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Insulin-Like Growth Factor 1 Stimulation of Androgen Receptor Activity Requires β_{1A} Integrins

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Abstract

Despite the findings that β_1 integrins play a vital role in the regulation of cell proliferation and survival, the mechanisms through which they operate and lead to cancer progression remain elusive. Previously, our laboratory has shown that β_{1A} integrins support insulin-like growth factor 1 (IGF1)-mediated mitogenic and transforming activities. Here we report that β_{1A} integrins regulate *basal* levels of IGF-IR, although they are not critical for maintaining cancer cell morphology. Upon transfection of β_{1A} siRNA and consequent downregulation of IGF-IR, we show inhibition of anchorage-independent growth of prostate cancer cells, a function which is dependent on IGF-IR expression. In addition, we demonstrate that IGF1-mediated activation of androgen receptor (AR), known to occur in prostate cancer cells, requires expression of β_{1A} integrins as evaluated by luciferase reporter assays and immunoblotting analysis. Since β_{1A} integrin levels are increased by R1881 or dihydrotestosterone (DHT), our results imply that β_{1A} integrins support an androgen-enhanced feedback loop that regulates the expression of IGF-IR. β_{1A} integrins also regulate *inducible* levels of IGF-IR in cells stimulated by androgen or by a combination of androgen and IGF1, as evaluated by flow cytometric analysis and immunoblotting. Furthermore, upon transfection of β_{1A} siRNA and consequent downregulation of IGF-IR, neither activation of AKT, an effector of IGF-IR, nor AR levels are affected. We conclude that β_{1A} integrin expression is critical for maintaining the regulatory crosstalk between IGF-IR and AR.

Keywords

β_{1A} integrins; IGF-IR; AR - mediated transcriptional activation; AKT; PSA

Introduction

Prostate cancer is one of the leading types of cancer in males in the United States (Jemal et al., 2010). In addition to the well-established role of genetic events, the interplay of extracellular matrix and growth factor receptor signaling contribute significantly to the development of this disease (Alam et al., 2007).

AR plays a central role in prostate carcinogenesis and appears to be required for the growth of prostatic adenocarcinoma, even in a hormone refractory state (Reddy et al., 2006). Experimental evidence indicates that prostate tumor growth *in vitro* and *in vivo* is inhibited by chemopreventive drugs or antisense oligonucleotides that downregulate androgen receptor (AR) expression (Culig and Bartsch, 2006). However, changes in AR activity and/

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or associated regulatory pathways are known to induce aberrant receptor activity and enable cancer to grow in a therapy-resistant manner (Knudsen and Penning, 2010). The role played by epithelial cell differentiation signals or growth factor receptors and their effect on AR activity might be crucial in prostate cancer development. Prostate gland development is known to involve IGFI and TGF β signaling and compromised receptor signaling may lead to disease progression (Prins and Putz, 2008; Kyprianou and Isaacs, 1988).

IGF-IR is a transmembrane glycoprotein, frequently upregulated in cancers. This receptor is known to play a critical role in cell survival and malignant transformation and its importance in oncology is highlighted by the use of antibodies targeting this receptor in several clinical trials (Baserga, 2009). Traditionally known to signal from the cell surface, IGF-IR was recently reported to be translocated to the nucleus (Aleksic et al., 2010; Sehat et al., 2010). Our understanding of the crosstalk between IGFI and AR signaling is limited and might be critical for regulating prostate cancer progression. Using exogenous induction of AR, Plymate et al. reported that IGFI enhanced DHT-stimulated AR transcriptional activity in M12-derived prostate cancer cells (Plymate et al., 2004). It was shown that IGF-IR signaling may modulate AR compartmentation and thus alter AR activity (Wu et al., 2006). The ability of IGFI to influence androgen signaling needs further investigation in order to identify molecular mediators of this crosstalk and potentially develop specific strategies for targeted therapy.

We have previously reported that the activity of IGF-IR in prostate cancer is regulated by β_{1A} integrins and that the expression levels of both β_{1A} integrins and IGF-IR are significantly upregulated in the TRAMP (transgenic adenocarcinoma of the mouse prostate) model of prostate cancer (Goel et al., 2005). Integrins are predominant receptors of extracellular matrix (ECM) proteins and aberrant integrin/ECM interactions are known to contribute to cancer progression (Boudreau and Bissell, 1998; Fornaro et al., 2001; Goel et al., 2008). These receptors are hetero-dimers consisting of α and β subunits; 18 α and 8 β subunits are known (Alam et al., 2007). Five β_1 variant subunits β_{1A} , β_{1B} , β_{1C} , β_{1C-2} and β_{1D} generated by alternative splicing have been described. Among these, two variants β_{1C} and β_{1A} , have been shown to be expressed in prostatic epithelium. β_{1A} is consistently detected in prostate cancer while β_{1C} is expressed in normal prostatic epithelial cells, but substantially downregulated in adenocarcinoma (Fornaro et al., 1999; Perlino et al., 2000). Since IGF-IR signaling has been reported to control the cellular localization of AR and its activity in prostate cancer cells (Wu et al., 2006), elucidating the crosstalk between integrins and IGF-IR in regulating AR activity remains critical.

Here, we demonstrate that the β_{1A} integrins are required to mediate IGF-IR regulation of AR activity in prostate cancer cells. This mechanism may thus have an important role in aberrant growth of prostate carcinoma cells.

Materials and Methods

Reagents and antibodies

The following reagents were used. RPMI-1640, Opti-Mem and oligofectamine (all from Invitrogen, CA), synthetic androgen R1881 (Perkin-Elmer, CA), DHT and proteinase inhibitors (Sigma, St Louis, MO), recombinant IGFI (R&D Systems, MN), Luciferase Assay Kit (Promega, Madison, WI). The following murine monoclonal antibodies (mAbs) were used: to β_1 integrins (BD Transduction Laboratories, San Jose, CA), to IGF-IR for flow cytometry (α IR-3, EMD, NJ). The following rabbit polyclonal Abs (pAbs) were used: to IGF-IR (IGF-IR- β sc713), AR (sc441), AKT, phospho AKT (Ser 473) and ERK1 (all from Santa Cruz, CA), and to prostate-specific antigen (PSA) (Dako Cytomation, Carpinteria, CA). siRNA oligonucleotides were purchased from Integrated DNA Technologies Inc. (IDT,

San Diego, CA). The sequence of siRNAs used are as follows: β_{1A} -1 integrin: 5'-AUGGGACACGGGUGAAAAUTT-3'; β_{1A} -2 integrin: 5'-AAUGUAACCAACCGUAGCATT-3'; β_{1A} -3 integrin: 5'-GGAACAGCAGAGAAGCUCATT-3'; and β_{1C} integrin: 5'-CCUCUGACUCCAGAUUCCTT-3'.

Cells

LNCaP and TRAMP-C2 cells were purchased from ATCC. LNCaP cells were grown at 37°C and 5% CO₂ in RPMI-1640 supplemented with 5% FBS and 1% (v/v) each of sodium pyruvate, HEPES and non-essential amino acids (Invitrogen). To evaluate the effect of agonists, cells were maintained in 2–5% charcoal-stripped serum containing medium throughout the duration of the experiment. TRAMP-C2 cells were cultured as described (Foster et al., 1997).

Transient siRNA transfection

Transfection of cells with siRNA oligonucleotides was performed as described (Goel et al., 2005).

Anchorage-independent growth assay

Cell growth in soft agar was assayed as described (Goel et al., 2005).

Immunoblotting

Cell lysates were used for immunoblotting as described (Goel et al., 2004).

Luciferase assay

Cells were plated in RPMI medium supplemented with 5% FBS in 60 mm dishes for 48 h and grown to 70% confluency prior to transfection. Cells were transfected with siRNA, as described above, and retransfected with the following vectors: pGL3-(ARE)4-luciferase carrying four contiguous androgen-response elements (ARE) fused to the luciferase gene (Lu et al., 2001), and pCMV- β -galactosidase reporter (Promega). The cells were then treated with 1 nM R1881, or vehicle control, for further 24 h and harvested for luciferase activity. Each sample was analyzed in triplicate using Luciferase Assay Kit and values were normalized against the β -galactosidase activity assayed using the Galacto-Lite Plus reagent (Tropix, Bedford, MA).

Quantitative real time PCR

Total RNA was isolated from cells by RNeasy mini kit (Qiagen, Valencia, CA). 2.5 μ g RNA were subjected to DNase treatment followed by conversion to cDNA using oligo dT, random hexamers, dNTPs and Superscript III (Invitrogen, Carlsbad, CA). The cDNA was diluted to 12.5 ng/ μ l final concentration and 50 ng of cDNA from each sample were subjected to real time PCR using sequence specific oligos (IDT), SYBR Green master mix (ABI, Carlsbad, CA) and ABI 7500 real time system. In order to ensure the specificity of oligos, the dissociation curves of PCR products, at the end of PCR, were studied before analyzing the real time data. ΔC_t values were calculated by subtracting the Threshold Cycle (C_t) values of β -actin from C_t values of target genes. Further, $\Delta\Delta C_t$ values were calculated either by subtracting the ΔC_t value of vehicle treated samples from those of agonist treated samples or in case of transfection by subtracting ΔC_t value of β_{1C} siRNA transfected samples from those of β_{1A} siRNA transfected samples. Relative expression was calculated by using the formula $2^{-\Delta\Delta C_t}$. Each reaction was carried out, at least, in triplicate; standard deviations and significance were calculated using Excel software. The sequences of oligos

used are as follows: PSA, (sense: AGGTCAGCCACAGCTTCCCA, antisense: GGGCAGGTCCATGACCTTCA), transmembrane protease serine 2 (TMPRSS2), (sense: GCAAGTGCTCCAACCTCTGGG, antisense: GTCGTCTTGGCACACAGGGT), IGF-IR, (sense: AATGAGTGCTGCCACCCCGA, antisense: ACACAGCGCCAGCCCTCAAA), β -actin, (sense: TCCATCATGAAGTGTGACGT, antisense: GGAGGAGCAATGATCTTGAT).

FACS analysis

The cells were harvested and stained with 1 μ g/ml Ab to IGF-IR or rabbit IgG as negative control, followed by staining with FITC-conjugated secondary Ab. Expression profiles were acquired using FACS Calibur instrument (BD) and data were analyzed by Flowjo software (Tree Star, Inc., OR).

Statistical analysis

Statistical significance (P value and t-test) between datasets was calculated using Excel software. A two-sided P-value of less than 0.02 was considered statistically significant. The results were plotted on a graph using DeltaGraph 4.5 (RockWare, Golden, CO) software.

Results

β_{1A} integrins regulate IGF-IR expression and anchorage-independent growth in prostate cancer cells

To determine the role of β_{1A} integrins on IGF-IR expression, LNCaP cells were transfected with β_{1A} integrin siRNA followed by treatment with R1881, a synthetic androgen. A significant decrease in IGF-IR protein levels is observed upon β_{1A} integrin downregulation but not upon transfection of a control siRNA to the β_{1C} integrin isoform, which is absent in LNCaP cells (Fig. 1A). Since β_{1A} integrins regulate cytoskeletal architecture, we studied the effect of their downregulation on cellular morphology. Phase contrast analysis shows that the morphology of LNCaP cells transfected with siRNA to β_{1A} integrins was comparable to cells transfected with a control siRNA to the β_{1C} integrin isoform (Fig. 1B). To study the effect of loss of the β_{1A} /IGF-IR complex on aberrant growth, β_{1A} integrin expression in LNCaP cells was downregulated and cells grown in soft agar until the formation of visible colonies (Fig. 1C). Counting in three representative optical fields indicate that β_{1A} integrin downregulation leads to more than 95% reduction in the number of colonies, whereas β_{1C} siRNA transfected cells showed a 20% reduction in average number of colonies when compared to untransfected cells. This significant decrease in colony formation upon abrogation of β_{1A} integrin expression suggests that the β_{1A} integrin/IGF-IR complex is critical for the anchorage-independent growth of prostate cancer cells.

The β_{1A} /IGF-IR complex synergistically regulates AR activity upon androgen stimulation

In order to study the effect of β_{1A} integrins on the functional interaction between IGF-IR and AR, promoter based AR reporter activity was evaluated following β_{1A} downregulation in LNCaP cells, which require androgen stimulation to elicit measurable AR activity. To start with, agonist-dependent AR activation was measured in LNCaP cells. Cells were transfected with pGL3-(ARE) 4-luciferase and pCMV- β -galactosidase plasmids, followed by treatments with R1881 and/or IGFI. A synergistic response to these agonists on AR activity was observed (Fig. 2A). The effect of β_{1A} integrins on AR activity was tested by analyzing PSA levels by immunoblotting and we observe a significant reduction in PSA levels upon β_{1A} integrin downregulation (Fig. 2B). To compare the extent of functional changes upon β_{1A} integrin downregulation, three independent β_{1A} -1, -2 and -3 siRNAs were transfected in LNCaP cells and evaluated for influencing AR functions in AR-

dependent reporter assay in presence of R1881. The data indicate that AR transcriptional activity was reduced by 75% or more by these siRNAs (Fig. 2C) and β_{1A} siRNA -1 was employed for all knockdown studies. To further confirm the regulation of AR activity by β_{1A} integrins, LNCaP cells were transfected with β_{1A} integrin siRNA and re-transfected with pGL3-(ARE) 4-luciferase and pCMV- β -galactosidase plasmids, followed by treatment with physiological androgen DHT and/or IGFI. The data shown in Fig. 2D, reproducibly demonstrate a synergistic increase in the transcriptional activity of AR upon treatment of cells with DHT and IGFI. Agonist-induced transcriptional activation is substantially alleviated upon abrogation of β_{1A} integrin expression. Similar results are observed using TRAMP-C2 cells (data not shown). These data demonstrate that β_{1A} integrins are essential for IGF-IR mediated regulation of AR.

Androgen upregulates IGF-IR and β_{1A} integrin expression

Agonist-induced expression of β_{1A} and IGF-IR was analyzed in LNCaP cells treated with R1881 or DHT. Our data indicate that the levels of IGF-IR and AR-regulated PSA and TMPRSS2 transcripts are induced upon androgen treatment, as determined by quantitative real time PCR (Fig. 3A). Real time data was validated by evaluating the expression profile in LNCaP cells by immunoblotting. Figs. 3B and 3C indicate that R1881 not only elicits a significant induction of IGF-IR and PSA protein levels, but induction of β_{1A} integrin subunit is also observed. The experiment was repeated in presence of the physiological androgen DHT and similar induction of PSA, IGF-IR and β_{1A} is observed in LNCaP cells upon DHT treatment (Fig. 3D). These data strongly point to the role of androgens in IGF-IR and β_{1A} integrin signaling in prostate cancer cells.

Androgen-mediated upregulation of IGF-IR cell surface expression depends on β_{1A} integrins

After demonstrating the effect of loss of β_{1A} integrins on total IGF-IR expression and AR signaling, we analyzed expression of IGF-IR at the cell surface. LNCaP cells were transfected with β_{1A} siRNA and treated with 1 nM R1881, with or without 100 ng/ml IGFI, for 24 h and the expression profile was studied by flow cytometry. R1881 significantly induces IGF-IR surface expression (Fig. 4A top and middle, left panels) which is lost after β_{1A} integrin downregulation (Fig. 4A top and middle, right panels). A similar result is observed using R1881 in combination with IGFI (Fig. 4A bottom panels). This implies that androgen stimulation elicits a β_{1A} integrin-dependent feedback loop to maintain IGF-IR expression at the cell surface.

Understanding the β_{1A} integrin/IGF-IR complex-mediated downstream signaling is critical to dissect the mechanism underlying their physiological functions. Since AKT is known to play a critical role in IGF-IR signaling (Metalli et al., 2010), we investigated if the loss of IGF-IR correlated with reduced AKT phosphorylation. We observe that protein levels of total AKT and p-AKT are not affected in the presence of androgen after β_{1A} integrin downregulation (Fig. 4B). We then, investigated whether β_{1A} integrin downregulation reduces AR levels and show that β_{1A} integrins do not modulate AR protein levels (Fig. 4C). Collectively, these data suggest that IGF-IR expression at the cell surface is regulated by β_{1A} integrins and imply that both of these receptors work as a complex to regulate downstream AR transcriptional activity.

Discussion

In this study, we demonstrate that the functional integrity of β_{1A} integrins is critical for maintaining IGF-IR expression and functions, specifically anchorage-independent growth and regulation of AR activity stimulated by IGFI. Our unique finding of a regulatory

crosstalk between β_{1A} integrins, IGF-IR and AR has important implications for cancer therapy.

Regulation of IGF-IR expression by β_{1A} integrins

The crosstalk between integrin receptors and activated growth factor receptors is likely to play a critical role in the initiation and progression of human cancer. β_{1A} integrins are known to associate with IGF-IR and mediate its localization in focal contacts (Goel et al., 2005). This relocalization of IGF-IR mediated by β_{1A} integrins sustains IGF-IR/IRS-1 signaling required for tumor progression. In this paper, we show that the expression of IGF-IR is tightly regulated by β_{1A} integrins. Whether this effect depends on changes in transcriptional regulation of IGF-IR remains to be established, although it is conceivable that β_{1A} integrin/IGF-IR reported colocalization in focal contacts may affect IGF-IR protein turnover.

Regulation of androgen receptor activity by the β_{1A} integrin/IGF-IR complex

Here, we demonstrate that β_{1A} integrins are essential for IGF-IR mediated AR signaling. Our novel data show a striking decrease in IGF-IR protein levels and a consequent decrease in AR transcriptional activity induced by IGFI upon β_{1A} integrin downregulation, and imply that this downstream physiological effect is a reflection of aberrant receptor expression levels. Our laboratory has previously reported that the expression of β_{1A} integrins is crucial for IGF-IR mediated mitogenic and transforming activities in prostate cancer (Goel et al., 2005). Using prostate tumor lysates from age-matched mice at different stages of prostate cancer, the protein levels of β_{1A} and IGF-IR were reported to be concurrently increased in early stages of neoplastic transformation. It could be speculated that this receptor upregulation might work as a mechanism to promote disease progression to a state of androgen independence. Activation of AR in the aggressive disease is known to occur as a result of increased sensitivity of AR, activating point mutations and in response to non-steroidal compounds (Culig, 2004). In these androgen-independent tumors alternative means of AR activation have been invoked. Overexpression of Her2/neu/cErbB2, a type I tyrosine kinase and a member of ErbB receptor family, has been implicated in the activation of AR through MAP kinase/AKT pathway leading to hormone-independent prostate cancer growth (Craft et al., 1999; Wen et al., 2000; Yeh et al., 1999). Besides, the pro-inflammatory cytokine, Interleukin 6 (IL-6) has been reported to regulate AR activity and cause growth of AR positive tumors (Culig et al., 2002; Malinowska et al., 2009). Synergizing with low levels of androgen, the neuropeptide growth factor bombesin, was reported to confer AR mediated transcriptional activation (Dai et al., 2002). In addition to recapitulating DHT mechanism, bombesin was shown to result in AR recruitment to specific ARE sites and assemble a divergent transcriptional complex (Desai et al., 2006). All these reports suggest unique mechanisms of AR activation, which are critical in promoting ablation resistant survival of prostate cancer cells. The synergistic involvement of IGF-IR and β_{1A} integrins in regulating AR function suggests that these receptors are likely to play a role in progression to androgen-independent disease.

Abrogating the expression of β_{1A} integrins elicits a striking decrease in IGFI-mediated AR targets as observed in human prostate cancer LNCaP and TRAMP-C2 cell systems. Several possibilities could account for the observed IGFI-mediated regulation of AR activity by β_{1A} integrins. We exclude the possibility that β_{1A} integrins might directly, or indirectly, regulate AR expression; our data show that AR protein levels are not affected upon β_{1A} integrin knockdown (Fig. 4C). Another possible explanation for the observed effect of β_{1A} integrins on AR activity may be via β_{1A} 's influence on selective recruitment of co-activators and/or co-repressors, to the AR-responsive gene promoters (Taplin and Balk, 2004). Several of these AR-associated proteins co-localize with AR in the nucleus and enhance, or suppress,

its activity. The examples are: Supravillin (Ting et al., 2002), Gelsolin (Nishimura et al., 2003), ARA55 (Rahman et al., 2003), and Hsp90 (Fang et al., 1996). As a result of altered co-factor recruitment, β_{1A} integrins may modulate AR cellular localization, and as described for other nuclear receptors (Singh and Kumar, 2005), the nuclear localization of AR may affect its transcriptional activity (Wu et al., 2006). The mechanisms regulating this pathway need to be further investigated.

Androgen signaling regulates β_{1A} integrin/IGF-IR levels: a feedback mechanism

Our data show that androgen signaling operates through a feedback mechanism to regulate IGF-IR and β_{1A} integrin levels. We demonstrate a significant androgen-mediated induction of IGF-IR, β_{1A} integrins and AR-regulated genes, underscoring the importance of IGF-IR and β_{1A} integrins in AR signaling. Our data are consistent with previous reports, whereby androgen treatment of prostate cancer cells was shown to modulate the expression levels of IGF-IR (Pandini et al., 2005). These authors reported that AR positive LNCaP cells and AR transfected PC-3 cells show increased IGF-IR protein levels upon androgen treatment. The data clearly suggest a functional crosstalk between AR and IGF-IR in prostate cancer, and we demonstrate that the concerted signaling by these receptors is, in turn, regulated by β_{1A} integrins. Androgen signaling not only regulates IGF-IR expression, it also maintains this feedback loop by enhancing expression of β_{1A} integrins (Fig. 5).

Androgen mediated regulation of IGF-IR might involve transcriptional and/or post-translational mechanisms. We did not observe any consensus androgen response elements in either IGF-IR or β_{1A} integrin promoters; however, typical and atypical half sites were observed which suggest potential transcriptional regulatory mechanisms. Transcriptional regulation of IGF-IR was reported recently when it was demonstrated that androgens induce cyclic AMP response-element binding protein (CREB) activation through c-Src-dependent ERK 1/2 activation, and a CREB-binding site was identified in the 5'-UTR region fragment of IGF-IR promoter (Genua et al., 2009). It has been reported that androgen induced upregulation of IGF-IR involves the activation of a Src-ERK pathway independent of AR binding to specific DNA response elements (Pandini et al., 2005). Post-translational mechanisms have also been reported; IGF-IR ubiquitination and degradation by the proteasome system has been shown to occur in melanocytes (Girnita et al., 2003). In another study, the adaptor protein Grb 10 in complex with E3 ligase family member Nedd4 was also reported to regulate ligand-induced ubiquitination and stability of IGF-IR (Monami et al., 2008; Vecchione et al., 2003). The transcriptional, translational or post-translational mechanisms underlying androgen signaling to IGF-IR and β_{1A} integrins are not known at this time.

We, surprisingly, observe that activation of AKT, an important effector of IGF-IR signaling (Peruzzi et al., 1999), is not affected by the downregulation of the β_{1A} /IGF-IR complex. Aberrant function of serine/threonine kinase AKT is central to the pathophysiology of prostate cancer (Manning and Cantley, 2007). This demonstrates that AKT, known to be constitutively active in prostate cancer cells and reported to mediate phosphorylation of AR at serine 210 and serine 790 residues leading to suppression of AR activity in prostate cancer PC3 cells (Lin et al., 2001), is not sufficient to mediate anchorage-independent growth in the absence of the β_{1A} /IGF-IR complex.

In conclusion, the novel findings presented here imply that the β_{1A} integrins are required to mediate IGF-IR regulation of AR activity in prostate cancer cells. This study highlights the importance of adhesion receptors in cancer and implies that the requirement of β_{1A} integrin signaling pathways may apply to several "oncogenes" critical in human cancers. These findings open interesting possibilities for dissecting the signaling mechanisms mediated by β_{1A} integrins and IGF-IR in initiation and progression of prostate cancer.

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Fig. 1. β_{1A} integrins are required for regulation of IGF-IR expression and anchorage-independent growth

A: LNCaP cells were transfected two times for a total of 48 h with 100 nM β_{1A} siRNA. β_{1C} integrin siRNA was used as a control. The cells were serum starved (i.e., grown in medium containing charcoal-stripped serum) for 24 h and treated with 1 nM R1881 for additional 24 h. Cell lysates were analyzed for expression of β_{1A} integrin subunit and IGF-IR by immunoblotting. ERK1 was used as a loading control. **B:** In order to test if the β_{1A} integrin downregulation results in any morphological changes, LNCaP cells were transfected and treated as above and live cells visualized using a phase contrast microscope. Cells transfected with either siRNA showed comparable morphology. **C:** LNCaP cells transfected and treated as in Fig. 1A were plated on soft agar for anchorage-independent growth assay. After 14 days, colonies larger than 100 μm were counted in three different optical fields in each sample and the bars represent mean number of colonies with different treatments. Triplicate observations in three independent experiments were carried out with similar results. Data from one representative experiment are shown here. Asterisk sign indicates statistical significance ($P<0.001$) relative to β_{1C} integrin siRNA treatment.

Fig. 2. IGF-I induces AR activity in a β_{1A} integrin- dependent manner

A: LNCaP cells transfected with pGL3-(ARE)4-luciferase and CMV- β -galactosidase plasmids for 24 h followed by serum starvation or treatment with vehicle, 1 nM R1881 and/or 100 ng/ml IGF-I for 24 h. Lysates were used to measure luciferase activity and values were normalized against the β -galactosidase activity in each sample. Each experiment was run in triplicate and the data are presented as mean RLU's of three measurements. Asterisk signs represent statistical significance of $P<0.01$. **B:** LNCaP cells were transfected with β_{1A} or β_{1C} siRNA, serum starved as in Fig. 1A and treated with 1 nM R1881 and 100 ng/ml IGF-I for 24 h. Total cell lysates were analyzed by immunoblotting using Abs to IGF-IR, PSA and ERK1. **C:** LNCaP cells were transfected with 100nM siRNAs to β_{1A} integrins: β_{1A} siRNA-1, β_{1A} siRNA-2 or β_{1A} siRNA-3. After 24 h, cells were re-transfected with pGL3-(ARE)-luciferase and CMV- β -galactosidase plasmids and cell lysates were processed for luciferase assay. Asterisk signs represent statistical significance of $P<0.001$. **D:** LNCaP cells were transfected with β_{1A} integrin siRNA and re-transfected with pGL3-(ARE)4-luciferase and CMV- β -galactosidase plasmids for 24 h followed by serum starvation for 24

h and treatment with either vehicle, 10 nM DHT and/or 100 ng/ml IGFI for additional 24 h. Lysates were used to measure luciferase activity and values were normalized against the β -galactosidase activity in each sample. Each experiment was run in triplicate and the data are presented as mean RLU's of three independent measurements. Asterisk signs represent statistical significance of $P < 0.01$.

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Fig. 3. Androgen-mediated induction of IGF-IR and β_{1A} Integrin

A: Serum-starved LNCaP cells were treated with vehicle, 1 nM R1881 or 10 nM DHT, for 24 h. RNA isolated from these cells was evaluated for transcript levels of IGF-IR, PSA and TMPRSS2 using quantitative real time PCR. Expression values were normalized with transcript levels of β -actin and data are presented as relative expression. Each reaction was run in triplicate and asterisk signs on treatment bars indicate statistical significance ($P < 0.01$), relative to vehicle treatments. **B:** LNCaP cells plated in charcoal-stripped serum containing medium were treated with 1 nM R1881 for 24 h and IGF-IR protein levels were analyzed by immunoblotting. ERK1 was used as a loading control. **C:** LNCaP cells plated and treated as in Fig 3B and levels of β_{1A} integrin subunit and PSA analyzed by immunoblotting. ERK1 was used as a loading control. **D:** Cells were plated as in Fig 3B, treated with 10 nM DHT for 24 h and lysates prepared for expression analysis of β_{1A} integrins, IGF-IR and PSA by immunoblotting. ERK1 was used as a loading control.

Fig. 4. Androgen and IGF1-induced increase of IGF-IR surface expression is dependent on β_{1A} integrins

A: Quantitation of IGF-IR surface expression by flow cytometry. LNCaP cells were transfected as in Fig. 1A and treated with 1 nM R1881, a combination of 1 nM R1881 and 100 ng/ml IGF1 for 24 h or left untreated. Cells were harvested and stained with rabbit IgG or Ab to IGF-IR (1 μ g/ml) and analyzed by FACS. Representative histograms depict IGF-IR dependent expression upon various treatments. **B:** Loss of the β_{1A} integrins/IGF-IR complex is not associated with reduced AKT phosphorylation. LNCaP cells were plated in charcoal-stripped serum containing medium, transfected with siRNA as in Fig. 1A, and treated with 1 nM R1881 for 24 h. Total cell lysates were analyzed by immunoblotting using Abs to β_{1A} integrin subunit, IGF-IR, p-AKT (Ser 473) and total AKT. ERK1 was used as a loading control. **C:** AR expression is not affected by β_{1A} integrin downregulation. LNCaP cells were transfected and treated as in Fig 1A and lysates were analyzed for AR protein expression by immunoblotting. ERK1 was used as a loading control.

Fig. 5. Schematic model depicting the β_{1A} integrin-mediated feedback loop that links IGF-IR and AR

β_{1A} integrins regulate the expression of IGF-IR and the β_{1A} /IGF-IR complex synergistically induces AR mediated transcription. Androgen signaling operates through a feedback mechanism to regulate the expression of IGF-IR and β_{1A} integrins which consequently modulates AR activity. Overall, these regulatory mechanisms are central in regulating anchorage-independent growth.