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Et al.

β_{1A} Integrin Expression Is Required for Type 1 Insulin-Like Growth Factor Receptor Mitogenic and Transforming Activities and Localization to Focal Contacts

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Abstract

The cells' ability to proliferate in response to growth factor stimulation is significantly altered during cancer progression. To investigate the mechanisms underlying these alterations in prostate cancer, the role and expression of β_{1A} integrin and type 1 insulin-like growth factor receptor (IGF-IR), known to contribute to cell proliferation and transformation, were analyzed. Using small interfering RNA oligonucleotides to down-regulate β_{1A}, we show that β_{1A} expression is required for IGF-IR–mediated prostate cancer cell proliferation and anchorage-independent growth. *In vivo*, using age-matched transgenic adenocarcinoma of mouse prostate (TRAMP) mice at different stages of prostate cancer [prostatic intraepithelial neoplasia, PIN; well-differentiated adenocarcinoma, WD; and poorly differentiated adenocarcinoma, PD], the expression of β_{1A} and of IGF-IR was studied. β_{1A} and IGF-IR expression levels were concurrently up-regulated in high PIN and WD, whereas their expression did not correlate in late-stage PD. In contrast to the up-regulated expression of β_{1A}, the levels of β_{1C}, a β_1 cytoplasmic variant that inhibits cell proliferation, were down-regulated in all stages of prostate cancer. A similar expression pattern was observed for a β_{1C} downstream effector, Grb2-associated binder-1 (Gab1) which is known to inhibit IGF-IR phosphorylation. To analyze *in vitro* the mechanistic implications of β_{1A}, β_{1C}, and Gab1 deregulation in prostate cancer, we investigated whether expression of either β_1 variant in β_1-null cells affected IGF-IR localization. We found that IGF-IR and β_{1A} were colocalized in highly specialized integrin signaling compartments, designated focal contacts. However, in the presence of β_{1C}, IGF-IR remained diffuse on the cell surface and did not localize to focal contacts. The findings that β_1 integrins and IGF-IR are concurrently deregulated and that expression of β_1 integrins is necessary to achieve appropriate IGF-IR intracellular distribution point to the important role that the cross-talk between these receptors may have during prostate cancer progression and will be helpful in formulating new therapeutic strategies. (Cancer Res 2005; 65(15): 6692-700)

Introduction

Prostate cancer remains a significant health issue for aging men and is the second leading cause of cancer death among American men (1). Whereas substantial advances have been made in the diagnosis and treatment of prostate cancer, the underlying causes of this disease remain to be fully investigated. The development of prostate cancer proceeds through a series of defined stages, including preinvasive disease (prostatic intraepithelial neoplasia, PIN), invasive cancer, and androgen-dependent or androgen-independent metastases (2, 3).

The ability of neoplastic prostate cells to grow and invade is mediated by interactions with the surrounding cells and extracellular matrix–mediated by integrins (4). Integrins are crucial regulators of differentiation, growth, survival, migration, and invasion (5, 6). In prostate cancer, tumor cells have a markedly different surrounding matrix than normal cells; thus, changes in the integrin profile may be functionally relevant and contribute to cancer progression (7, 8). A number of studies have reported changes in integrin expression as prostate cancer progresses to an advanced stage (7, 9, 10).

Integrins are heterodimers consisting of an α and β subunit (6). The cytoplasmic domain of the β_1 subunit, in its canonical form (β_{1A}), is highly conserved and is a positive regulator of cell proliferation, development, migration, and integrin localization (11, 12). The β_{1C} integrin is an alternatively spliced variant of the β_1 subfamily that contains a unique sequence in its cytoplasmic domain (12). Previous studies have shown that either full-length β_{1C} or its cytoplasmic domain inhibits prostate cancer cell proliferation (12). β_{1C} is expressed in the nonproliferative, differentiated epithelium and is selectively down-regulated in prostatic adenocarcinoma (13–16).

Several reports in the literature suggest that there are physical and functional interactions between integrins and components of growth factor signaling pathways, including insulin-like growth factor (IGF) or its downstream signaling proteins (17). However, the role of these interactions between type 1 IGF receptor (IGF-IR) and integrins in regulating the progression of prostate cancer is still not clear. The IGF axis is an important modulator of growth and development, and changes in this axis may have important implications in malignant growth (18, 19). The IGF and its receptor (IGF-IR) are found in human prostate stroma and epithelial cells (20). There is, however, no consensus regarding the relative levels of IGF-IR expression in benign and malignant prostate epithelium and the role of IGFs in metastases. In 1996, Tennant et al. reported that IGF-IR expression is significantly lowered at the protein and RNA level in malignant versus benign prostate epithelium (21). Similarly, Chott et al. reported that IGF-IR is expressed in luminal and basal epithelium in nonneoplastic prostate but is lost in metastatic androgen-independent prostate cancer (22). However, Kaplan et al. did fail to show a significant increase in IGF-IR mRNA during primary prostate tumor progression in transgenic adenocarcinoma

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of mouse prostate (TRAMP) mice, indicating the possibility of posttranscriptional regulation of IGF-IR but showed reduced levels of IGF-IR mRNA in metastatic lesions and in androgen-independent disease (23). Subsequently, Nickerson et al. reported that progression to androgen independence is associated with increased expression of IGF-I and IGF-IR as well as increased tyrosine phosphorylation of IGF-IR (24). Finally, in 2002, another study has shown that IGF-IR was significantly up-regulated at the protein and mRNA level in primary prostate cancer compared with benign prostatic epithelium (25).

Most signaling pathways stimulated by receptor tyrosine kinases use scaffolding adaptors like insulin receptor substrate (IRS) or Gab known to be involved in IGF signaling axis (26, 27). Upon the addition of growth factors, Grb2-associated binder (Gab1) becomes tyrosine phosphorylated and recruits Src homology-2 domain-containing tyrosine phosphatase 2 (Shp2; ref. 28). In our previous study, we have shown that the Gab1/Shp2 complex is recruited to the cell surface by integrins. Upon recruitment to cell surface, Gab1/Shp2 complex causes IGF-IR dephosphorylation and causes change in cell adhesion to laminin in response to IGF-I (29). Previous studies have shown that overexpression of Gab1 increases growth factor responsiveness and tumorigenicity in NIH3T3 cells (26). A recent study has shown that overexpression of Gab1 lacking the pleckstrin homology domain enhances (by 3-fold), whereas wild-type Gab1 inhibits, epidermal growth factor–induced soft agar colony formation of preneoplastic Syrian hamster cells (30). However, studies carried out to investigate whether changes in expression levels of Gab1 occur during cancer progression have not been done.

In the present study, we show that IGF-IR–mediated prostate cancer cell proliferation and anchorage-independent growth is inhibited by β_{1A} down-regulation. Our study highlights a role for the β_{1A} integrin as modulator of IGF-IR–mediated functions in prostate cancer.

Materials and Methods

Antibodies. The following mouse monoclonal antibodies (mAb) were used: to all human or mouse β_1 integrins, Clone-18 (BD Bioscience, San Jose, CA); to human β_1 integrin (K-20; Immunotech, Marseille, France); to phosphatidylinositol 3-kinase (PI3-K; p85; BD Bioscience); mouse IgM specific to β_{1C} integrin, 7D5/BF10 (31); to vinculin (Chemicon, Temecula, CA). The following rabbit polyclonal antibodies were used: to all β_1 integrins (M-106; Santa Cruz Biotechnology, Santa Cruz, CA); to Akt (Cell Signaling, Beverly, MA); to extracellular signal-regulated kinase-2 (ERK-2, Santa Cruz Biotechnology); to cytoplasmic domain of β_{1A} integrin (kindly provided by Dr. E. Ruoslahti, Cancer Research Center, The Burnham Institute, La Jolla, CA); antibody to IGF-IR− (Santa Cruz Biotechnology); to β_5 integrin (Chemicon); to β_{1C} integrin (13); to Gab1 (Upstate Biotechnology, Lake Placid, NY). Normal mouse IgM (ni-Ab) and normal rabbit IgG (rIgG) were from Calbiochem (La Jolla, CA).

Transgenic mice. TRAMP mice on a pure C57BL/6 background were bred in the colony at the University of Alabama at Birmingham School of Medicine. Transgenic females were bred with nontransgenic littermates (NT) males because transgenic males tend to develop prostate tumors. All mice were maintained in a climate-controlled environment with a 12-hour light/12-hour dark cycle and diet and water supplied *ad libitum*. Breeders were fed standard pellet mouse feed (Harlan Teklad 7012, Madison, WI). Following weaning at 3 to 4 weeks of age, the gender of offspring was determined, males were separated from females, and a tail biopsy was collected from each mouse. Tail DNA, isolated by standard procedures, was used for determination of transgene incorporation by PCR as described previously (32–34). All TRAMP mice develop premalignant changes resembling human PIN, which ultimately progress to poorly differentiated (PD) prostatic adenocarcinomas that metastasize to distant sites, primarily the lymph nodes, bones, and lungs (33). The present study focused on PD (grade 6) as an example of advanced prostate cancer, WD (grade 4) as an example of an early-stage of adenocarcinoma, prostates grade 3 as an example of PIN, and normal prostate (grade 1) from age-matched NT littermates.

Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the University of Alabama Animal Care Committee.

Preparation and analysis of tissues. At necropsy, the urogenital tract, consisting of the prostate, the seminal vesicles, and the emptied bladder, was weighed. A fragment of the DLP, as well as periaortic lymph nodes, were rapidly frozen in liquid nitrogen and stored at −80°C, until further processed. The rest of the DLP was fixed in an acid alcohol solution containing 96% ethanol, 1% glacial acetic acid, and 3% distilled water, as described (35). Lymph nodes were fixed in 10% neutral buffered formalin phosphate. The fixed tissues were then embedded in paraffin and 4- to 5-μm sections were mounted on Colorfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were stained with H&E (lymph nodes) or with Gomori trichrome staining (DLP). DLP sections were evaluated blindly and scored using established criteria (32, 34) as normal prostate (score 1), low PIN (score 2), high PIN (score 3), well-differentiated (WD, score 4), moderately differentiated (MD, score 5), or poorly differentiated (PD, score 6). Prostate sections from each mouse were evaluated blindly by Dr. Isam A. Eltoum, a certified Pathologist (Department of Pathology, University of Alabama at Birmingham, Birmingham, Al; refs. 32, 34). Tumors showing grade 2 to 5 were heterogeneous and the pathologist graded them depending on the type of tissue that is most prevalent. The MMHCC Pathology Committee has published very elaborate guidelines in Cancer Research (36). Dr. Isam A. Eltoum, the pathologist who has examined these sections has followed those guidelines during his assessments of the tumor grade.

Data analysis. Analysis of the expression levels of IGF-IR and β_1 integrin was done using SigmaStat, version 3.0 (SysStat, Inc., Evanston, IL). When the effect of one variable was examined, one-way ANOVA was done if data in multiple groups were normally distributed with equal variance. The Kruskall-Wallis one-way ANOVA on ranks was used if three or more groups were compared and the data were not normally distributed with equal variance. If the latter two tests indicated that the groups were significantly different, a Tukey or Dunn test was carried out, respectively, to determine which of the groups were significantly different from the others. P values for the appropriate tests are given with the results.

Prostate tissue lysis. TRAMP mice were maintained as described above until they were 28 weeks of age. DLPs were collected at necropsy. In this study, we used frozen prostate samples from TRAMP graded 3, 4, and 6 as well as prostate from age-matched NT. Tumor specimens were extracted as follows: specimens were homogenized on ice in a Dounce homogenizer in 100 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% Triton X-100, 1 mmol/L benzamidine, 10 μg/mL soybean trypsin inhibitor, 10 μg/mL leupeptin, 1 mmo/L phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin A, 1 μmol/L calpain inhibitor, 1 mmol/L Na_3VO_4, and 1 mmol/L $Na_4O_7P_2$. SDS was then added to a final concentration of 5%, after which the extracts were boiled for 5 minutes. After boiling, samples were centrifuged at 14,000 × g for 20 minutes, and supernatant was collected for protein determination using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

Immunoblot analysis. Proteins from TRAMP prostate tissue lysate (PIN, WD, and PD) or from NT were electrophoresed using 7.5% SDS-PAGE under reducing condition and transferred onto polyvinylidene difluoride membranes, then probed sequentially with mAb to β_1 integrins (Clone-18) or to PI3-K or to IGF-IR-β, mouse IgM to β_{1C} integrin (7D5/BF10; ref. 31), normal mouse IgM, rabbit polyclonal antibody to β_{1C} integrin or Gab1, or to β_5 integrin. Proteins were detected using enhanced chemiluminescence.

Immunofluorescence microscopy. GD25 murine cells transiently transfected with human β_{1A} or β_{1C} integrin or GD25 cells stably transfected with human β_{1A} integrin using LipofectAMINE 2000 (Invitrogen, San Diego, CA)

were cultured as described previously (37). Cells were seeded onto 10 μg/mL fibronectin-coated coverslips and processed for immunofluorescence microscopy as described previously (38). The primary antibodies used were K-20 to human β_1 integrin or a mAb to vinculin or a polyclonal antibody to IGF-IR-β. The secondary antibodies used were goat anti-rabbit IgG coupled to FITC or goat anti-mouse IgG coupled to rhodamine (The Jackson Laboratory, West Grove, PA). One hundred cells per experiment to study the colocalization of IGF-IR and β_1 integrins were examined and experiments were done >10 times.

Small interfering RNA–mediated down-regulation of β_{1A} integrin. This study used small interfering RNA (siRNA) to target a region specific to the β_{1A} transcript. The siRNA was designed using conventional criteria (39) and corresponded to nucleotides 2425 to 2443 of the coding region in human β_{1A} integrin. The sequence of siRNA was sense strand 5′-AUGGGAC-ACGGGUGAAAAUTT-3′ and antisense strand 5′-AUUUUCACCCGUGUCC-CAUTT-3′. These sequences were submitted to BLAST search to ensure that only the β_{1A} integrin was targeted by the β_{1A} integrin siRNA and that control sequences were not homologous to any known genes. siRNA specific to β_{1A} integrin or control were synthesized by Dharmacon (Lafayette, CO). Mouse β_{1A} integrin also have the same last 18 nucleotides in its coding regions as the human β_{1A} integrin and correspond to nucleotides 2502 to 2519 of mouse β_{1A} integrin coding region.

PC3, R−, or R+ cells were cultured as described before (29). TRAMP-C2 (American Type Culture Collection, Manassas, VA) cells were grown in DMEM, 2 mmol/L L-glutamine, 5 mg/mL bovine insulin, 10 nmol/L dihydrotestosterone, 5% fetal bovine serum (FBS), and 5% Nu-Serum (BD Biosciences, Bedford, MA). TRAMP-C2, R+, R−, or PC3 cells were transfected with siRNA (100 nmol/L) using oligofectamine (Invitrogen) in serum-free medium. After a 4-hour incubation, cells were fed with complete growth medium described above and incubated for additional 48 hours. After incubation, cells were lysed and proteins were separated on SDS-PAGE under reducing conditions. Proteins were immunoblotted with a mAb to β_1 integrins (Clone-18) or to Akt or to ERK.

Sulforhodamine B assay. Proliferation was measured using sulforhodamine B (SRB) assay. PC3, R+, or TRAMP-C2 cells were transfected with siRNA to β_{1A} integrin or control siRNA or oligofectamine alone for 48 hours. Cells were detached and seeded on 96-well cell culture plate (7.5×10^3 cells per well) and allowed to attach for 5 hours in the presence of serum. After attachment, cells were washed and incubated in the presence or in the absence of IGF-I (100 ng/mL) in serum-free medium for 48 hours. After

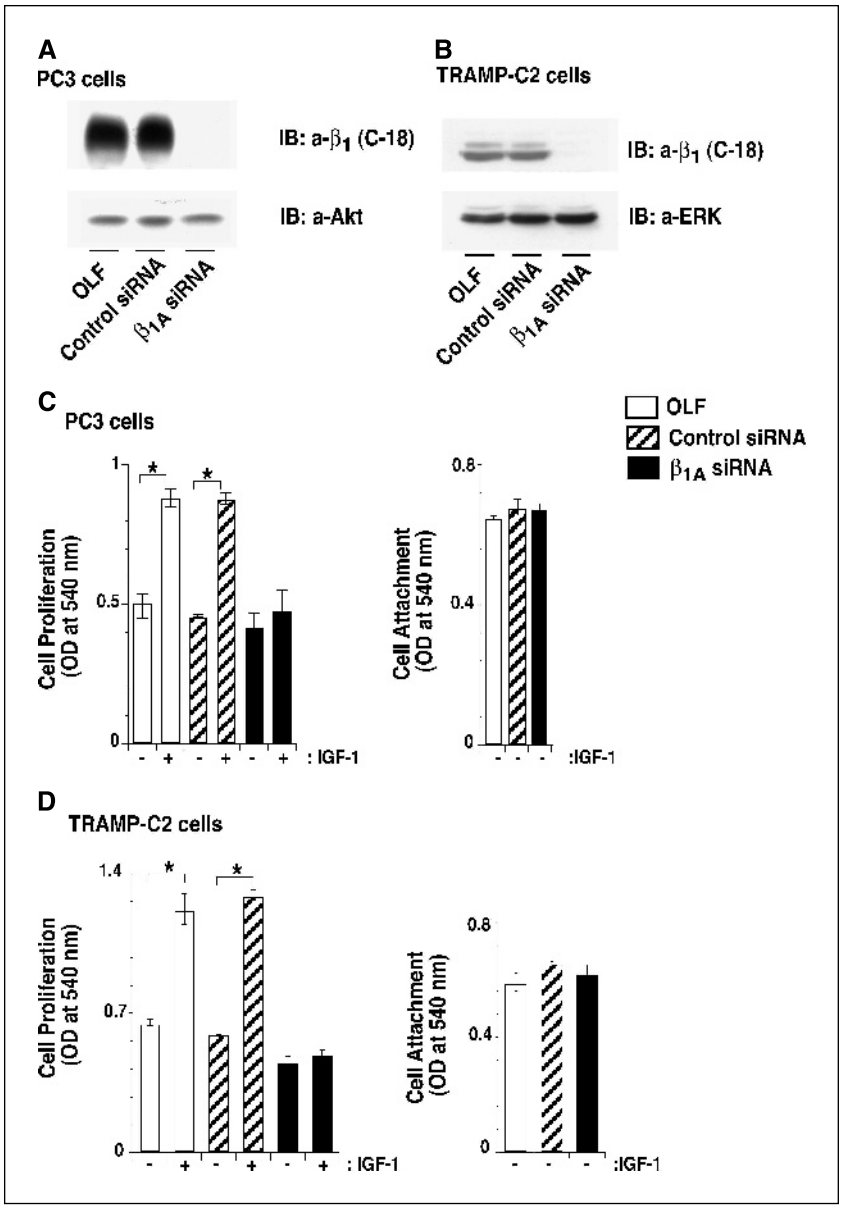

Figure 1. β_{1A} expression is essential for IGF-I-mediated prostate cancer cell proliferation. PC3 (*A* and *C*) and TRAMP-C2 (*B* and *D*) cells were transfected with 100 nmol/L β_{1A} integrin siRNA, control siRNA or oligofectamine alone (*OLF*). *A-B,* after 48 hours, cells were detached, lysed, and proteins were separated using SDS-PAGE and immunoblotted with mAb to β_1 integrins (Clone-18) or antibody to Akt or antibody to ERK. *C-D,* cells were plated in 96-well tissue culture plates in the presence of 10% FBS for 5 hours. Cells were washed, incubated for 48 hours in serum-free medium in the presence or in the absence of IGF-I (100 ng/mL), fixed, and stained with SRB (*left*). As plating control, one plate was fixed and stained with SRB after 5 hours in the presence of serum (*right*). In (*C*) and (*D*), the differences in cell proliferation in the presence or in the absence of IGF-I are statistically significant. *, $P \leq 0.01$.

incubation, cells were fixed and stained with SRB as described before (29). As a plating control, one set of plates after 5 hours of cell attachments was washed, fixed, and stained with SRB.

Anchorage-independent growth assay. Cell growth in soft agar was assayed by scoring the number of colonies formed in medium (DMEM supplemented with 10% FBS) containing 0.3% agarose, with a 0.5% agarose medium underlay as described (40). R− or R+ (2×10^3) or DU145 (5×10^3) cells were seeded on 60-mm diameter plates in triplicate in the presence of IGF-I (100 ng/mL). Cells were fed with 1.5 mL of suspension medium (DMEM supplemented with 10% FBS) every 3 days. The number of colonies larger than 100 μm were counted after 14 days (R+ cells) or after 21 days (DU145 cells). Cell viability was measured 48 hours after siRNA transfection using trypan blue exclusion and plotted as percentage of total viable cells.

Immunohistochemistry. Paraffin-embedded prostate tissue sections from eight mice were baked for 2 hours at 60°C and washed thrice with xylene to deparaffinize the slides. The sections were rehydrated using ethanol and then distilled water. The sections were boiled in sodium citrate using a steamer at 100°C to enhance antigen retrieval. Endogenous peroxidase was quenched with 3% H_2O_2 for 5 minutes. Blocking was achieved with 50% normal goat serum in TBS containing 0.2% bovine serum albumin for 20 minutes at room temperature. The sections were then incubated with primary antibodies (rabbit polyclonal antibody to β_1 integrin, M-106, 0.8 μg/mL or ni-rIgG, 0.8 μg/mL) overnight at 4°C, followed by biotinylated goat anti-rabbit IgG (3 μg/mL, Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. The sections were incubated with streptavidin-peroxidase complex (5 μg/mL, Vector Laboratories) for 30 minutes at room temperature. Each incubation step was followed by three washes with TBS. Finally, 3,3′-diaminobenzidine tetrahydrochloride dehydrate was used as chromogen (Sigma, St. Louis, MO). Sections were then stained using hematoxylin for 1 minute. Tissue sections were examined on an Olympus BX41 microscope and photographed using Olympus DP12 camera. The immunostaining of β_{1A} integrin was independently evaluated by four investigators, including Dr. David S. Garlick (DVM, Pathologist, Department of Cancer Biology, University of Massachusetts Medical School), H.L.G., M.B., and L.R.L. The expression of β_1 integrin in prostate tissue sections of NT or TRAMP was evaluated and recorded as a separate observation.

Results

β_{1A} expression is essential for insulin-like growth factor-I–mediated prostate cancer cell proliferation. To investigate the role of β_{1A} in IGF-I stimulated prostate cancer cell proliferation, we down-regulated the expression of β_{1A} in PC3 or TRAMP-C2 cells using siRNA specific to β_{1A} integrin. As shown in Fig. 1*A* and *B*, *B*, transfection of β_{1A} siRNA significantly down-regulated β_{1A} expression compared with control siRNA, without affecting the expression of other molecules such as Akt (Fig. 1*A*), ERK (Fig. 1*B*), or cell viability (data not shown). To investigate the role of β_{1A} in IGF-I stimulated cell proliferation, we transfected PC3 (Fig. 1*C*) or TRAMP-C2 (Fig. 1*D*) cells with β_{1A} siRNA or control siRNA and measured their proliferative response in the presence or in the absence of IGF-I. Cells transfected with β_{1A} siRNA did not respond to IGF-I (Fig. 1*C* and *D, left*), whereas IGF-I increased cell proliferation when the cells were transfected with control siRNA

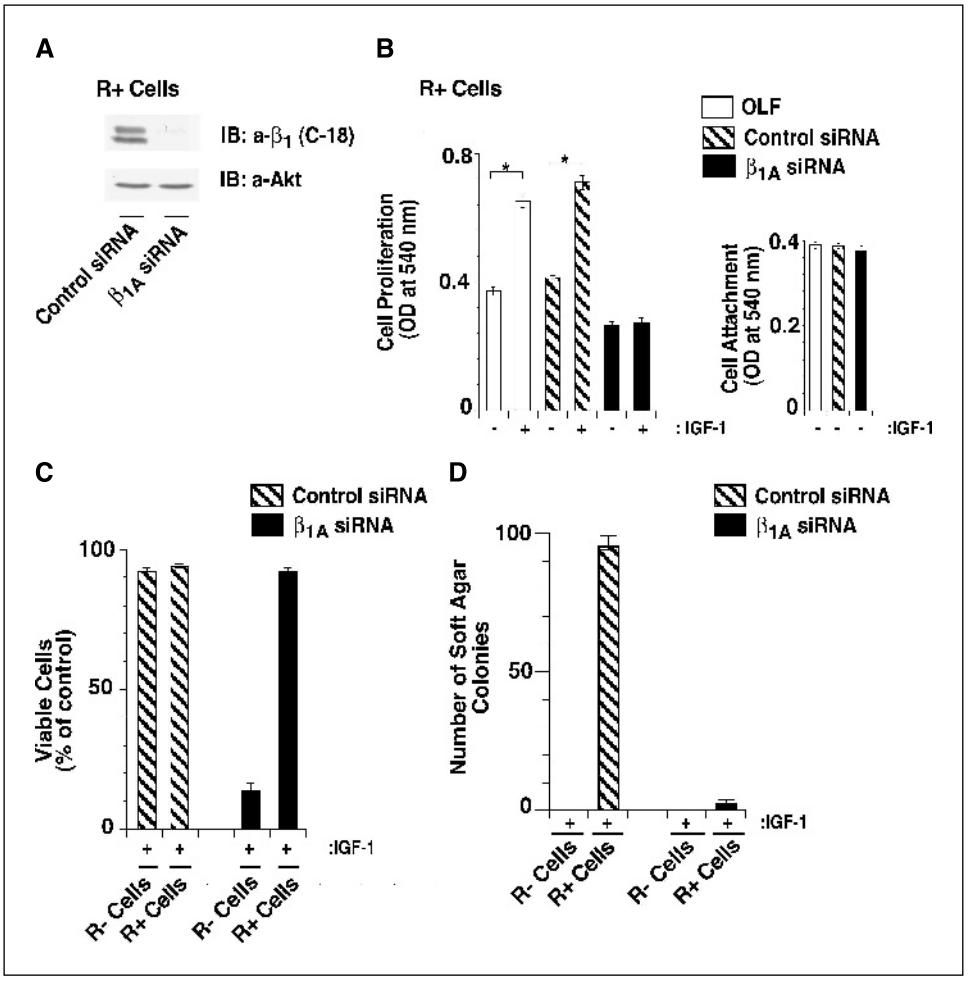

Figure 2. β_{1A} is required for IGF-IR mediated anchorage-independent growth. *A-B,* R+ cells were transfected with 100 nmol/L β_{1A} integrin siRNA, control siRNA or oligofectamine alone (*OLF*). *A,* after 48 hours, cells were detached, lysed and proteins were separated using SDS-PAGE and immunoblotted with a mAb to β_1 integrins (Clone-18) or antibody to Akt. *B,* cells were plated on 96-well tissue culture plates in the presence of 10% FBS for 5 hours. Cells were washed, incubated for 48 hours in serum-free medium in the presence or in the absence of IGF-I (100 ng/mL), fixed, and stained with SRB (*left*). As plating control, one plate was fixed and stained with SRB after 5 hours in the presence of 10% FBS (*right*). In (*B*), the differences in cell proliferation in the presence or in the absence of IGF-I are statistically significant. *, $P \leq 0.01$. *C,* R− or R+ cell viability after transfection with control siRNA or β_{1A} integrin siRNA was measured. The number of viable cells and of total cells was counted and the percentages of viable cells were calculated. *D,* R− or R+ (2×10^3) cells were suspended in 1.5-mL medium containing 0.3% agarose and 10% FBS, with a 0.5% agarose medium underlay in 60-mm diameter plates in the presence of IGF-I (100 ng/mL). Cells were fed with 1.5-mL suspension medium (DMEM supplemented with 10% FBS) every 3 days. The number of colonies larger than 100 μm were counted after 14 days. Triplicate observations in three separate experiments were done. Data from one representative experiment.

(Fig. 1*C* and *D, left*). Cells transfected with control siRNA or β_{1A} siRNA did attach to tissue culture plates in a comparable manner; therefore, the effect observed was not a consequence of a differential ability of these cells to adhere to the plate (Fig. 1*C* and *D, right*). Based on these results, we conclude that β_{1A} integrin expression is essential for IGF-I-mediated prostate cancer cell proliferation.

β_{1A} is required for type 1 insulin-like growth factor receptor–mediated cell anchorage-independent growth. IGF-I binds to its receptor, IGF-IR, and stimulates tumor cell growth and cell survival (18). Cells devoid of IGF-IR are unable to undergo transformation, suggesting a crucial role for IGF-IR in the establishment and maintenance of the transformed phenotype (41). To investigate the role of β_{1A} in IGF-IR-mediated prostate cancer cell transformation, we used R− or R+ cells. R− cells are 3T3-like fibroblasts established from mouse embryos with a targeted disruption of *igf-ir* gene and cannot be transformed by SV40 T antigen, or an activated Ha-ras, or a combination of both (42–44). R+ cells were generated by transfecting wild-type IGF-IR in R− cells (42–44). We down-regulated β_{1A} in R+ cells using siRNA specific to β_{1A} integrin and measured the effect of IGF-I on cell proliferation. We found significant down-regulation of β_{1A} integrin upon siRNA transfection in R+ cells (Fig. 2*A*). As shown in Fig. 2*B*, β_{1A} down-regulation prevented IGF-I stimulated R+ cell proliferation (Fig. 2*B, left*), but it did not affect cell attachment (Fig. 2*B, right*). In R− cells, we observed significant cell death (90%) after transfection with β_{1A} siRNA as compared with R+ cells, indicating a crucial role for β_{1A} integrin in cell survival in the absence of IGF-IR (Fig. 2*C*). An analysis of the effect of β_{1A} integrin down-regulation on the cells' ability to grow in anchorage-independent manner shows that R+ cells formed colonies in soft agar when transfected with control siRNA, but their ability to grow in soft agar was completely inhibited by β_{1A} down-regulation (Fig. 2*D*). R− cells did not form colonies in soft agar (Fig. 2*D*) confirming a previous published observation that IGF-IR is required for anchorage-independent growth (40). Similar results were obtained in human prostate cancer cells, DU145, whose viability was not affected by β_{1A} integrin down-regulation, although a complete inhibition of anchorage-independent growth was achieved (data not shown). These results show that β_{1A} integrin expression plays a crucial role in IGF-IR-mediated cell transformation.

β_{1A} and type 1 insulin-like growth factor receptor expression is up-regulated in prostatic intraepithelial neoplasia and well-differentiated prostate carcinoma. Because we have shown that the β_{1A} integrin regulates IGF-IR-mediated transformation (29), we investigated whether the expression levels of β_{1A} and IGF-IR correlate during prostate cancer progression *in vivo*. We studied the expression profile of the β_{1A} integrin and IGF-IR in prostate tissues from TRAMP mice or from NT. We have analyzed five prostates showing tumor grade 3, five prostates showing tumor grade 4, and four prostates showing tumor grade 6. As shown in Fig. 3*A-B*, we found a significant increased expression of IGF-IR and β_{1A} integrin in all prostate tissues showing grades 3 and 4 compared with prostates from NT mice ($P < 0.011$). The concurrent up-regulation of β_{1A} and IGF-IR in the early stages of neoplastic transformation in TRAMP mice is consistent with our model that β_{1A} and IGF-IR form a functional complex. In PD tumors, however, the expression of β_{1A} was significantly decreased relative to WD tissues, whereas levels of IGF-IR were comparable with WD tissues, suggesting that

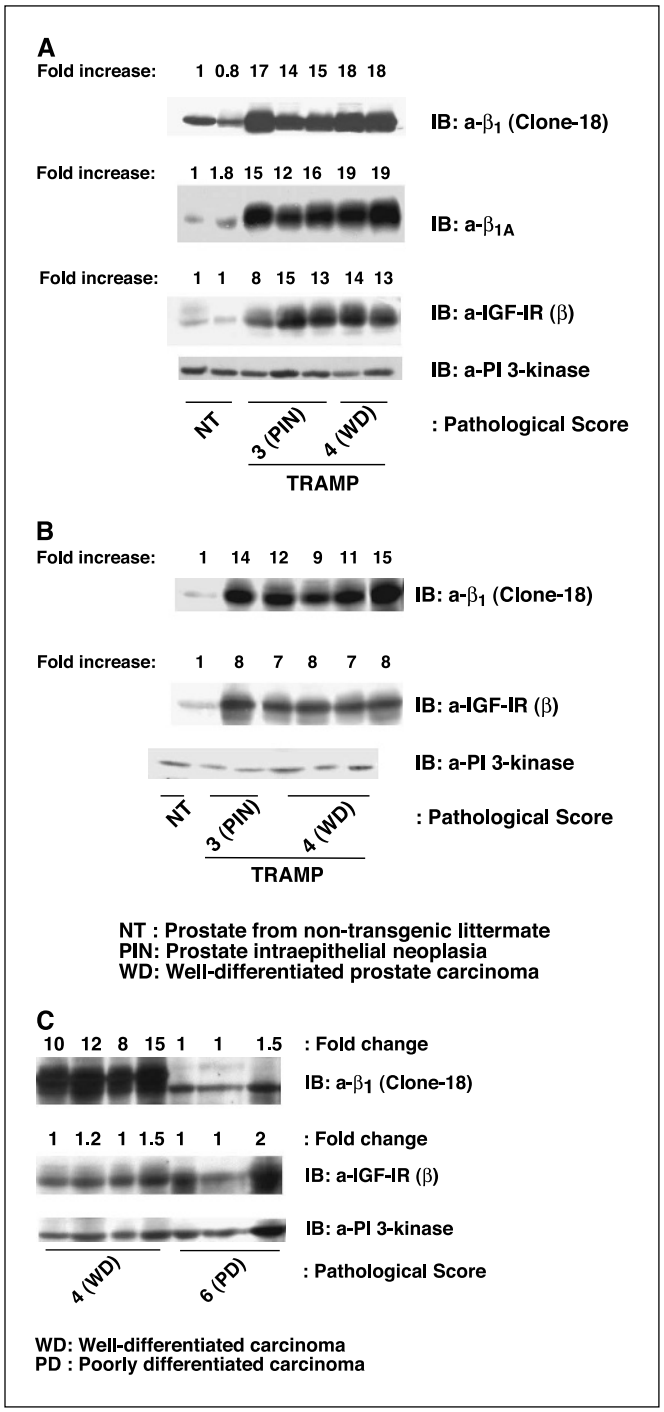

Figure 3. β_{1A} integrin and IGF-IR are upregulated in high-grade PIN and WD TRAMP prostatic adenocarcinoma. Prostate tumor lysates from 28-week-old TRAMP mice and their corresponding NT were separated using SDS-PAGE and proteins were immunoblotted with mAb to β_1 integrins (Clone-18), rabbit polyclonal antibody to β_{1A} integrin, to IGF-IR or, as loading control, mAb to PI3-K (p85). *A-B,* NT, grade 3 (PIN), grade 4 (WD). *C,* grade 6 (PD). The results are presented as fold increase obtained using densitometric analysis upon normalization. Two sets of prostate tissues in two separate gels.

enhanced expression of β_{1A} is not required to maintain the proliferative capacity of late-stage tumor cells (Fig. 3*C*). Immunohistochemical analysis of TRAMP prostate tissue sections was done in a qualitative manner to determine the localization of β_1 integrin. As shown in Fig. 4, we consistently found that the

β_1 integrin was expressed in epithelial and stroma cells in WD but was down-regulated in PD tissue specimens. These results indicate that a concurrent expression of IGF-IR and β_1 occurs in PIN and WD but not in PD prostate cancer in TRAMP mice.

Down-regulation of β_{1C} integrin and Gab1 expression in prostate carcinoma. The β_{1C} integrin is expressed in human nonproliferative, differentiated epithelium and is selectively down-regulated in prostatic adenocarcinoma (13–16). Similarly, we show here using the TRAMP model of progressive prostate carcinoma that β_{1C} is expressed in normal mouse prostate (Fig. 5*A*), but its expression is down-regulated in PIN, WD, and PD adenocarcinomas (Fig. 5*B*). Detergent lysates of PNT1A cells were used as a positive control (Fig. 5*B, lane 1*). In contrast, β_5 integrin expression was found in normal (NT) and TRAMP prostates at similar levels (Fig. 5*B*). These data indicate that selective down-regulation of β_{1C} is an early event during prostate cancer progression.

In our previous study, we have shown that β_{1C} recruits Gab1 to the cell surface and that Gab1 mediates β_{1C}-negative regulation of IGF-IR signaling (29). A potential correlation of the expression of β_{1C} and Gab1 was investigated. We analyzed Gab1 expression in tissue lysates from NT, and from PIN, WD and PD TRAMP prostates using an antibody to Gab1. Consistent with the expression profile of β_{1C} integrin, Gab1 seems down-regulated in all TRAMP prostate specimens of grades 3, 4, and 6 compared with normal (NT) prostate tissue specimens (Fig. 5*C*). These results indicate that expression of β_{1C} integrin and Gab1 correlates in both normal and neoplastic prostate tissues.

β_{1A} integrin recruits type 1 insulin-like growth factor receptor to focal contacts. In our previous study, we have shown that β_{1A} but not β_{1C} associates with IGF-IR and that β_{1C} expression down-regulates IGF-IR-mediated signaling (29). To investigate the mechanism through which β_1 integrin regulates IGF-IR signaling, we hypothesized that β_1 might regulate the localization of IGF-IR in specific cellular signaling compartments. The experiments were done using GD25 cells that do not express β_1 integrin, stably or transiently transfected with β_{1A} or β_{1C}. In β_{1A} stable transfectants, we found that IGF-IR was localized in focal contacts, where vinculin (Fig. 6*A-C*), β_{1A} and several signaling molecules, such as Src and focal adhesion kinase (45), are known to be localized in spread cells. In transiently transfected cells, β_{1A} and IGF-IR colocalized in focal contacts (Fig. 6*D-F*), whereas upon β_{1C} expression, IGF-IR was not found to be localized in focal contacts (Fig. 6*G-I, arrow*). Moreover, in nontransfected GD25 cells, the IGF-IR was found to be diffuse and not localized to focal contacts, suggesting that β_{1A} integrin is essential for the localization of IGF-IR to focal contacts (Fig. 6*G-I, arrowhead*). In conclusion, these data indicate that IGF-IR localization is controlled by specific β_1 integrins.

Discussion

The novel observation of this study is that down-regulation of the β_{1A} integrin via siRNA oligonucleotides results in inhibition of IGF-IR-mediated cell proliferation and anchorage-independent growth. It is also shown that whereas β_{1A} and IGF-IR are up-regulated, the β_{1C} integrin cytoplasmic variant and Gab1, a β_{1C} downstream effector, are down-regulated during prostate cancer progression. Finally, β_{1A}'s ability to modulate IGF-IR localization in focal contact signaling compartments, is shown to be the mechanism through which β_{1A} controls IGF-IR signaling.

This is the first study that uses siRNA oligonucleotides to integrins to prove a role for the integrin/growth factor receptor cross-talk in prostate cancer and suggests the use of siRNA to down-regulate integrins as a novel therapeutic approach for this disease. The results show that IGF-mitogenic and transforming activities but neither basal proliferation nor cell viability are prevented by siRNA to β_{1A} integrin. Based on this observation, we conclude that β_{1A} expression is essential to activate IGF-IR.

It is well established that activated IGF-IR sends signals required for growth and transformation (41). Our results indicate that the synergistic action of IGF-IR and β_{1A} in stimulating mitogenic signals during initiation of carcinogenesis is required. In conclusion, a model can be formulated in which differential expression and function of β_1 integrins in prostate cancer modulates cell responses to IGF via IGF-IR. We suggest a paradigm shift that changes the point of view that (a) β_1 integrins per se contribute to tumor progression (46, 47) and that (b) the IGF-IR per se contributes to tumor progression (18). By elucidating the players that contribute to prostate cancer progression, future studies will provide new insights into the current therapeutic approaches that target either integrins or IGF-IR.

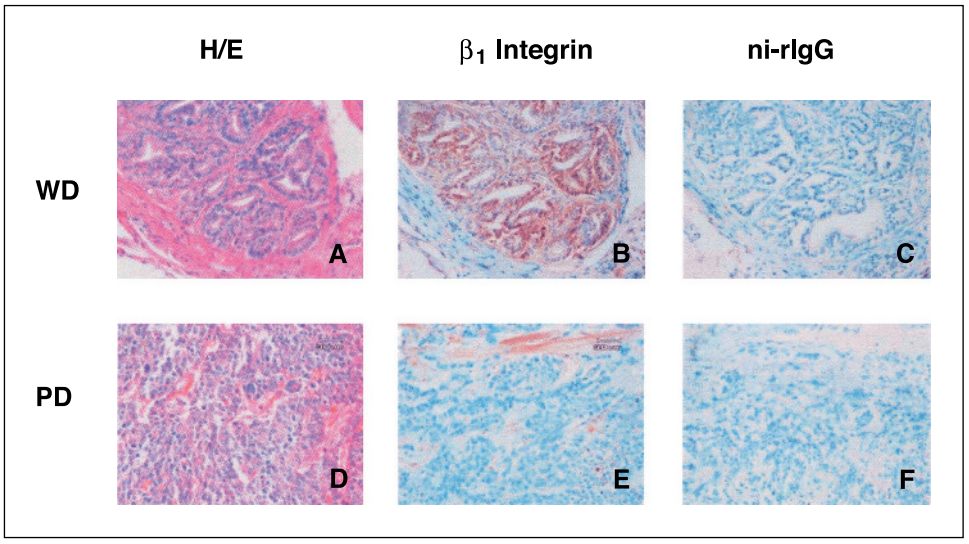

Figure 4. β_1 integrin is down-regulated in PD TRAMP prostatic adenocarcinoma. Paraffin sections (4-5 μm) of DLP from 28-week-old TRAMP mice were stained with H&E (*A* and *D*), antibodies against β_1 integrin (*B* and *E*) or ni-rIgG (*C* and *F*). *A-C,* grade 4 (WD); *D-F,* grade 6 (PD). Magnification, 20×.

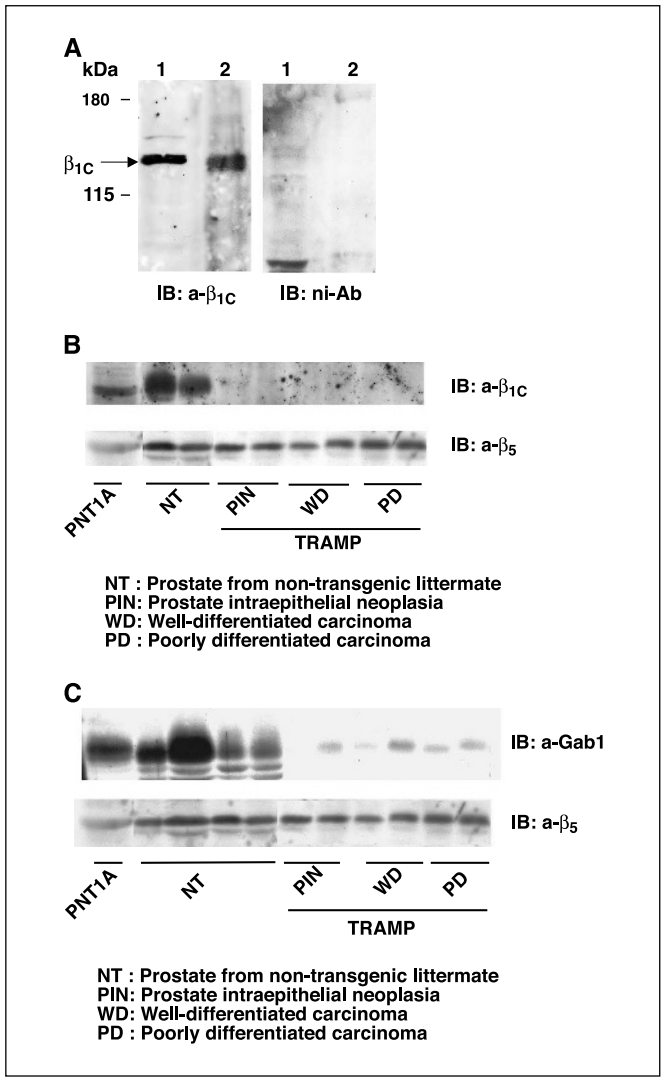

Figure 5. Down-regulation of β_{1C} integrin and Gab1 in PIN, WD, and PD TRAMP prostate tissues. *A,* PNT1A cell lysate (*lane 1*) or tissue extract from normal mouse prostate (*lane 2*) were separated using a 7.5% SDS-PAGE and proteins were immunoblotted with mAb to β_{1C} integrin or nonimmune antibody (ni-Ab). *B,* PNT1A cell lysate or prostate tumor lysates from 28-week-old TRAMP mice and their corresponding NT were separated using SDS-PAGE and proteins were immunoblotted with rabbit polyclonal antibody to β_{1C} integrin or β_5 integrin. *C,* PNT1A cell lysate or prostate tumor lysates from 28-week-old TRAMP mice and their corresponding NT were separated using SDS-PAGE and proteins were immunoblotted with rabbit polyclonal antibody to Gab1 or β_5 integrin.

The molecular basis and the cross-talk between the β_1 integrins and IGF-IR in prostate cancer progression have not been investigated. Our previous study has unraveled a novel mechanism mediated by β_1 integrin cytoplasmic variants that differentially regulates IGF-mediated prostate cell adhesion to a basement membrane protein, laminin (29). The relevance of this observation to prostate cancer, where up-regulation of the IGF-IR has been described (24), is that β_{1A} by limiting cancer cell adhesion to basement membrane proteins and promoting cell proliferation in response to IGF presumably allows the tumor mass to expand and invade. In contrast, β_{1C}, a cytoplasmic variant known to be down-regulated in prostate cancer, has the opposite effect; it completely prevents IGF-mediated cell proliferation and tumor growth, and promotes cell adhesion to laminin. Interestingly, the loss of IGF-IR in the adult prostate seems to cause the cells to become greatly disorganized and loose attachment to basement membrane while remaining viable.[4] Consistent with the hypothesis that β_{1A} integrin and IGF-IR act as a complex that promotes cell proliferation but inhibits cell adhesion to basement membrane, in this study, β_{1A} and IGF-IR are found to be concurrently up-regulated in TRAMP prostate tissues in the early stages of neoplastic transformation, when disruption of the basement membrane occurs.

We describe a concurrent increase in expression of both β_{1A} integrin and IGF-IR in PIN and WD tumors in a defined model of prostate cancer, designated TRAMP; in contrast, PD tumors, where β_{1A} is down-regulated, no longer show this correlation. As previously described for human prostate cancer (7), the present study shows that the expression of β_1 (β_{1A} and β_{1C}) is altered during cancer progression in TRAMP mice. The findings show, specifically, that β_{1A} integrin expression is up-regulated, whereas β_{1C} is down-regulated during prostate tumor progression. This suggests that up-regulated expression of β_{1A} and IGF-IR might contribute to uncontrolled cell division during initiation of prostate cancer; however, increased levels of both molecules are not required in the late stages of tumor progression. It is, thus, expected that lack of expression of β_{1A} will adversely affect initiation of prostate cancer growth. This is likely to be the result because, similarly, targeted disruption of β_1 has been recently shown to prevent initiation of mammary tumorigenesis *in vivo* in a transgenic mouse model (47).

The mechanism by which the β_1 integrin cytodomain differentially modulates prostate cancer cell functions in response to IGF and allows IGF-IR to dissect downstream signaling pathways occurs via differential recruitment of IGF-IR downstream effectors to the plasma membrane. A specific association between integrins and IGF-IR effector molecules, specifically β_{1A} and IRS-1, β_3 integrins and IRS-1, β_3 integrins and DOK1, and β_{1C} and Gab1/Shp2-phosphatase complex, is likely to explain many activities attributed to IGF-IR (29, 48–50). In this study, we show that both β_1 integrins and IGF-IR are found to be colocalized in focal contacts where a significant amount of integrin downstream signaling is coordinated. In contrast, expression of β_{1C} that is known to remain diffuse on the cell surface, prevents, or is not able to support IGF-IR localization in focal contacts. The relevance of these findings to prostate cancer is high because both β_{1C} and Gab1 are down-regulated in prostate cancer when β_{1A} is up-regulated. Presumably, in normal tissues, by preventing IGF-IR localization to focal contacts, β_{1C} potentiates Gab1/Shp2 signaling that in turn keeps IGF-IR in a nonphosphorylated state. In contrast, in neoplastic tissues, an appropriate localization of IGF-IR in focal contacts mediated by β_{1A} association with IGF-IR maintains IRS-1 signaling active and supports tumor progression.

In conclusion, deregulated expression of integrins and of IGF-IR and deregulation of their cross-talk is likely to be responsible for several alterations of cell functions and intracellular signaling in prostate cancer. Therefore, these alterations should be regarded as highly significant events in the search for novel therapeutic targets in prostate cancer.

[4] N.M. Greenberg, unpublished results.

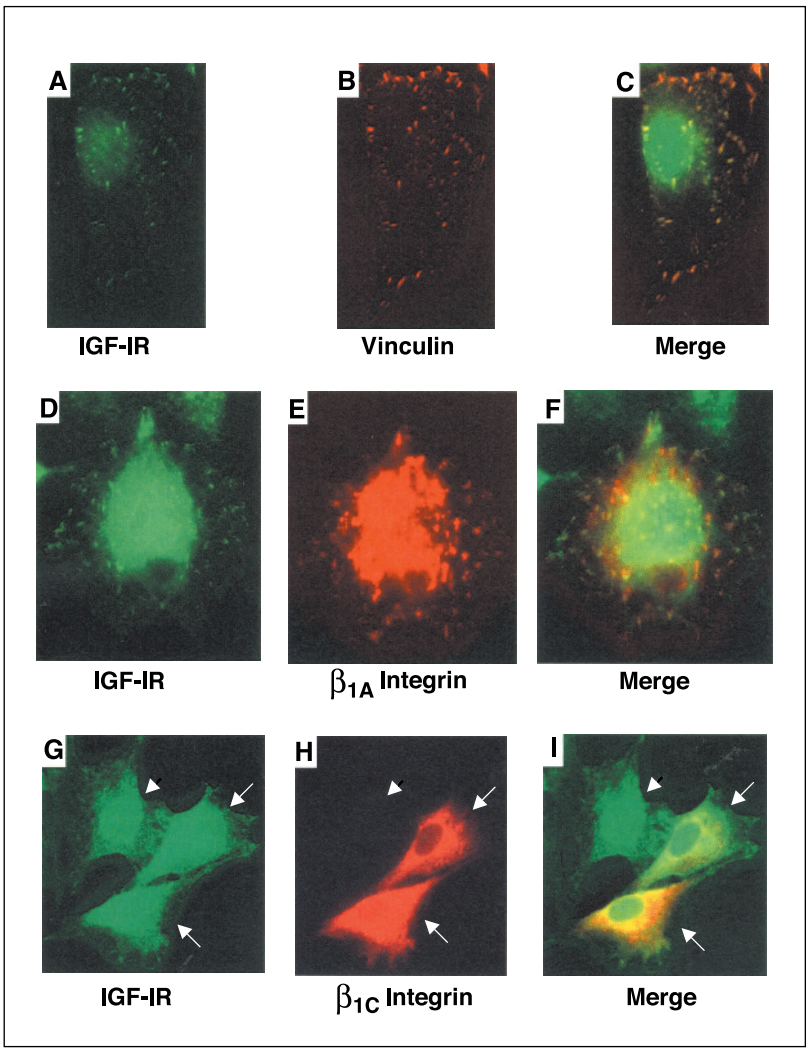

Figure 6. β_{1A} integrin but not β_{1C} recruits IGF-IR to focal contacts. *A-C,* GD25 cells stably transfected with human β_{1A} integrin were stained with antibodies to IGF-IR (*green*) or vinculin (*red*); image-merging analysis of vinculin and IGF-IR (*yellow*). *D-I,* GD25 cells transiently transfected with human β_{1A} (*D-F*) or β_{1C} (*G-I, arrow*) integrin were stained with antibodies to IGF-IR (*green*) or to β_1 integrins (*red*); image-merging analysis of β_1 integrins and IGF-IR (*yellow*). *G-I,* nontransfected GD25 cell (*arrowhead*). The experiments were repeated 10 times with similar results.

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