF-IR and stimulate several signaling pathways including phosphatidylinositol 3-kinase (PI 3-kinase) and MAPK (Valentinis and Baserga, 2001; LeRoith and Roberts, 2003); both pathways are also known to be activated by integrin engagement (Damsky and Ilic, 2002). IGF-I induces association of β_1 integrins and IGF-IR and increases adhesion of myeloma cells to fibronectin (FN; Tai et al., 2003). It has also been shown to increase adhesion of breast cancer cells to LN and this increase is inhibited by α-IR3, an antibody to the IGF-IR (Dunn et al., 1998).

The present work unravels a novel mechanism that regulates cell adhesion to LN-1 in response to IGF without affecting cell proliferation or tumor growth and is mediated by the association of β_1 integrins with IGF-IR and with IGF-IR downstream effectors.

Results

IGFs stimulate adhesion to LN-1 of β_{1C}-, but not of β_{1A}-expressing cells

To investigate the effect of β_1 integrins on IGF-IR–mediated functions, we used CHO and PC3 cells expressing either β_{1A} or β_{1C} integrin cytoplasmic variants under the control of a tetracycline (tet)-regulated promoter (Fornaro et al., 2000, 2003). As shown in Fig. 1 (A and B), addition of IGF-II caused a eightfold increase in adhesion to LN-1 of CHO cell transfectants expressing β_{1C} (−tet) compared with cells that did not express β_{1C} (+tet). IGF-II had no effect on cell adhesion to FN. Preincubation of cells with Ab to IGF-II prevented IGF-II mediated increased adhesion to LN-1 in β_{1C} expressing cells (Fig. 1 A). In contrast, IGF-II had no effect on cell adhesion in the presence of exogenously expressed β_{1A} integrin (Fig. 1, A and B). Comparable levels of surface expression of exogenous β_{1A} and β_{1C} evaluated using an mAb to human β_1, TS2/16, were consistently observed (Fig. 1 C). Flow cytometry (FACS®) analysis performed us-

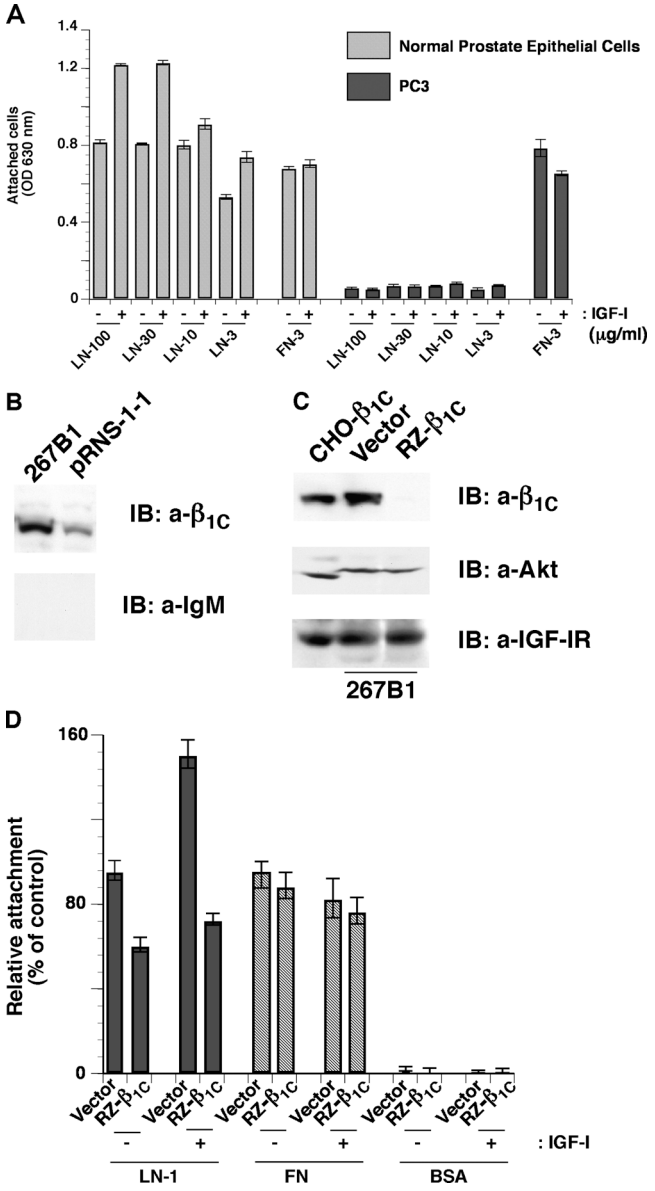

Figure 2. **Down-regulation of β_{1C} expression in 267B1 cells inhibits IGF-I–stimulated cell adhesion to LN-1.** (A) PrEC and PC3 cells were serum starved for 12 h, detached, and plated on LN-1 or FN at 37°C for 2 h in the presence or in the absence of IGF-I. Cell adhesion was analyzed by crystal violet staining. (B) pRNS-1-1 and 267B1 cells were lysed and immunoblotted with mAb to β_{1C} or normal IgM. (C) 267B1 cells were transiently transfected with pBJ1-RZ-β_{1C} or vector alone, lysed, and immunoblotted with mAb to β_{1C}, Ab to Akt, or Ab to IGF-IR. CHO-β_{1C} cell lysate was used as a positive control. (D) 267B1 cells were transiently transfected with pBJ1-RZ-β_{1C} or vector alone were detached and seeded on BSA or LN-1 or FN-coated plates at 37°C for 2 h in the presence or in the absence of IGF-I and stained with β-gal. Cell attachment was expressed as percentage of cells transfected with vector and attached to LN-1 in the absence of IGF-I, set at 100. Data are expressed as mean ± SEM. The experiments were repeated at least twice with similar results.

ing mAb 7E2 (Brown and Juliano, 1985) revealed comparable levels of expression of endogenous β_1 integrin in the β_{1A} and β_{1C} transfectants (Fig. 1 C), thus suggesting that the observed effect on cell adhesion to LN-1 was not due to changes in endogenous β_1 integrin levels.

Because our previous studies have shown that the β_{1C} variant is down-regulated in prostate carcinoma (Fornaro et al., 1999; Moro et al., 2004), we analyzed the ability of normal or neoplastic prostate cells to adhere to LN-1 in response to IGF-I. IGF-I stimulated adhesion to LN-1 of normal human prostate epithelial cells (PrEC), but did not increase adhesion to LN-1 of human prostate cancer, PC3, cells (Fig. 2 A). To further confirm that IGF-stimulated cell adhesion to LN-1 is mediated by β_{1C} integrin in prostate epithelial cells, we used 267B1, a nontumorigenic prostate epithelial cell line, that expresses β_{1C} (Fig. 2 B) and adheres to LN-1 (not depicted). We down-regulated the expression of β_{1C} in 267B1 cells using a ribozyme (RZ) specific for β_{1C} (RZ-β_{1C}; Fig. 2 C). The RZ specifically inhibited β_{1C} expression but did not have any effect on the levels of other proteins, such as Akt or IGF-IR (Fig. 2 C). As shown in Fig. 2 D, down-regulation of β_{1C} integrin completely prevented IGF-I stimulated cell adhesion to LN-1, but did not have any effect on cell adhesion to FN.

Re-expression of β_{1C} integrin in PC3 cells caused a significant increase in cell adhesion to LN-1 in the presence of IGF-II (Fig. 3 A) and IGF-I (Fig. 3 C). In contrast, IGF-II had no effect on cell attachment to LN-1 in β_{1A} expressing cells (Fig. 3 B). Similarly, IGF-I caused an increase in cell adhesion to LN-1 in the presence of β_{1C}, but not of β_{1A} integrin (Fig. 3 C). Because both IGF-I and IGF-II bind IGF-IR (Baserga, 2000) and gave comparable results (not depicted), they were used interchangeably. Cell adhesion to LN-1 stimulated by IGF-I was inhibited by an Ab to LN-1 (Fig. 3 D) and no differences between β_{1C} and β_{1A} expressing cells were observed when the cells attached to FN (Fig. 3, A–D). In conclusion, cells expressing elevated levels of β_{1C} show a small, enhanced adhesion to LN-1, which is significantly stimulated in the presence of IGF-I or IGF-II. In contrast, cells expressing β_{1A} retain the low baseline level of adhesion to LN-1, and their adhesion is not responsive to IGFs.

The $\alpha_6\beta_1$ integrin is the major LN-1 receptor and inhibition of $\alpha_6\beta_1$ functions prevents acinar morphogenesis in prostate epithelial cells (Cooper et al., 1991; Shaw and Mercurio, 1993; Mercurio, 1995; Bello-DeOcampo et al., 2001). As shown in Fig. 3 E, addition of an inhibitory Ab to α_6 (GoH3; Sonnenberg et al., 1987) prevented IGF-I mediated increased cell adhesion to LN-1 in PC3-β_{1C} transfectants. Neither GoH3 nor normal rat IgG (rtIgG) inhibited cell adhesion to FN (Fig. 3 F). Comparable levels of surface expression of exogenous β_{1A} or β_{1C} in PC3 cells were consistently observed in all experiments after tet removal (Fig. 3 G). As shown in Fig. 3 G, induction of exogenous β_{1A} or β_{1C} expression did not change the levels of endogenous α_6 integrin as analyzed by FACS® using GoH3. In conclusion, the α_6 integrin is the predominant receptor that mediates IGF-stimulated cell adhesion to LN-1 of β_{1C} expressing cells.

Expression of β_{1C} inhibits IGF-stimulated cell proliferation and tumor growth

We investigated whether β_1 integrins could interfere with PC3 cell proliferative response to IGFs. We found that both IGF-I and IGF-II increased PC3 cell proliferation in the presence of β_{1A}, whereas expression of β_{1C} prevented IGF-I or

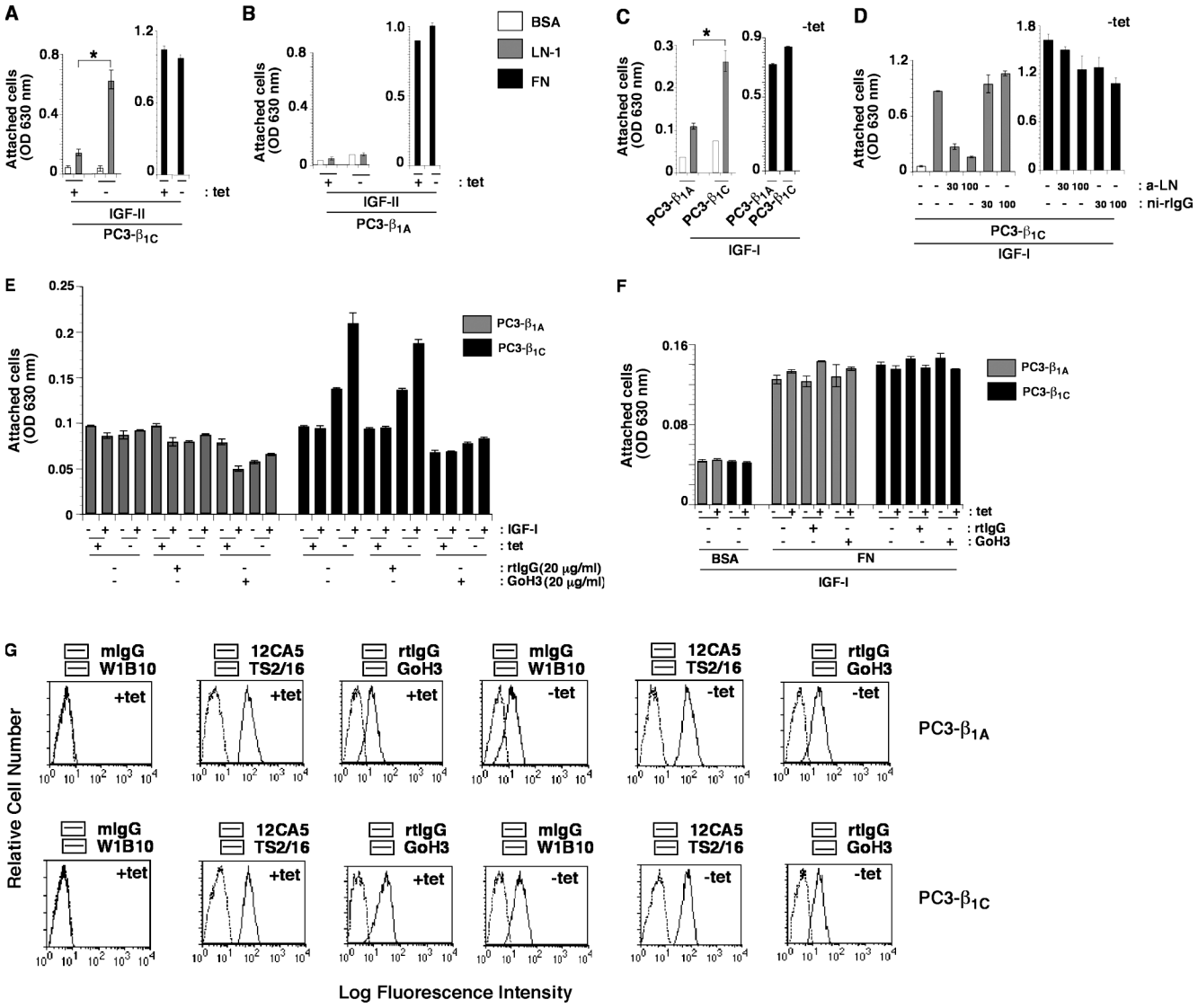

Figure 3. **β_{1C} enhances IGF-I– and II–mediated PC3 cell adhesion to LN-1.** (A–D) β_{1A}- and β_{1C}-PC3 clones were cultured in the presence or in the absence of tet for 48 h. Cells were detached and plated on BSA or LN-1 or FN at 37°C for 2 h in the presence of IGF-II (A and B), of IGF-I (C and D), or of Ab to LN-1 (D) or ni-rIgG (D). In A, the differences in cell adhesion to LN-1 in the presence or in the absence of tet are statistically significant (*$P \leq 0.03$). In C, the differences in cell adhesion to LN-1 between β_{1A} and β_{1C} expressing cells are statistically significant (*$P \leq 0.024$). The experiments were repeated at least three times with similar results using two clones each of β_{1A}- and β_{1C}-PC3 cells. (E and F) β_{1A}- and β_{1C}-PC3 clones were cultured in the presence or in the absence of tet for 48 h. Cells were serum starved for 12 h, detached, and plated on LN-1 (E), FN or BSA (F) in serum-free medium at 37°C for 2 h in the presence or in the absence of IGF-I or GoH3 or rtIgG. Cell adhesion was analyzed by crystal violet staining. Data are expressed as mean ± SEM. (G) Surface expression of endogenous or exogenous β_{1A} or β_{1C} integrin or endogenous α_6 integrin was analyzed in PC3 cells by FACS® using Ab to human β_1, TS2/16, chicken β_1, W1B10, α_6, GoH3; or, as negative controls, mIgG, rtIgG, or 12CA5.

IGF-II stimulation of cell proliferation (Fig. 4 A). Based on the finding that β_1 integrin is essential for tumor growth (Bloch et al., 1997) and IGF-IR promotes transformation (Baserga, 2000), we hypothesized that β_{1C} would prevent tumor growth given its ability to inhibit IGF-stimulated cell proliferation. Because prostate cancer cells form tumors in an IGF-IR–dependent manner when injected subcutaneously in nude mice (Burfeind et al., 1996; Reiss et al., 1998), we injected PC3 cell transfectants expressing either β_{1A} or β_{1C} and followed tumor growth. Tumor formation by PC3-β_{1A} cells was not affected by the presence or absence of tet in the drinking water and there were no differences in tumor formation and growth at all the examined time points (Fig. 4 B). The tumors that formed in the tet-deprived animals injected with PC3-β_{1C} cells were significantly smaller in size compared with those in animals given tet (Fig. 4 C). PC3-β_{1C} cells showed tumor formation at significantly later time points indicating that the cells were alive (not depicted). These data show that β_{1C} significantly reduced prostate cancer cell proliferation and tumor growth, in vivo.

IGF-IR and β_{1C} act synergistically to support cell adhesion to LN-1

To analyze the role of IGF-IR in mediating cell adhesion to LN-1, we used R− cells derived from mouse embryos with a targeted disruption of the IGF-IR gene, and R+ cells are ob-

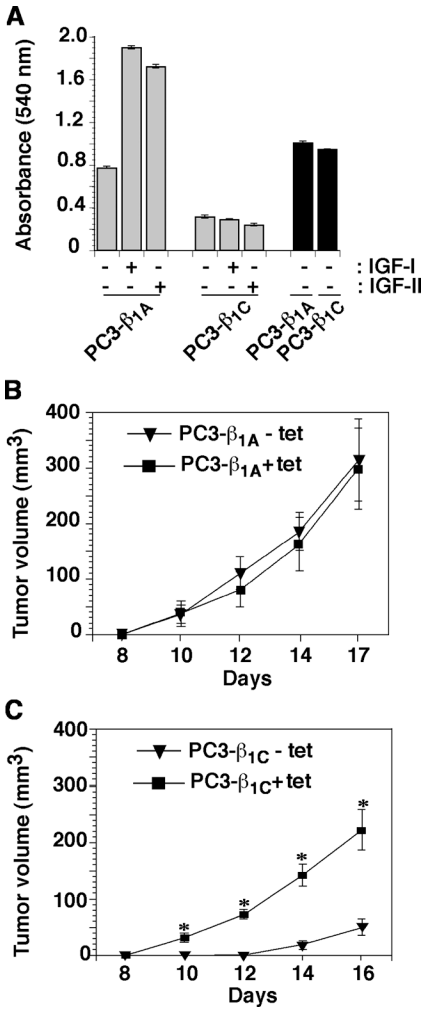

Figure 4. **β_{1A} and β_{1B} differentially affect IGF-mediated cell proliferation and tumor growth.** (A) β_{1A}- and β_{1C}-PC3 clones were cultured in the absence of tet for 48 h. Cells were plated on a 96-well plate in serum-free medium with or without IGF-I or IGF-II at 37°C. After 72 h of incubation, cell proliferation was analyzed using SRB. As control, sulforhodamine B (SRB) incorporation of cells attached to FN-coated substrates was measured in parallel (black bars). (B and C) PC3-β_{1A} (B) and PC3-β_{1C} (C) clones were injected subcutaneously in athymic male Balb/c mice. Mice were given water supplemented with either 5% sucrose to induce β_{1A} or β_{1C} expression, or 5% sucrose plus 100 μg/ml tet. The graphs show kinetics of tumor growth. Tumor growth is expressed as tumor volume in cubic millimeters. Data are the mean ± SEM of 10 animals per group. *$P \leq 0.001$ at 10, 12, 14, and either 16 or 17 d from injection. These experiments were repeated three times with similar results.

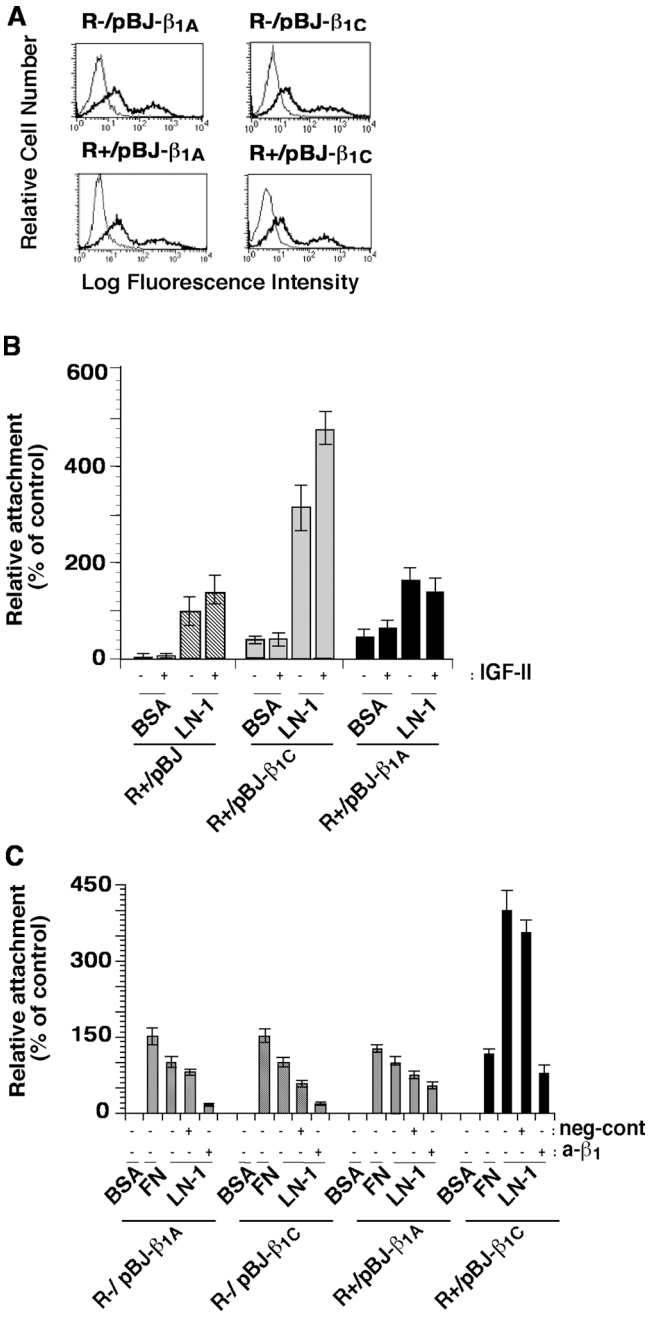

Figure 5. **IGF-IR and β_{1C} act synergistically to support cell adhesion to LN-1.** (A) R− and R+ cells were transiently transfected with human β_{1A} or β_{1C}. Surface expression of β_{1A} or β_{1C} was analyzed by FACS® using TS2/16 or 12CA5 as a negative control. Thick line, TS2/16; thin line, 12CA5. (B) R+ cells transiently transfected with β_{1A} or β_{1C} were detached and seeded on BSA or LN-1–coated plates at 37°C for 2 h in the presence or in the absence of IGF-II and stained with β-gal. (C) R− and R+ cells (10^6) transiently transfected with β_{1A} or β_{1C} were incubated with or without P4C10 (a-β_1) or 1C10 (neg-cont) on ice for 1 h. Cells were plated on BSA or LN-1 or FN at 37°C for 2 h and stained with β-gal. Attachment of cells transfected with β_1-integrin cDNA was expressed as percentage of cells transfected with pBJ (B) or pBJ-β_{1A} (C) that were attached to LN-1, set at 100. The experiments were repeated at least twice with similar results. Data are expressed as mean ± SEM.

tained by transfection of IGF-IR in R− cells (Sell et al., 1994). R− and R+ cells were transiently transfected using human β_{1A} or β_{1C} integrin cDNA. The expression levels of the transfected integrins were analyzed by FACS® and found to be comparable (Fig. 5 A). Expression of β_{1C}, but not of β_{1A} integrin in R+ cells significantly increased cell adhesion to LN-1 and this increase was further stimulated by IGF-II (Fig. 5 B). Transfection of β_{1C} integrin in R− cells showed no increase in cell adhesion to LN-1, suggesting that β_{1C} integrin alone is unable to bind to LN-1 (Fig. 5 C). Comparing cell adhesion of R− and R+ cells to LN-1 and FN, revealed that these cells adhere equally well to LN-1 and FN (not depicted). This excludes the possibility that IGF-IR alone is sufficient to stimulate cell adhesion to LN-1. These results show that neither β_{1A} alone, nor β_{1C} alone, nor IGF-

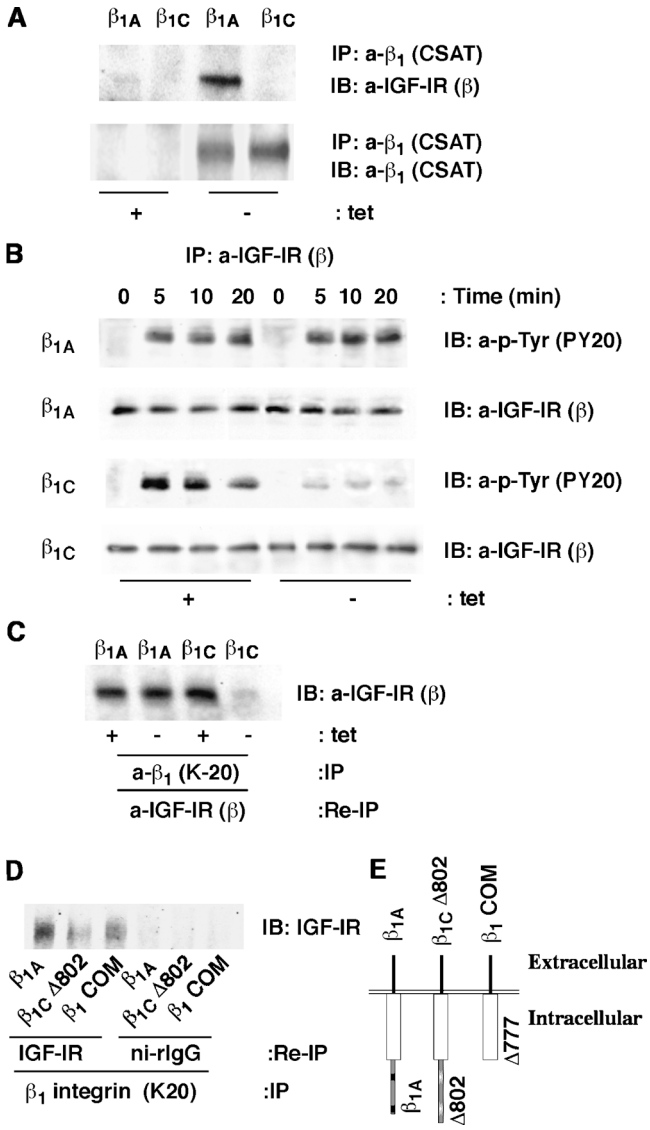

Figure 6. **β_{1C} inhibits IGF-IR tyrosine phosphorylation and β_{1A} association with IGF-IR.** β_{1A}- and β_{1C}-PC3 clones were cultured in the presence or in the absence of tet for 48 h, stimulated with IGF-I for 10 min at 37°C, lysed. Exogenous β_1 was immunoprecipitated using CSAT (A) and endogenous β_1 using K-20 (C). In A, immunoblotting (IB) was performed using CSAT or Ab to IGF-IR-β. (B) Cells were incubated with or without IGF-I for 0, 5, 10, and 20 min in suspension at 37°C. Immunoprecipitation was performed using Ab to IGF-IR-β. Immunoprecipitates were separated on SDS-PAGE and immunoblotted with PY20 or Ab to IGF-IR-β. In C, the endogenous β_1 immunoprecipitates were reprecipitated and immunoblotted with Ab to IGF-IR-β. (D) CHO clones expressing the full-length β_{1A} integrin or β_1COM or $\beta_{1C}\Delta802$ truncated mutant were cultured for 48 h. Cells were lysed and proteins were immunoprecipitated with K-20. The immunocomplexes were dissociated and proteins were reimmunoprecipitated with Ab to IGF-IR or ni-rIgG. The immunoprecipitates were separated using SDS-PAGE and immunoblotted with Ab to IGF-IR. (E) Schematic representation of β_{1C} cytoplasmic domain deletion mutants. The experiments were repeated at least twice with similar results.

IR alone, nor IGF-IR and β_{1A} support cell adhesion to LN-1, and that the synergistic activity of both IGF-IR and β_{1C} is required to support cell adhesion to LN-1.

β_{1C} decreases IGF-stimulated tyrosine phosphorylation of IGF-IR and β_{1A}–IGF-IR association

Because β_1 integrins have been shown to associate with IGF-IR (Tai et al., 2003), we investigated whether β_{1A} or β_{1C} would differentially associate with IGF-IR using PC3 transfectants. The results showed that β_{1A}, but not β_{1C} associates with IGF-IR (Fig. 6 A). IGF-IR belongs to the family of receptor tyrosine kinases that undergo tyrosine phosphorylation upon ligand binding (LeRoith and Roberts, 2003). IGF-I increased IGF-IR tyrosine phosphorylation in β_{1A} expressing PC3 cells, whereas expression of β_{1C} significantly prevented IGF-stimulated tyrosine phosphorylation of IGF-IR resulting in inhibition of association between β_{1A} and IGF-IR (Fig. 6, B and C).

To analyze whether the β_{1C} integrin unique 48–amino acid cytoplasmic domain has an inhibitory effect on IGF-IR–β_{1A} association, we used CHO transfectants expressing full-length β_{1A} integrin or the β_1COM mutant, that preserves the sequence shared by β_{1A} and β_{1C} integrins or the $\beta_{1C}\Delta802$ truncated mutant shown in Fig. 6 E. As expected, expression of full-length β_{1A} integrin showed a strong association with IGF-IR (Fig. 6 D). The β_1COM mutant retained the property to associate with IGF-IR showing that the common sequence between β_{1A} and β_{1C} integrin is only partially responsible for this association. The Δ802 significantly inhibited the association with IGF-IR, showing that the 778–802 domain mediates the β_{1C} inhibitory effect on the association between IGF-IR and β_{1A}. The results suggest an important role of the β_1 cytoplasmic domain in the regulation of IGF-IR tyrosine phosphorylation and of β_{1A}–GF-IR association.

β_{1A} associates with and supports tyrosine phosphorylation of IRS-1, whereas β_{1C} associates with and supports tyrosine phosphorylation of Gab1 and Shp2

Upon tyrosine phosphorylation, IGF-IR becomes the docking site for SH2 domain-containing proteins (LeRoith and Roberts, 2003), such as insulin receptor substrate-1 (IRS-1). IRS-1 is known to mediate several functions, predominantly proliferation and transformation, stimulated by the IGF-IR (Surmacz, 2003). Grb2-associated binder1 (Gab1) is another downstream effector of IGF-IR, as well as of the insulin receptor, and shares functional and structural homology with IRS-1 (Winnay et al., 2000; Gu and Neel, 2003). To investigate whether the β_1 cytoplasmic domain would affect the downstream signaling proteins via direct association with β_{1A} or β_{1C}, we used CHO transfectants, where β_{1A} was found to be associated with the IGF-IR (Fig. 7 A, top). Our results showed that IRS-1 associates with β_{1A} and that β_{1C} associates with Gab1 (Fig. 7 B). Cell transfectants expressed similar levels of exogenous β_1 integrins (Fig. 7 A, bottom), IRS-1, or IGF-IR (not depicted).

To study the effect on IRS-1 or Gab1 tyrosine phosphorylation of the differential association of β_{1A} and β_{1C} with IRS-1 or Gab1 respectively, we used CHO transfectants. As shown in Fig. 7 C, IGF-I increased the tyrosine phosphorylation of IRS-1 in β_{1A} expressing CHO cells, whereas expression of β_{1C} significantly prevented IGF-stimulated tyrosine phosphorylation of IRS-1. In contrast, we found that expres-

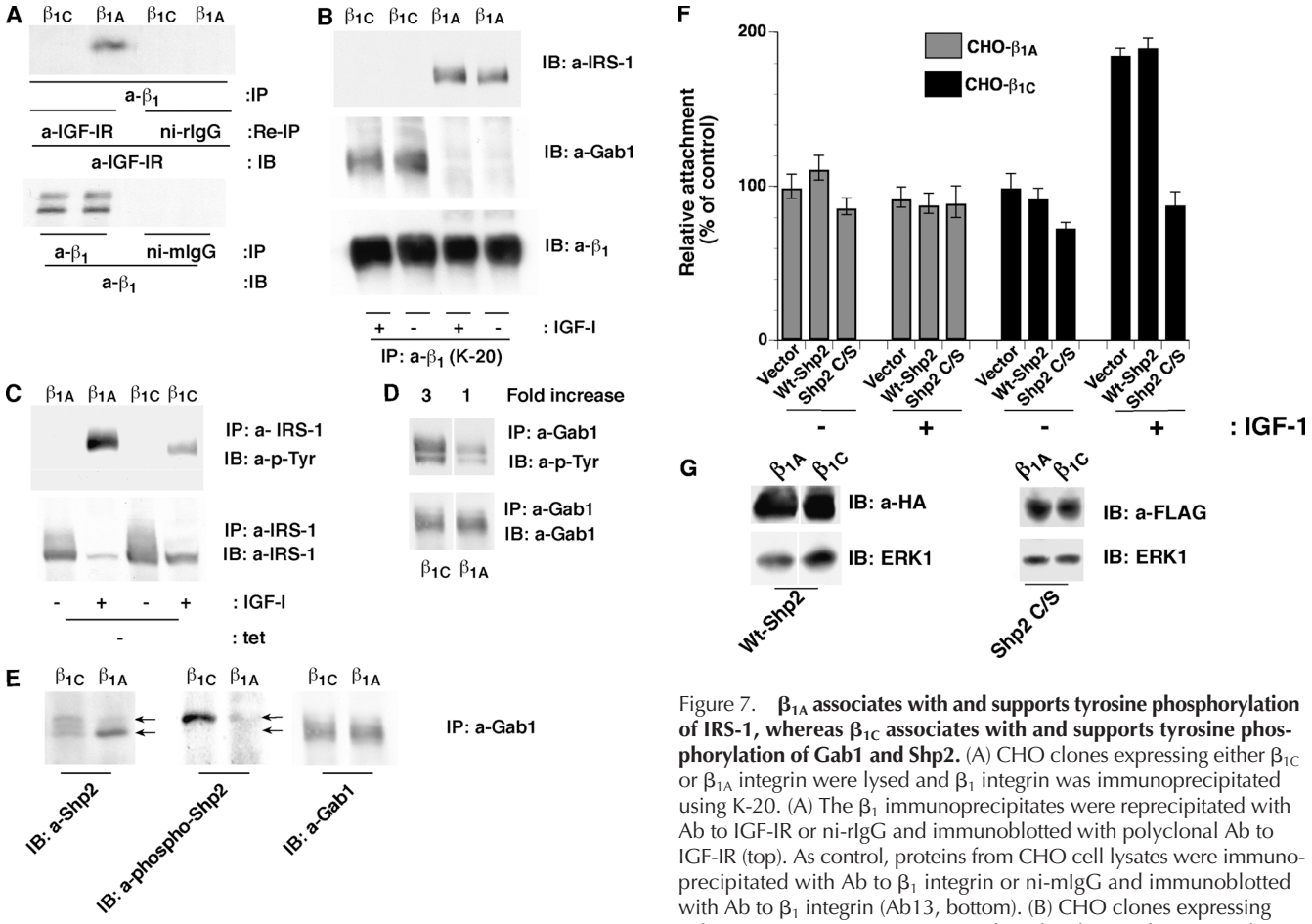

Figure 7. **β_{1A} associates with and supports tyrosine phosphorylation of IRS-1, whereas β_{1C} associates with and supports tyrosine phosphorylation of Gab1 and Shp2.** (A) CHO clones expressing either β_{1C} or β_{1A} integrin were lysed and β_1 integrin was immunoprecipitated using K-20. (A) The β_1 immunoprecipitates were reprecipitated with Ab to IGF-IR or ni-rIgG and immunoblotted with polyclonal Ab to IGF-IR (top). As control, proteins from CHO cell lysates were immunoprecipitated with Ab to β_1 integrin or ni-mIgG and immunoblotted with Ab to β_1 integrin (Ab13, bottom). (B) CHO clones expressing either β_{1C} or β_{1A} integrin were incubated with or without IGF-I for 10 min, lysed, and proteins were immunoprecipitated with Ab to β_1 integrins (K-20) and immunoblotted with Ab to IRS-1 (top), Gab1 (middle), or β_1 integrin (bottom). (C–E) CHO clones expressing either β_{1C} or β_{1A} integrin were incubated with or without IGF-I for 10 min, lysed, and proteins were immunoprecipitated with Ab to IRS-1 (C) or Gab1 (D and E). Immunoprecipitates were separated using SDS-PAGE and immunoblotted with Ab to p-Tyr (PY20) (C and D), IRS-1 (C), Gab1 (D and E), Shp2 (E), or phospho-Shp2 (E). (F) β_{1A}- and β_{1C}-CHO clones were transiently transfected with wt-Shp2 or Shp2 C/S or vector alone. Cells were then cultured in the absence of tet for 48 h, plated on LN-1 and incubated for 2 h at 37°C in the presence or in the absence of IGF-I. After incubation, cells were fixed and stained with β-gal. Attachment of cells transfected with wt-Shp2 or Shp2 C/S cDNA was expressed as percentage of the number of attached cells transfected with vector alone, set at 100 in the absence of IGF-I. Data are expressed as mean ± SEM. (G) β_{1A}- and β_{1C}-CHO clones were transiently transfected with either HA-tagged wt-Shp2 or FLAG-tagged Shp2 C/S or vector alone. ERK1 was used as loading control. Cells were lysed after 48 h and immunoblotted with Ab to HA for wt-Shp2 or Ab to FLAG for Shp2 C/S. The experiments were repeated at least three times with similar results. White lines indicate that intervening lanes have been spliced out.

sion of β_{1C} significantly increased tyrosine phosphorylation of Gab1 (Fig. 7 D). Gab1, upon tyrosine phosphorylation, recruits and activates SH2-containing protein-tyrosine phosphate 2 (Shp2) phosphatase that causes dephosphorylation of Shp2 substrates including IGF-IR (Neel et al., 2003). In β_{1C}, but not in β_{1A} expressing cells, Gab1-associated Shp2 showed increased tyrosine phosphorylation levels (Tyr542) as evaluated by mobility shift assay and using a phospho-Ab specific to Shp2 (Fig. 7 E).

To explore the role of Shp2 in IGF-I mediated cell adhesion to LN-1, CHO cells expressing β_{1A} or β_{1C} were transiently transfected with either HA-tagged wt-Shp2 or FLAG-tagged Shp2 C/S, a dominant negative form of Shp2 (Zhang et al., 2002). As shown in Fig. 7 F, enhanced cell adhesion to LN-1 in the presence of IGF-I in β_{1C} expressing CHO cells was completely prevented by transfection of Shp2 C/S, as compared with vector alone or wt-Shp2. The expression levels of the transfected wt-Shp2 or Shp2 C/S cDNAs were analyzed in cell lysates and found comparable in β_{1A} and β_{1C} expressing cells (Fig. 7 G).

Overall, these data show that β_{1A} and β_{1C} differentially associate with IRS-1 and Gab1 and that Shp2 activation is required for cell adhesion to LN in response to IGFs.

PI 3-kinase mediates IGF-stimulated adhesion to LN-1

PI 3-kinase is a downstream effector that mediates IGF-IR as well as integrin-stimulated signaling; PI 3-kinase activation requires Shp2 in some pathways activated by receptor-tyrosine kinases such as IGF-IR and PDGF receptor, but not by others such as EGF receptor (Reiss et al., 2001; Neel et al., 2003; Tai et al., 2003). We investigated the role of PI 3-kinase in cell adhesion stimulated by IGF-II in cell transfectants expressing β_{1C} integrin. We transiently transfected CHO clones expressing β_{1C} integrin with dominant nega-

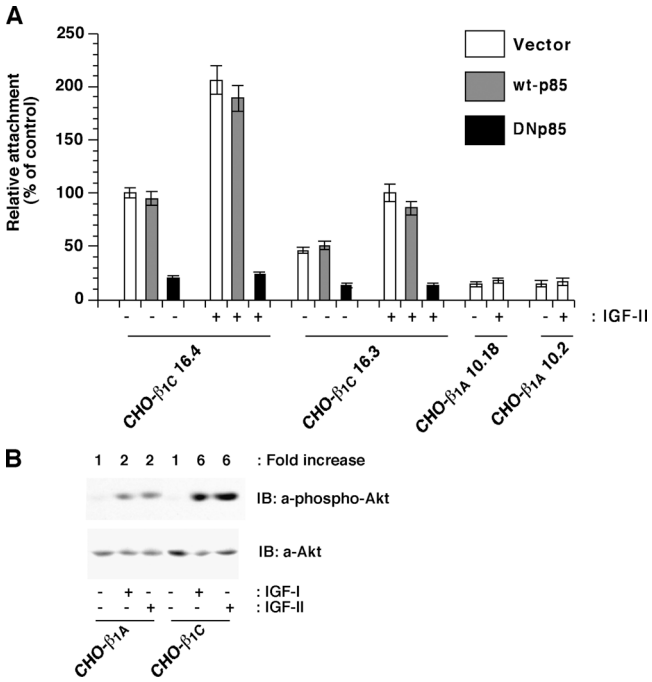

Figure 8. **PI 3-kinase mediates IGF-stimulated adhesion to LN-1.** (A) β_{1C}-CHO clones were transiently transfected with vector alone (pcDNA3), wt-p85, or DNp85. Cells were cultured in the absence of tet for 48 h. 1.5×10^5 cells were plated per well coated with LN-1 and incubated for 2 h at 37°C in the presence or in the absence of 100 ng/ml IGF-II. Cells were washed, fixed, and stained with β-gal. Attachment of cells transfected with PI 3-kinase cDNA was expressed as percentage (average and SD) of the number of attached cells that were transfected with vector alone, set at 100. Data are expressed as mean ± SEM. (B) β_{1A}- or β_{1C}-CHO clones were stimulated with IGF-I or IGF-II for 10 min, lysed and immunoblotted with Ab to Akt or Ab to phospho-Akt. Experiments were repeated at least twice with similar results. Results using representative clones are shown.

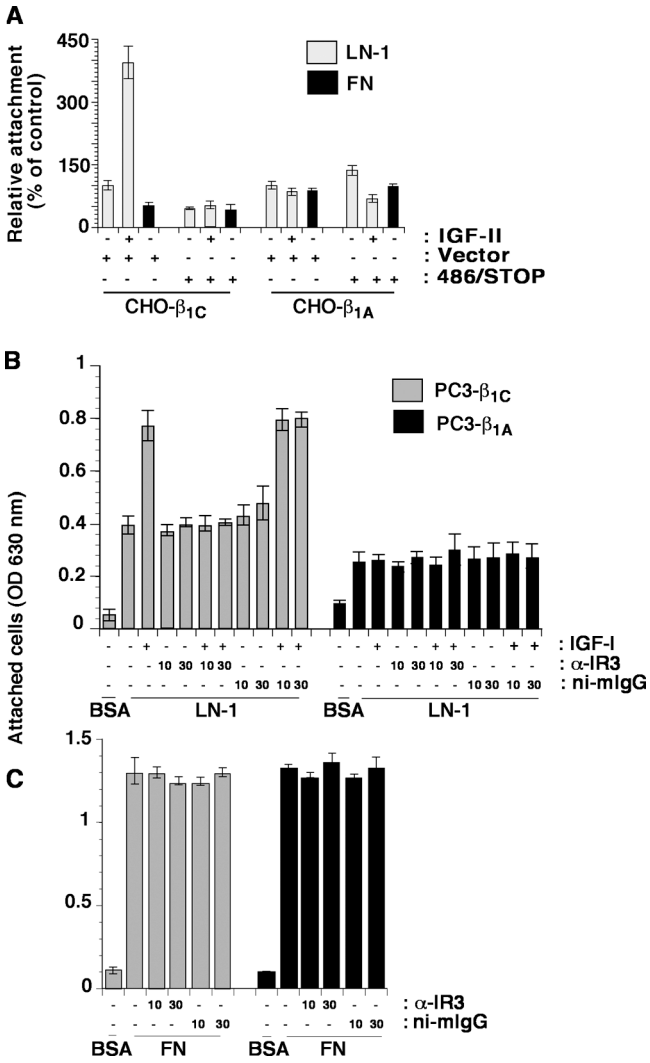

Figure 9. **IGF binding to IGF-IR mediates adhesion of β_{1C} expressing cells to LN-1.** (A) β_{1A}- and β_{1C}-CHO clones were transiently transfected with 486/STOP or vector alone. Cells were cultured in the absence of tet for 48 h. Cells were plated on either LN-1 or FN and incubated for 2 h at 37°C in the presence or in the absence of IGF-II. After incubation, cells were fixed and stained with β-gal. The number of attached cells transfected with 486/STOP cDNA was expressed as percentage of the number of attached cells transfected with vector alone, set at 100. (B and C) β_{1C}- and β_{1A}-PC3 clones were cultured in the absence of tet for 48 h. Cells were plated on BSA or LN-1 (B) or FN (C) at 37°C for 2 h in the presence or in the absence of IGF-I or α-IR3 or ni-mIgG. The experiments were repeated at least three times with similar results. Data are expressed as mean ± SEM.

tive form of p85 (DNp85), wild-type p85 (wt-p85), or vector alone. As shown in Fig. 8 A, IGF-II stimulated cell adhesion to LN-1 in β_{1C} expressing cells in either vector or wt-p85 transfected cells, but cell adhesion to LN-1 was significantly inhibited upon transfection of DNp85. In our previous work, we have shown that antibody-mediated engagement of β_1 integrins activates the PI 3-kinase/Akt pathway (Fornaro et al., 2000). We found threefold higher activation of Akt, a PI 3-kinase downstream signaling molecule, in response to IGF-I or IGF-II stimulation in β_{1C} expressing cells as compared with β_{1A} expressing cells (Fig. 8 B). Our results indicate that PI 3-kinase mediates β_{1C} integrin effect on cell adhesion to LN-1 in response to IGF.

Inhibition of IGF-IR prevents IGF-stimulated cell adhesion to LN-1

To confirm a direct involvement of IGF-IR in IGF-stimulated cell adhesion to LN-1 in the presence of β_{1C} integrin, CHO cells expressing β_{1A} or β_{1C} were transiently transfected with 486/STOP, a dominant negative form of IGF-IR, known to inhibit IGF-IR signaling (Dunn et al., 1998). As shown in Fig. 9 A, enhanced cell adhesion to LN-1 in the presence of IGF-II in β_{1C} integrin expressing CHO cells was completely prevented by transfection of 486/STOP, as compared with vector alone. The expression level of the mutant receptor was analyzed in culture medium and found comparable in β_{1A} and β_{1C} expressing cells transfected with 486/STOP (unpublished data). Additional evidence confirming the involvement of IGF-IR in adhesion to LN-1 of PC3 cells expressing β_{1C} was that an antibody known to inhibit IGF-IR, α-IR3, prevented cell adhesion to LN-1 in the presence of IGF-I, whereas ni-mIgG had no effect (Fig. 9 B). This effect was specific to LN-1 because no differences were observed when the cells were attached to FN (Fig. 9 C). In conclusion, IGF-IR binding to IGF is

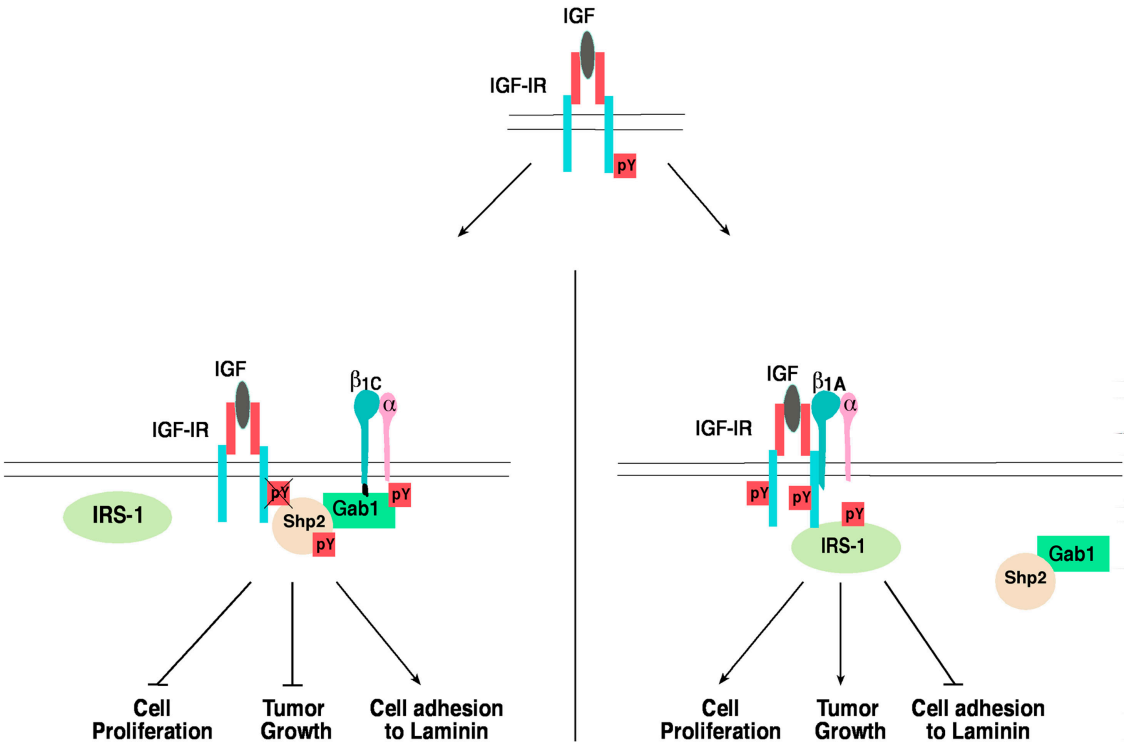

Figure 10. **A model for the biological effects of the cross-talk between IGF-IR and β_1 integrins.** This model shows that the β_{1C} integrin forms a complex with and activates Gab1/Shp2; this results in recruitment of Shp2 to IGF-IR and consequently, IGF-IR dephosphorylation. The signaling events stimulated by β_{1C} expression results in increased cell adhesion to laminin, reduced cell proliferation and inhibition of tumor growth. When down-regulation of β_{1C} occurs, IGF-IR remains tyrosine phosphorylated and associated with β_{1A} integrin, and this results in increased tumor growth and cell proliferation, but in reduced cell adhesion to LN.

necessary to achieve maximal levels of cell adhesion to LN-1 in response to IGFs.

Discussion

The novelty of this work is that β_1 integrin modulation of IGF-IR functions is controlled by the β_1 cytodomains that recruit specific IGF-IR downstream effectors, either IRS-1 or the Gab1–Shp2 phosphatase complex, to the cell surface.

The finding that the association between β_{1A} integrins and IGF-IR regulate cell adhesion to a basement membrane protein in response to IGF stimulation without affecting cell proliferation or tumor growth, is novel. Although it was known that integrins and growth factor receptors act synergistically and are associated, it was not known that their cross-talk determines the specificity of their activities (Yamada and Even-Ram, 2002). The relevance of this work is that the β_{1A}–IGF-IR complex by limiting cancer cell adhesion to the basement membrane, presumably allows the tumor mass to expand and invade. A direct involvement of IGF-IR in cell adhesion to LN has been shown earlier (Dunn et al., 1998) supporting the evidence of a synergistic and overlapping activity of integrins and IGF-IR in cell adhesion. Our discovery was made possible by the serendipitous finding that β_{1C}, a β_{1A} cytoplasmic variant that does not associate with IGF-IR, increases cell adhesion to LN-1. This variant form causes disruption of the β_{1A}–IGF-IR association via a 25–amino acid domain which is uniquely found in the β_{1C} integrin. Several studies have demonstrated that structural differences in the intracellular domains are expected to be important determinants of the specificity of a variety of integrin-mediated events (Fornaro and Languino, 1997). Although the cytodomain was not believed to affect ligand specificity, our data prove that the integrin cytodomain affects ligand specificity in a substrate-dependent manner. We show an inhibitory effect on IGF-IR activation without a wide change in integrin ligand binding, because cell adhesion to FN is unaffected in response to β_{1C} expression.

Here, as shown in Fig. 10, we demonstrate that the β_{1A}–IGF-IR complex preserves IGF-IR phosphorylation and association with IRS-1, a molecule necessary to promote stimulation of IGF-IR signaling pathways important for cell proliferation and transformation (Reiss et al., 2000). In contrast, β_{1C} does not associate with either IGF-IR or with IRS-1 and this results in failure to respond to a canonical IGF stimulation. The β_{1C} integrin inhibits IGF-stimulated tyrosine phosphorylation of IGF-IR and cell proliferation, as well as tumor growth by associating with Gab1, and increasing phosphorylation of Gab1. Gab1, a member of the IRS-1 family of adaptor proteins, is known to be phosphorylated in response to several growth factor receptors' activation, enhances cell growth and cell transformation, but has also a negative role on cell survival through interaction with the Shp2 tyrosine phosphatase (Gu and Neel, 2003; Holgado-Madruga and Wong, 2003). β_{1C} is likely to achieve the goal of preventing IGF-IR and IRS-1 tyrosine phosphorylation via activation of Gab1 and consequent recruitment of a

Gab1 binding protein, Shp2 (Gu and Neel, 2003), to IGF-IR and IRS-1 (Myers et al., 1998). Gab1 activates PI 3-kinase, a molecule known to mediate cell adhesion by inhibiting the tyrosine phosphorylation of IRS-1 (Reiss et al., 2001). PI 3-kinase is a downstream effector that mediates IGF-IR as well as integrin-stimulated signaling; PI 3-kinase activation requires Shp2 in some pathways activated by receptor-tyrosine-kinases such as IGF-IR and PDGF receptor, but not by others such as EGF receptor (Reiss et al., 2001; Neel et al., 2003; Tai et al., 2003). Our data show that both Shp2 and PI 3-kinase mediate β_{1C}-Gab1 effect on cell adhesion. On the same line, a previous set of studies by Clemmons's group had shown that inhibition of $\alpha_V\beta_3$ integrin binding to its ligands prevents IGF-stimulated tyrosine phosphorylation of IGF-IR and IRS-1 as well as cell proliferation to occur via Shp2 activation (Zheng and Clemmons, 1998). In contrast, this is the first work showing Gab1 recruitment to the cell surface by integrins, because Gab1 has only been shown to bind different growth factor receptors (Gu and Neel, 2003).

Neoplastic cells have a surrounding matrix markedly different from the ECM surrounding normal cells (Parise et al., 2000). In prostate cancer, like in breast carcinoma, a complete loss of basement membranes has been described even in well-differentiated tumors affecting its various components including LN-1 (Fuchs et al., 1989; Nerlich et al., 1998). LN-1 is found in normal human prostate glands (Cress et al., 1995; Davis et al., 2001; Brar et al., 2003) and in adult mouse prostate (Falk et al., 1996), but its expression is lost in basement membranes surrounding primary carcinoma and metastatic lymph node lesions (Brar et al., 2003). Although normal tissues display expression of both β_{1C} and LN-1, down-regulation of both molecules occurs during prostate cancer progression (Fornaro et al., 2001b), indicating that disruption of basement membranes and dysregulation of integrin variant expression are coordinated events in neoplastic transformation and that their expression is essential to sustain IGF stimulation of cell adhesion. Indeed, assembly of LN polymers requires expression of the LN receptor β_1 integrin (Bouvard et al., 2001).

Further elucidation of the role of integrin–IGF-IR association as well as of their downstream signaling pathways will provide a better understanding of the mechanisms that contribute to prostate cancer progression.

Materials and methods

Reagents and antibodies

Mouse LN-1 and lipofectamine 2000 were purchased from Invitrogen; BSA was purchased from Sigma-Aldrich; recombinant human IGF-I was purchased from R&D Systems; and IGF-II was purchased from Austral Biologics. Human FN was purified as described previously (Fornaro et al., 2003).

The following murine mAbs were used: to human β_1 integrin TS2/16 (American Type Culture Collection); K-20 (Immunotech); P4C10 (CHEMICON International, Inc.); 13 and clone 18 to β_1 integrin (BD Biosciences); 7D5/BF10 to β_{1C} integrin (Fornaro et al., 2001a); to chicken β_1 integrin W1B10 (Sigma-Aldrich); CSAT (Developmental Studies Hybridoma Bank [DSHB]); to hamster β_1 integrin 7E2 (DSHB). In addition, mAb to FLAG (Sigma-Aldrich); mAb 12CA5 to HA (American Type Culture Collection); 1C10 to vascular endothelial surface protein (Life Technologies); α-IR3 to IGF-IR (Oncogene); PY20 to phosphotyrosine (Santa Cruz Biotechnology, Inc.); rat mAb to α_6 GoH3 (CHEMICON International, Inc.). The following rabbit polyclonal Abs were used: to IGF-IR-β (Santa Cruz Biotechnology, Inc.); to Gab1 and to IRS-1 (Upstate Biotechnology); to hIGF-II (PeproTech); to Shp2 (Santa Cruz Biotechnology, Inc.); to phospho-Akt (Ser 473), to Akt, to phospho-Shp2 (Tyr542; Cell Signaling); to ERK1 (Santa Cruz Biotechnology, Inc.); to LN-1 described previously (Tsiper and Yurchenco, 2002; provided by P. Yurchenco, Robert Wood Johnson Medical School, Piscataway, NJ). Non-immune Abs were ni-mIgG (Pierce Chemical Co.), ni-rIgG and mIgM (Sigma-Aldrich), and rtIgG (Cappel).

Cell lines and transfectants

Normal human PrEC cells were obtained from Clonetics and maintained as the manufacturer recommended. SV40 immortalized nontumorigenic human prostate epithelial 267B1 and pRNS-1-1 cells were grown in keratinocyte serum-free medium with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract (Parda et al., 1993; Peehl et al., 1997).

CHO stable cell transfectants expressing either human β_{1A} or β_{1C} integrin were cultured as described previously (Fornaro et al., 2000). PC3 stable cell transfectants expressing chimeric β_{1A} (clones 8 and 11) or β_{1C} (clones 17 and 19) integrin (chicken extracellular and human intracellular) were generated using the tet-regulated expression system and cultured as described previously (Fornaro et al., 2003). CHO-β_{1A}, CHO-β_{1C}, PC3-β_{1A}, and PC3-β_{1C} clones were cultured for 48 h in growth medium in the absence of 1 μg/ml tet to induce the expression of β_{1A} or β_{1C} integrin. R− cells, mouse embryo fibroblasts obtained from IGF-IR knockout mice; and R+ cells, previously established by stably transfecting R− cells with the human wild-type IGF-IR cDNA (Sell et al., 1994), provided by R. Baserga (Thomas Jefferson University, Philadelphia, PA).

Integrin surface expression was analyzed by FACS® as described previously (Fornaro et al., 2000, 2003). The pECE-β_1COM plasmid containing the β_1 integrin area of the cytoplasmic domain shared by β_{1A} and β_{1C} truncated at threonine residue 777, has been described previously (Retta et al., 1998; provided by G. Tarone, University of Torino, Torino, Italy). CHO cells were electroporated using 10 μg pECE-β_1COM along with 1 μg pFneo. G418-resistant clones were pooled and analyzed for cell surface expression of human β_1COM integrin by FACS® using TS2/16 or 12CA5, as a negative control, as described previously (Fornaro et al., 2000).

The β_{1C} nucleotide sequence (Languino and Ruoslahti, 1992) was used to design the RZ specific for the β_{1C} cytoplasmic domain. A double-stranded DNA encoding the RZ-β_{1C} was obtained by annealing two synthetic single-stranded oligodeoxyribonucleotides containing flanking ClaI restriction sites and spanning a 55-bp sequence. The resulting double-stranded DNA encoding the RZ-β_{1C} was subcloned into the ClaI site of the mammalian expression vector pBJ-1 and its sequence is as follows: 5′-cgCGGTTTACCCTGTGCAAAGCAGGAGTGCCTGAGTAGTCAGAGAGACAGCGGGT-3′. Lowercase letters correspond to the restriction sites, whereas the underlined sequence corresponds to β_{1C} regions (2420-2434 and 2437-2451) flanking the sequence encoding the catalytic domain of the RZ. The RZ activity was initially tested in in vitro cell-free assays using ^{32}P-labeled β_{1C} mRNA.

Prostate tumor growth

PC3-β_{1A} and PC3-β_{1C} transfectants were induced to express β_{1A} or β_{1C} integrin. Cells were detached using 0.05% trypsin/0.53 mM EDTA, washed, and resuspended in RPMI. Cells (10^6) were inoculated subcutaneously into the flank of 6–8-wk-old male athymic Balb/c mice (Charles River Laboratories). Mice were given water supplemented with 5% sucrose or 5% sucrose plus 100 μg/ml tet to regulate the exogenous β_1 integrin expression. Tumor size was determined using a caliper every other day. Tumor volume was calculated using the formula (vol = $0.5236 \times [\text{width}]^2 \times [\text{length}]$) and expressed as tumor volume in cubic millimeters. 10 mice/group were used in each experiment.

Transient transfection

All transient transfections were performed using Lipofectamine 2000. R− and R+ cells were transiently transfected with 2 μg pCMV-β-galactosidase (β-gal) and either 10–30 μg pBJ-β_{1A} or 20–30 μg pBJ-β_{1C}. CHO clones expressing β_{1A} or β_{1C} were transiently transfected with 2 μg β-gal and either 20 μg pCMV-HA-wt-Shp2 or 20 μg pCMV-FLAG-Shp2 C/S (Zhang et al., 2002) cDNA, provided by B. Neel (Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA). β_{1C}-CHO clones were transiently transfected with 2 μg β-gal and 20 μg of vector alone (pcDNA3), 20 μg DNp85 (pSRα-Δp85α) or 20 μg wt-p85 (Hara et al., 1994) cDNA, provided by R. Kalb (Yale University School of Medicine, New Haven, CT). Cells were cultured in the absence of tet in growth medium for 48 h. In a separate set of experiments, β_{1A}- and β_{1C}-CHO clones were transiently transfected with 2 μg β-gal and either 20 μg of vector alone (pcDNA3) or 20 μg 486/STOP (pCVNIGFIRsol cDNA; provided by R. Baserga). Cells

were cultured in the absence of tet in growth medium for 48 h. 267B1 cells were transiently transfected with 2 μg β-gal and either 20 μg pBJ1 or 20 μg pBJ1-RZ-β_{1C} cDNA. Cells were harvested 48 h after transfection and used in adhesion assays as described below. In parallel, transfected cells were seeded on 48-well plates and stained for β-gal expression to determine transfection efficiency as described previously (Manes et al., 2003).

Cell adhesion assay

CHO cell adhesion to LN-1 or BSA (100 μg/ml), or 10 μg/ml FN was performed as described previously (Languino et al., 1993) in the presence or absence of 100 ng/ml IGF-II. Where specified, cells were incubated with either Ab to IGF-II or, as a negative control, 0.1 μg/ml ni-rIgG for 1 h on ice.

Alternatively, cell adhesion assays of 267B1, CHO clones, R− and R+ cells to BSA or LN-1 (100 μg/ml), or 10 μg/ml FN after being transiently transfected with cDNA constructs were performed by incubating cells with the coated substrates for 2 h at 37°C in the presence or in the absence of IGF-II or IGF-I (100 ng/ml). After adhesion, β-gal staining was performed as described previously (Manes et al., 2003).

For PC3 clones and PrEC, 80,000 cells were allowed to adhere to plates coated with different substrates as indicated in each figure legend for 2 h at 37°C in the presence or in the absence of IGF-I or IGF-II (100 ng/ml), α-IR3 or ni-mIgG (10 or 30 μg/ml), Ab to LN-1 or ni-rIgG (30 or 100 μg/ml), or GoH3 or rtIgG (20 μg/ml). Cells were fixed and stained with crystal violet (0.5%) and OD was measured at 630 nm (Manes et al., 2003).

Analysis of β_1 integrin association with IGF-IR, IRS-1, or Gab1

PC3 and CHO clones were induced to express β_{1A} or β_{1C} integrin. Cells were detached and stimulated with 100 ng/ml IGF-I for 10 min, washed and lysed. Proteins were immunoprecipitated by incubating with K-20 and protein A-Sepharose. Immunocomplexes were dissociated and reprecipitated with Ab to IGF-IR or ni-rIgG as described previously (Fornaro et al., 2000). Proteins were separated by SDS-PAGE and immunoblotted using Abs to β_1 integrin (clone 18), to IGF-IR-β, to IRS-1 or to Gab1. As control, expression levels of IRS-1 or β_1 integrin or IGF-IR were analyzed using respective Abs. Immunoprecipitation of chicken β_1 integrin was performed using CSAT Ab as described previously (Marcantonio and Hynes, 1988). CHO clones expressing wild-type full-length β_{1A} or different truncated forms of β_1 integrin were allowed to grow to 70% confluency. The cells were lysed and association between β_1 integrin and IGF-IR was studied as described above.

Analysis of tyrosine phosphorylation of IGF-IR, IRS-1, or Gab1

PC3 or CHO clones were detached and trypsin was neutralized with soybean trypsin inhibitor. Cells were stimulated with 100 ng/ml IGF-I, washed, lysed, and proteins were immunoprecipitated with IGF-IR, IRS-1 or Gab1 Abs and protein A-Sepharose. Immunoprecipitates were then separated by SDS-PAGE under reducing conditions and immunoblotted with PY20, IGF-IR-β, IRS-1 or Gab1, and visualized by ECL.

Immunoblotting

pRNS-1-1 and 267B1 cells were grown to 70% confluency, lysed, and immunoblotted with mAb to β_{1C} or mIgM (5 μg/ml) as described previously (Fornaro et al., 2001a). 267B1 cells transiently transfected with RZ-β_{1C} or vector were lysed and immunoblotted with mAb to 5 μg/ml β_{1C} or Ab to Akt or Ab to 0.2 μg/ml IGF-IR (Fornaro et al., 2000). CHO clones expressing β_{1A} or β_{1C} were starved overnight, stimulated with IGF-I or IGF-II for 10 min, lysed, and immunoblotted with Ab to Akt or Ab to phospho-Akt (Fornaro et al., 2000). CHO clones expressing β_{1A} or β_{1C} were transiently transfected with HA-tagged wt-Shp2 or FLAG-tagged Shp2 C/S. Cells were lysed and immunoblotted with Ab to 0.5 μg/ml HA or Ab to 0.5 μg/ml FLAG or Ab to 0.2 μg/ml ERK1.

Analysis of Gab1 association with either Shp2 or phospho-Shp2

CHO clones were induced to express β_{1A} or β_{1C} integrin. Cells were detached and stimulated with 100 ng/ml IGF-I for 10 min, washed and lysed; proteins were immunoprecipitated by incubating with Ab to 1 μg Gab1 and protein A-Sepharose (Fornaro et al., 2000). Proteins were separated by SDS-PAGE and immunoblotted with Ab to 0.2 μg/ml Gab1 or 0.2 μg/ml Shp2 or phospho-Shp2 (1:1,000 dilution).

SRB assay

Proliferation was measured using SRB assay. PC3 clones were induced to express β_{1A} or β_{1C} integrin, serum starved for 12 h, detached, and seeded on 96-well plates (7.5×10^3 cells per well) in the presence or in the absence of IGF-I or IGF-II (100 ng/ml). Cells were incubated for 72 h without a medium change. After incubation, cells were fixed with 10% TCA at 4°C for 1 h, washed five times with tap water and stained with 0.2% SRB for 15 min. The plates were then washed five times with 1% acetic acid, dried, and stained cells in each well were solubilized with 10 mM Tris base. The absorbance in each well was measured at 540 nm. SRB results were confirmed by cell counting.

Statistical analysis

Differences in cell adhesion to LN were measured using *t* test. Tumor volume was measured for each mouse on multiple days; to analyze the data, a mixed-effect general linear model for repeated measurements was applied. Analysis was operated using SAS (version 8.2; SAS Institute, Inc.).

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