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Androgen receptor expression in prostate carcinoma cells suppresses alpha6beta4 integrin-mediated invasive phenotype / BONACCORSI L; CARLONI V; MURATORI M; SALVADORI A; GIANNINI A; CARINI M; SERIO M; G. FORTI; BALDI E.. - In: ENDOCRINOLOGY. - ISSN 0013-7227. - STAMPA. - 141:(2000), pp. 3172-3182.

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# Androgen Receptor Expression in Prostate Carcinoma Cells Suppresses $\alpha 6\beta 4$ Integrin-Mediated Invasive Phenotype\*

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## ABSTRACT

Prostate cancer cells may lose androgen-sensitivity after androgen ablation therapy, becoming highly invasive and metastatic. The biological mechanisms responsible for higher tumorigenicity of androgen-independent prostate carcinomas are not entirely known. We demonstrate that androgen receptor regulation of adhesion and invasion of prostate cancer cells through modulation of  $\alpha 6\beta 4$  integrin expression may be one of the molecular mechanisms responsible of this phenomenon. We found that protein and gene expressions of  $\alpha 6$  and  $\beta 4$  subunits were strongly reduced in the androgen-sensitive cell line LNCaP respect to the androgen-independent PC3 and that trans-

fection of PC3 cells with a full-length androgen receptor expression vector resulted in a decreased expression of  $\alpha 6\beta 4$  integrin, reduced adhesion on laminin, and suppressed Matrigel invasion. Growth in soft agar was also suppressed in androgen receptor-positive PC3 clones. Treatment of androgen receptor positive clones with the synthetic androgen R1881 further reduced  $\alpha 6$  and  $\beta 4$  messenger RNA expression as well as adhesion on laminin and Matrigel invasion. Our results indicate that androgens regulate cell-extracellular matrix adhesion and invasion by modulation of integrin expression and function, thus keeping a low invasive phenotype of prostate cancer cells. (*Endocrinology* **141**: 3172–3182, 2000)

PROSTATE carcinoma is the most common malignancy and the second leading cause of cancer-related deaths in American men (1, 2). Because the vast majority of malignant prostatic tumors are androgen dependent, androgen ablation represents the primary therapy of locally advanced and metastatic tumors. However, approximately 20% of patients are refractory to the therapy, and, after some time of treatment, androgen-independent clones develop in most of the responsive patients within few months. Androgen-independent carcinomas are characterized by higher invasive and metastatic properties and are rapidly fatal. Elucidating the molecular basis that lead to loss of androgen-dependence as well as the pathways that are specifically regulated by this hormone has become one of the major tasks of current research on prostate carcinoma. By using the androgen-sensitive human prostate adenocarcinoma cell line LNCaP or androgen-receptor transfected cells, some androgen-dependent biological events, besides regulation of growth, have been described (3–8), but the full complexity of regulation of prostate cancer cell biology by androgens remains to be elucidated. A major point concerns the role of androgens in the regulation of interaction between prostate carcinoma cells

and extracellular matrix (ECM) as well as ability to invade the basement membrane, two processes that play an important role in modulating proliferation, invasion, and metastasis of cancer cells. Interaction between cell and ECM is mediated by a family of transmembrane glycoproteins termed integrins, heterodimers composed of noncovalently associated  $\alpha$  and  $\beta$  subunits (9). In the last few years, compelling evidence emerged on the role of integrins in the regulation of proliferation and invasion of carcinomas (10). In particular, the integrin  $\alpha 6\beta 4$ , one of the receptor for laminins, has been shown to play a pivotal role in migration and invasion of carcinoma cells (11–14). Indeed, while in epithelia,  $\alpha 6\beta 4$  mediates the formation of stable adhesive structures termed hemidesmosomes (15) that link intermediate filaments with extracellular matrix, in carcinoma cells, this integrin promotes the migration on laminin through association with actin cytoskeleton (11). Few studies have been performed so far on  $\alpha 6\beta 4$  integrin expression in prostate carcinoma cells (16–19).

In this study, we investigated whether androgens are involved in the regulation of the expression and function of  $\alpha 6\beta 4$  integrin in human prostate carcinoma cells. The effects of androgen on integrin expression and cell adhesion properties were studied in the androgen-sensitive carcinoma cell line LNCaP and in the androgen-independent PC3 cells transfected with a full-length human androgen receptor complementary DNA (cDNA). Our data demonstrate that expression of androgen receptor in transfected PC3 cells results in decreased levels of  $\alpha 6\beta 4$ , providing a possible explanation for the lower malignant phenotype (lower in-

Received January 7, 2000.

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\* This work was supported by Associazione Italiana Ricerca sul Cancro (AIRC, Milan), University of Florence, Ministry of University and Scientific Research (Progetto 40%), and Zeneca SpA (Basiglio, Milan, Italy).

vasion and anchorage-independent growth) observed in the cell line after transfection.

## Materials and Methods

### Antibodies and chemicals

Polyclonal antiandrogen receptor antibody (N-20) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse mAbs P1E6 and P1B5, directed against  $\alpha 2$  and  $\alpha 3$  were obtained from Life Technologies, Inc. (Gaithersburg, MD). Rat mAb anti- $\alpha 6$  (GoH3) was from Immunotech (Marseille, France). Mouse mAb UM-A9 (anti  $\beta 4$ ) was kindly provided by Dr. T. E. Carey (University of Michigan, Ann Arbor, MI) and rat mAb AIB2 (anti  $\beta 1$ ) by Dr. C. Damski (University of California, San Francisco, CA). Matrigel was from Collaborative Biomedical Products (Bedford, MA), and type I collagen was provided by Dr. D. Schuppan (Free University, Berlin, Germany). The antibiotic G418 (Geneticin) was obtained from Calbiochem (La Jolla, CA). Other reagents not specified were from Sigma (St. Louis, MO).

### Cell culture and transfection

Cell lines (LNCaP, DU145, and PC3) were obtained from American Type Culture Collection (Manassas, VA) and maintained respectively in RPMI, DMEM, and HAM-F12 Coon supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% glutamine. Before stimulation with androgens, cells were kept for 24 h in serum- and phenol red-free medium. PC3 cells were transfected with human full-length androgen receptor construct (p5HbhAR) or vector alone (PC3-NEO cells) by electroporation method using 10  $\mu$ g of plasmid DNA. Cells were then selected in the presence of 0.5 mg/ml geneticin (G418, Calbiochem). To obtain stable colonies, individual clones were isolated by limiting dilution and tested for the presence of the androgen receptor both by Northern and Western analysis.

### Flow cytometry analysis

Flow cytometry was performed as described (20). Cells were grown on Petri dish until confluence, washed with PBS, detached with 0.1% trypsin-EDTA, and resuspended in PBS supplemented with 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ . Cells were then incubated for 30 min at 4 C with the different antiintegrin antibodies, or nonspecific IgG as control, washed three times with PBS and further incubated with FITC-conjugated goat antimouse or antirat secondary antibody (1:200) for 30 min. After washing three times, cells were fixed with 3% paraformaldehyde in PBS and analyzed by FACScan/Lysys II software (Becton Dickinson and Co., Mountain View, CA).

### Northern blot analysis

RNA extraction and Northern blotting were performed as reported previously (21). Briefly, total cell RNA was prepared by hot phenol method, 20  $\mu$ g were then fractionated in 1% agarose-formaldehyde gel and transferred overnight onto nylon membranes. After prehybridization for 1 h, membranes were hybridized with 3.3-kb  $\alpha 6$  HindIII cut cDNA, or 2.98-kb  $\beta 1$  EcoRI cut cDNA, or 5.5-kb  $\beta 4$  EcoRI cut cDNA radiolabeled with [ $\alpha$ - $^{32}$ P]dCTP by random priming. The membranes were then washed three times and submitted to autoradiography using Kodak X-Omat AR films and Kodak X-Omatic regular intensifying screen at  $-80$  C.

### Mitogenic assays

[ $^3$ H]thymidine (NEN Life Science Products, Boston MA), incorporation was evaluated as described previously (21). Briefly, cells were plated in 24-well plates in complete culture medium. After 2 days of culture, cells were kept in serum- and phenol red-free medium for 24 h before treatments. Cells were then treated with R1881 (0.1 and 1 nM) in phenol red- and serum-free medium for the indicated times.

For experiments on the effect of R1881 on cell number, cells were plated at low density in 6-well plates, treated exactly as for [ $^3$ H]thymidine incorporation assay, and counted with a Coulter Counter (Coulter Electronics, Ltd., Luton, UK) after trypsinization.

### Western blot analysis

Protein extraction and Western blot analysis were performed as previously described (22). Briefly, cells were scraped in PBS supplemented with 1 mM  $\text{Na}_3\text{VO}_4$  centrifuged, and resuspended in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.25% NP-40, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM PMSF). After measurement of proteins, cell extracts were diluted in Laemmli sample buffer, boiled at 90 C for 5 min and loaded on 8% polyacrylamide-bisacrylamide gels. Proteins were transferred to nitrocellulose membrane and incubated with antiandrogen receptor antibody (1:1000 dilution) for 1 h, washed, and incubated with secondary antibody (peroxidase-conjugated antirabbit antiserum, 1:4000) for 1 h. After washing, the blots were incubated with enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) detection reagent and exposed to film.

### Adhesion assay

Adhesion assays were performed as described previously (20). Briefly, flat 96-multiwell plates were coated with 100  $\mu$ g/ml poly-lysine for 10 min at 37 C or with 10  $\mu$ g/ml laminin-1 or 10  $\mu$ g/ml type-I collagen or 20  $\mu$ g/ml fibronectin overnight at 4 C in PBS. After washing with PBS,  $10^5$  cells/well were added in triplicate and incubated for the indicated times. Wells were then washed three times with PBS, and cells were fixed in formaldehyde for 10 min and stained with 0.5% solution of crystal violet. Adhesion was quantitated by measuring the absorbance at 600 nm. The values are reported as the percent of poly-L-lysine (considered as 100% of cellular adhesion).

### Invasion assay

Invasion assays were performed as described previously (20) according to Albini *et al.* (23) using the Boyden chambers equipped with 8  $\mu$ m porosity polyvinylpyrrolidone-free polycarbonate filters. A thin layer of Matrigel solution (50  $\mu$ g/ml) was overlaid on the upper surface of the filter and allowed to gel by incubating the filters at 37 C for 30 min. Cell ability to invade the substrate was assessed by using epidermal growth factor (EGF). EGF (100 ng/ml in DMEM) was added to the bottom well of the Boyden chambers.  $10^5$  cells were added to the top of the chambers and incubated for 24 h at 37 C. Migrated cells were quantitated by counting cells with a Carl Zeiss microscope (Oberkochen, Germany) equipped with brightfield optics (40 $\times$  magnification). Results are expressed as the number of migrated cells per high-power field.

### Anchorage-independent growth assay

Anchorage-independent growth was determined as the ability of the cells to form colonies in soft agar (20). Cells were suspended in 2 ml of 0.3% select agar in medium containing 20% charcoal-treated FBS in presence or absence of 1 nM R1881, and plated into six-well plates. After 15 days, growth was examined with an inverted microscope and individual colonies (10 cells or more) were blindly counted by two different investigators.

### Statistical analysis

Statistical analysis was performed by paired (when applicable) and unpaired Student's *t* test.

## Results

### Expression of integrins and adhesion properties of prostate cancer cell lines LNCaP, and PC3

The best studied integrins that function as laminin receptors on carcinoma cells are  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ , and  $\alpha 6\beta 4$ . Surface expression of  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 1$ , and  $\beta 4$  integrin subunits was determined in two human prostate carcinoma cell lines by flow cytometric analysis: the androgen-sensitive LNCaP and the androgen-independent PC3, characterized respectively by absent and high ability to develop tumor in SCID mice (16). As shown in Fig. 1, LNCaP cells expressed little or none surface  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 6$ , and  $\beta 4$  integrins compared with PC3,

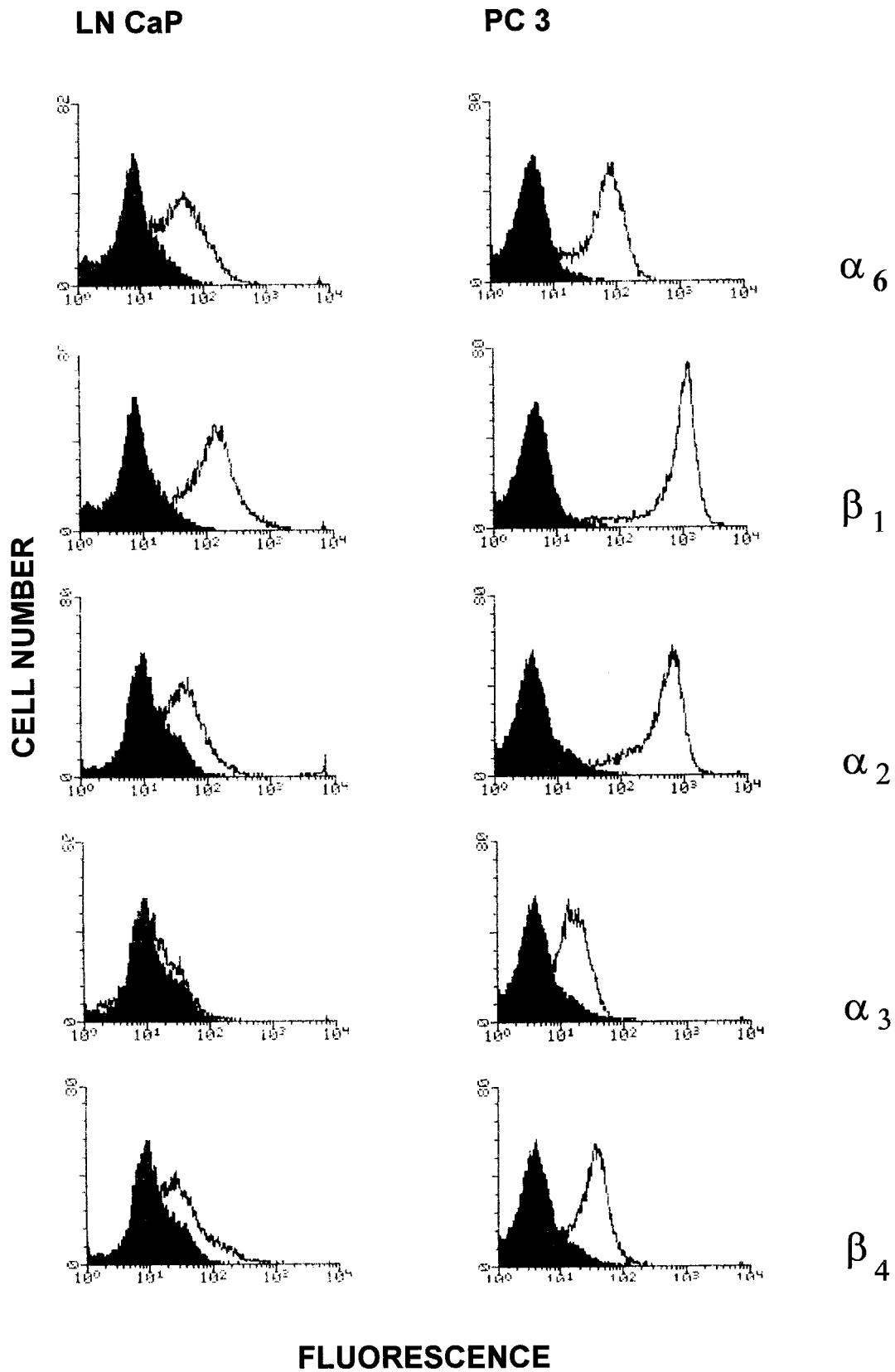


FIG. 1. Flow cytometry analysis of integrin subunit expression in LNCaP and PC3 cells. Cells were incubated with a nonspecific IgG as control (*left peak*) or antibodies specific for the indicated integrin subunit (*right peak*). Representative of two similar experiments for  $\alpha_2$  and  $\alpha_3$  and of at least three similar experiments for  $\alpha_6$ ,  $\beta_1$ , and  $\beta_4$  integrins.

whereas  $\beta 1$  expression was similar in the two cell lines, confirming previous results (16, 18–19). Northern blot analysis of messenger RNA (mRNA) expression for  $\alpha 6$ ,  $\beta 4$ , and  $\beta 1$  in the two cell lines confirmed the results of flow cytometric analysis. The highest mRNA levels of  $\alpha 6$  and  $\beta 4$  integrin subunits were found in PC3 cells, whereas LNCaP showed low ( $\alpha 6$ ) or undetectable ( $\beta 4$ ) transcripts of the two integrins (Fig. 2). Conversely,  $\beta 1$  mRNA levels were similar in the two cell lines (Fig. 2). Because LNCaP cells retain androgen sensitivity, we examined whether treatment of these cells with DHT affected mRNA expression of  $\alpha 6$ ,  $\beta 4$ , and  $\beta 1$  integrin subunits. mRNA expression for  $\alpha 6$  was reduced by treatment with 0.1 nM DHT (D, Fig. 2), whereas no effects were observed on  $\beta 1$  mRNA. Similarly,  $\beta 4$  mRNA levels remained undetectable also after treatment with DHT (Fig. 2). These data indicate a possible regulation of  $\alpha 6$  gene expression by androgens in LNCaP cells. As expected, no effects of DHT were observed in PC3 cells (Fig. 2). Similar results were obtained with the synthetic androgen R1881 (not shown).

Adhesion on laminin, collagen, and fibronectin at 30, 60, and 90 min of LNCaP and PC3 cells was also examined. As shown in Fig. 3, adhesion on laminin (panel A) and on col-

lagen (panel B) of LNCaP cells was significantly lower at all time points compared with PC3. Conversely, adhesion on fibronectin did not differ between LNCaP and PC3 cell lines (Fig. 3C). In keeping with their reduced tumorigenicity (16), LNCaP cells were also characterized by reduced ability to form colonies in soft agar respect to PC3 cells (data not shown).

#### *$\alpha 6\beta 4$ integrin expression and adhesion properties of PC3 cells transfected with an androgen receptor expression vector*

To examine the role of androgen in the modulation of integrin expression and cell adhesion in prostate cancer cells, we transfected PC3 cells with a full-length human androgen receptor cDNA vector. We reasoned that PC3 cells, due to their high expression of  $\alpha 6$  and  $\beta 4$  integrin subunits, could represent a suitable *in vitro* model to study regulation of these integrins by androgens. After selection and cloning of PC3 transfected cells, we evaluated the presence of androgen receptor by both Northern blot (not shown) and Western blot analysis (Fig. 4). Several positive clones, showing a protein band of 110 kDa exactly migrating as the androgen receptor from LNCaP cells, were obtained. The protein band was absent (Fig. 4) in androgen-receptor negative cell lines PC3WT, PC3 Mock-transfected (PC3-Neo) and DU145 (16). In some clones, the level of androgen receptor expression was lower than in LNCaP cells, whereas in clones 6 and 13 a similar level of expression was observed (Fig. 4), and thus further studies were conducted in these two clones. Because loss of the transcript may occur in stable transfected cells, androgen receptor expression in positive clones was frequently checked by Western blot analysis. To ascertain whether the androgen receptor in clone 6 and 13 cells was functional, we evaluated the effect of R1881 (0.1 and 1 nM for 6 days) on [ $^3$ H]thymidine incorporation. As shown in Fig. 5, 1 nM R1881 determined a significant decrease of [ $^3$ H]thymidine incorporation in the two clones, whereas no effects were observed in PC3-Neo cells. Accordingly, number of cells was also decreased in the two clones, but not in PC3-Neo cells, by treatment with 1 nM R1881 (not shown). At 0.1 nM, a significant effect of R1881 was observed only in clone 13 cells (Fig. 5). As shown in Fig. 5, the effects of R1881 were antagonized by the androgen receptor antagonist bicalutamide in both clones, indicating that the effects on cell growth of the synthetic androgen are mediated by binding to the androgen receptor. These results are in agreement with previous findings on the effect of androgen on growth of androgen receptor transfected PC3-cells (24, 25).

To investigate androgen regulation of  $\alpha 6\beta 4$ , we measured mRNA expression and surface distribution of the integrin in androgen receptor positive clones in basal conditions and after treatment with R1881 (1 nM, 3 days). As shown in Fig. 6, basal (lanes marked as C)  $\alpha 6$  and  $\beta 4$  mRNA expression was reduced in clones 6 and 13 with respect to PC3-Neo or PC3-WT cells. In addition, mRNA expression for both integrin subunits was further reduced in the two clones by treatment with 1 nM R1881 for 3 days (Fig. 6 lanes marked as T). Densitometric analysis of mRNA bands in the film performed by Image quest IQ Base software (Hamamatsu Pho-

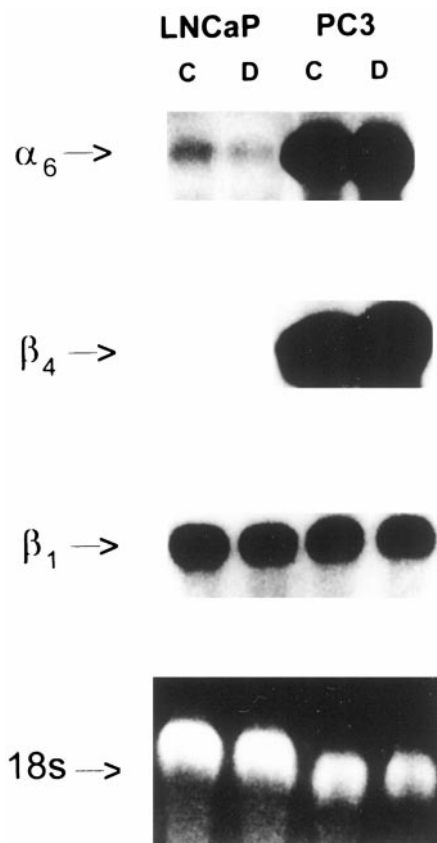


FIG. 2. mRNA expression of  $\alpha 6$ ,  $\beta 4$ , and  $\beta 1$  subunits in LNCaP and PC3 cells in basal conditions and after treatment with DHT. Treatments were performed in serum-free medium for 3 days with (D) or without (C) DHT (0.1 nM). Each lane contains 20  $\mu$ g of total RNA. The membrane was hybridized consecutively with  $^{32}$ P-labeled cDNA probes for  $\alpha 6$ ,  $\beta 4$ , and  $\beta 1$ . Ethidium bromide staining of total RNA loaded in each lane is shown in the lowest panel. Representative of at least three similar experiments.

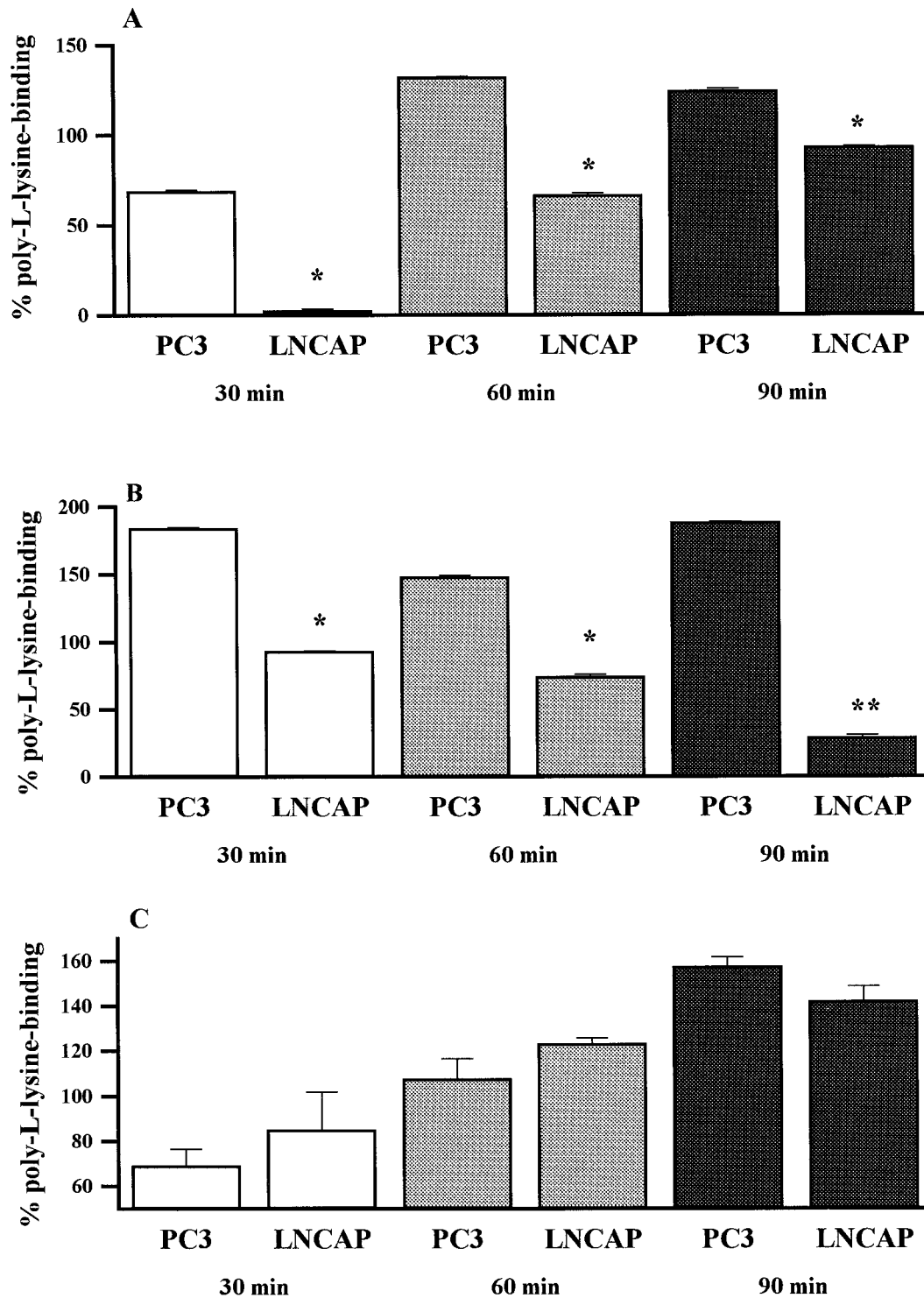


FIG. 3. Time course of adhesion of LNCaP and PC3 cells to laminin, collagen I, and fibronectin. Cells were assayed for their ability to adhere to 10  $\mu\text{g/ml}$  laminin-1 (A), 10  $\mu\text{g/ml}$  collagen I (B), or 20  $\mu\text{g/ml}$  fibronectin (C) for the indicated times. Values shown are expressed as the percentage of cells bound to poly-L-lysine (mean  $\pm$  SEM of triplicates of three similar experiments). \*,  $P < 0.01$  vs. PC3; \*\*,  $P < 0.001$  vs. PC3.

tonics, Tokyo, Japan) as previously described (22), revealed that basal mRNA for  $\alpha 6$  in clone 6 and 13 was, respectively,  $46 \pm 14.4\%$  and  $52.6 \pm 13.5\%$ , whereas for  $\beta 4$  was  $42 \pm 26.5\%$  and  $59 \pm 17\%$  respect to PC3-Neo ( $n = 3$  experiments). Conversely,  $\beta 1$  mRNA expression in androgen receptor pos-

itive clones was similar to PC3-Neo cells and was unaffected by treatment with R1881 (not shown). In agreement with these results, surface expression of  $\alpha 6$  and  $\beta 4$  subunits, analyzed by FACScan, was lower in clones 6 and 13 compared with PC3-Neo cells, whereas  $\beta 1$  expression remained un-

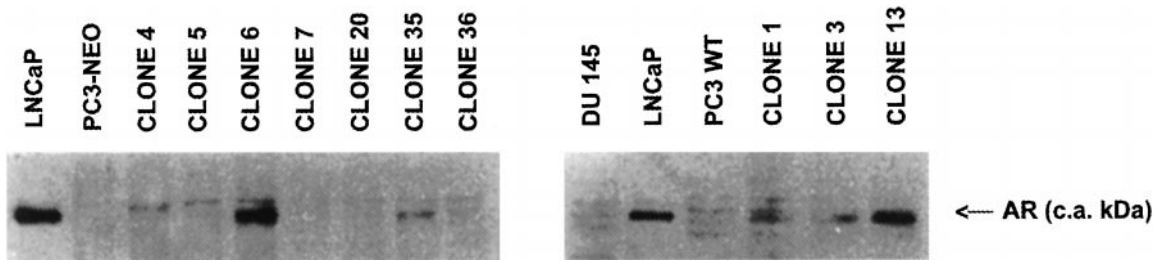


FIG. 4. Western blot analysis of human androgen receptor expression in stable PC3-transfected clones. Total cell lysates (50  $\mu$ g) were extracted from the indicated cell lines and PC3 clones and analyzed by Western blot for human androgen receptor protein expression. LNCaP cell lysates were run as positive controls, PC3WT and DU145 as negative controls. The arrow indicates the androgen receptor (AR). Representative of at least five similar experiments.

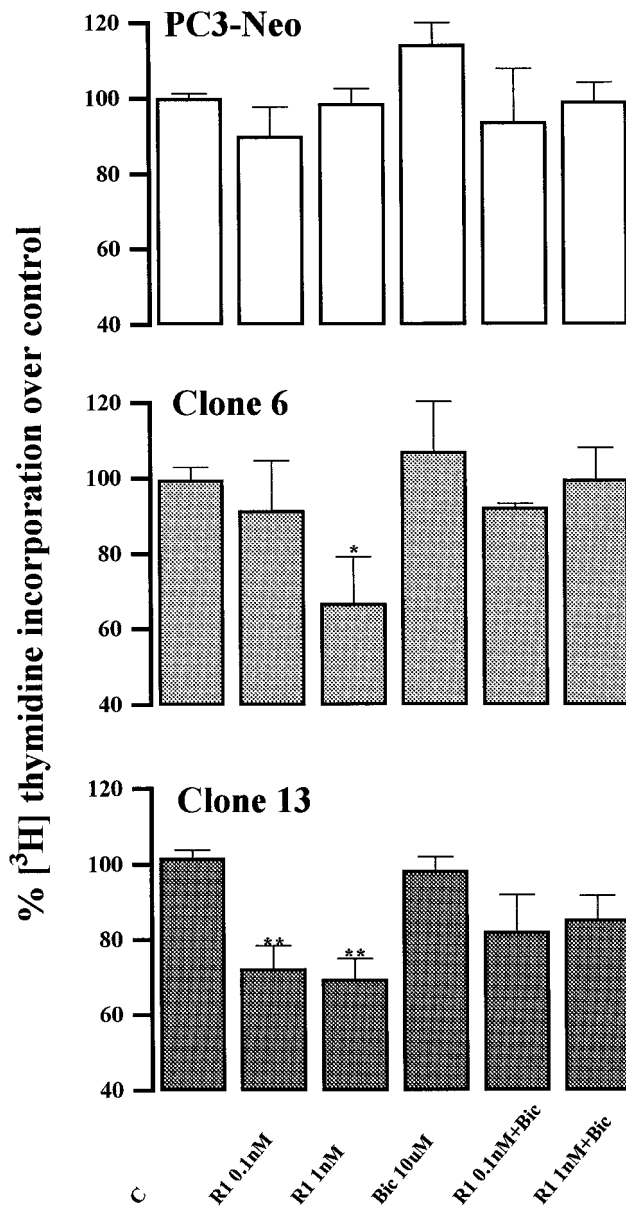


FIG. 5. Effect of androgen stimulation on [ $^3$ H]thymidine uptake of androgen receptor-transfected PC3 and PC3-Neo cells. Cells were incubated for 6 days with indicated treatments. Values represent the mean  $\pm$  SEM of four different experiments performed in triplicates. C, Control; R1, R1881; Bic, bicalutamide. \*,  $P < 0.005$  vs. C; \*\*,  $P < 0.001$  vs. C.

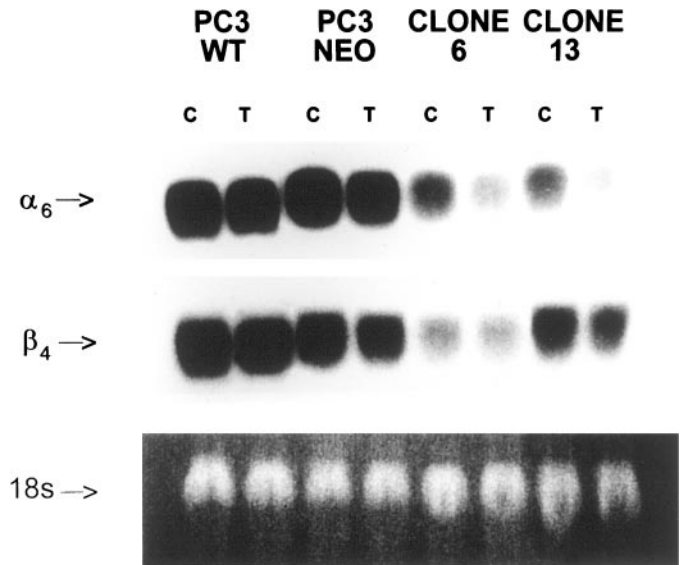


FIG. 6. Basal and R1881-stimulated mRNA expression of  $\alpha 6$  and  $\beta 4$  subunits in PC3-WT, PC3-Neo, and PC3-transfected clones 6 and 13. Cells grown in serum-free medium for 24 h, were incubated in 1 nM R1881 (T) or in serum free medium alone (C) for 3 days. Each lane contains 20  $\mu$ g of total RNA. The membrane was hybridized consecutively with  $^{32}$ P-labeled cDNA probes for  $\alpha 6$  and  $\beta 4$ . Ethidium bromide staining of total RNA loaded in each lane is shown in the lowest panel. Representative of three similar experiments.

changed (Fig. 7). In addition, the two clones were distinguished from PC3-Neo cells by the presence of a cell population expressing  $\alpha 6$  and  $\beta 4$  at the same levels of negative histograms (Fig. 7). Based on the relative mean fluorescence intensity, the percent decrease respect to PC3-Neo cells for  $\alpha 6$  and  $\beta 4$  expressions were respectively of  $63 \pm 15$  and  $55 \pm 12$  for clone 6 and  $35 \pm 9$  and  $53 \pm 18$  for clone 13 (mean  $\pm$  SEM,  $n = 3$ ). Despite the reduction of mRNA expression of both  $\alpha 6$  and  $\beta 4$  after treatment with R1881 (Fig. 6), no substantial quantitative modifications were observed on surface protein expression of the two integrins, evaluated by FACSscan, in both androgen-positive clones (not shown). Similar results were obtained also in LNCaP cells, where the inhibitory effect of androgens on mRNA expression of  $\alpha 6$  integrin (Fig. 2) did not result in changes in surface protein expression as analyzed by FACSscan (not shown).

To ascertain whether the observed modifications of integrin pattern in transfected cells corresponded to different adhesion properties, cells were plated on laminin- and col-

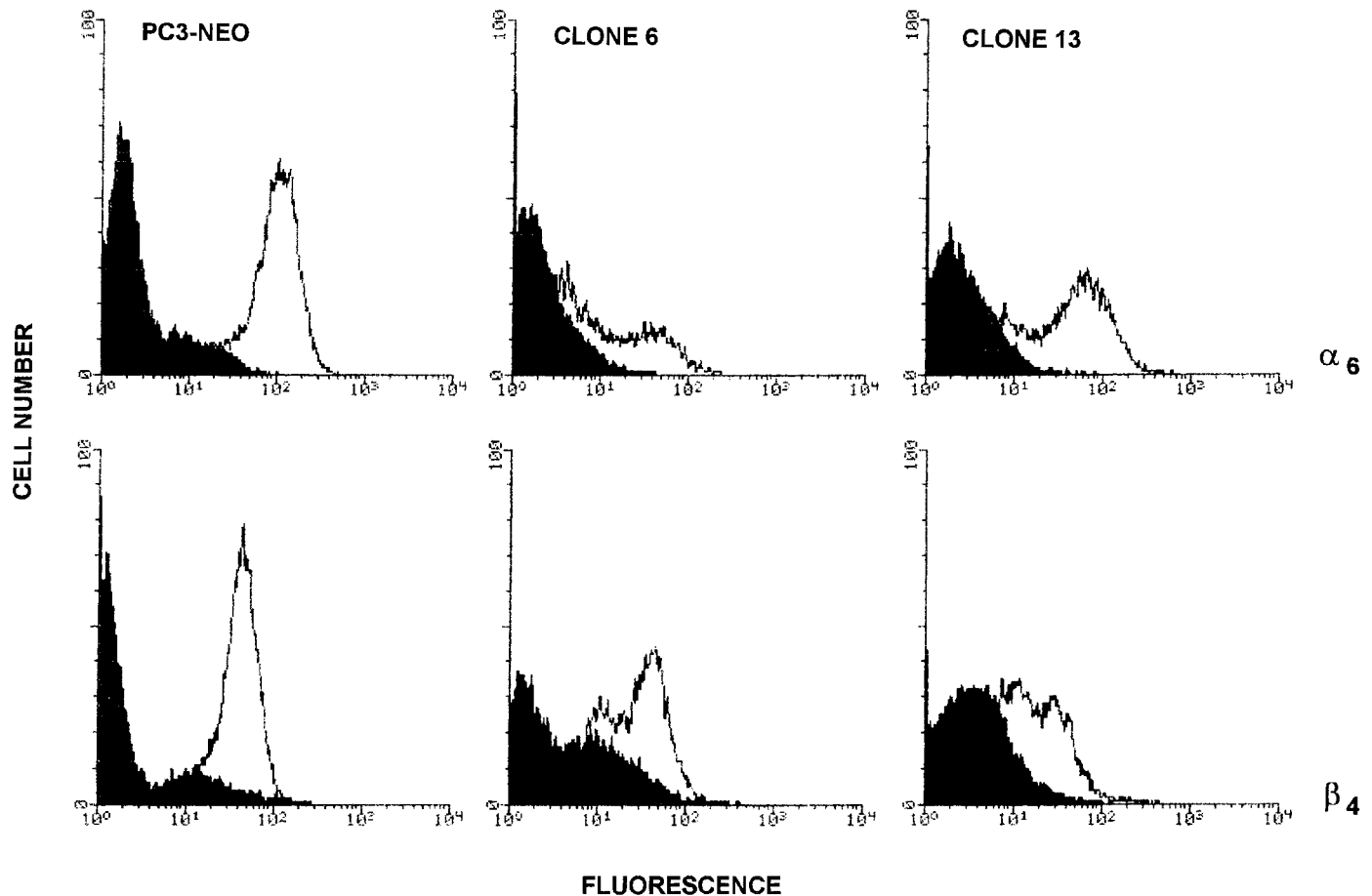


FIG. 7. Expression levels of  $\alpha 6$ ,  $\beta 4$ , and  $\beta 1$  integrin subunits in PC3-Neo and PC3-transfected clones. PC3-Neo (*left panel*), clone 6 (*central panel*), and clone 13 (*right panel*) cells were stained with specific antibodies for the  $\beta 4$ ,  $\alpha 6$ , and  $\beta 1$  integrin subunits (*right peak*) followed by a nonspecific IgG as control (*left peak*). The cells were analyzed by flow cytometry. Representative experiment out of three.

lagen-coated wells for different times and adhesion evaluated. Clone 6 and 13 transfected cells showed significantly lower adhesion to laminin at all time points when compared with PC3-Neo (Fig. 8A), whereas adhesion on collagen was similar in the three cell lines (Fig. 8B). Treatment with R1881 (1 nM, 3 days) further reduced adhesion to laminin at 15 min of clone 6 and 13, whereas that of PC3-Neo cells was unaffected (Fig. 8C). A similar reduction of adhesion to laminin was observed in LNCaP cells after treatment with R1881 (Fig. 8C).

We next evaluated the ability of transfected cell clones to invade a gel composed of the reconstituted basement membrane (Matrigel). This assay has been shown to correlate with the ability of tumor cells to invade the basement membrane *in vivo* (23). As shown in Fig. 9, clone 6 and 13 cells invaded significantly less respect to PC3-Neo cells both in basal conditions and after treatment with EGF (100 ng/ml). Invasion of clone 6 and 13 cells was further reduced significantly by treatment with 1 nM R1881 for 3 days. No invasion was observed in LNCaP cells either in basal conditions or following treatment with R1881 (Fig. 9).

The ability to form colonies in soft agar was reduced in clones 6 and 13 with respect to PC3-Neo cells (Fig. 10), indicating that restoration of androgen sensitivity and decreased expression of  $\alpha 6\beta 4$  result also in a lower anchorage-

independent growth. The presence of 1 nM R1881 in the medium determined a further decrease in the number of colonies that, however, was statistically significant in clone 6 but not in clone 13 cells (Fig. 10).

### Discussion

The results provided in this study define a novel mechanism of regulation of  $\alpha 6\beta 4$  integrin expression and function. We demonstrate that restoration of androgen sensitivity in an androgen-independent prostate carcinoma cell line suppresses  $\alpha 6\beta 4$ -mediated invasion by modulating the expression of this integrin.

The invasive phenotype requires specific quantitative and qualitative expression, on the cell surface, of various molecules that participate in the interaction between cancer cells and extracellular matrix. In the case of prostate carcinomas, highly invasive tumors develop after some time of androgen-ablation therapy probably due to selection of clones that have bypassed hormone dependency for proliferation and progression (3), suggesting that androgen may have specific modulating effects on molecules that regulate the invasive phenotype. Along this line, it has been recently demonstrated that androgens regulate neutral endopeptidase (NEP), which is not expressed in cells lines lacking androgen receptor such

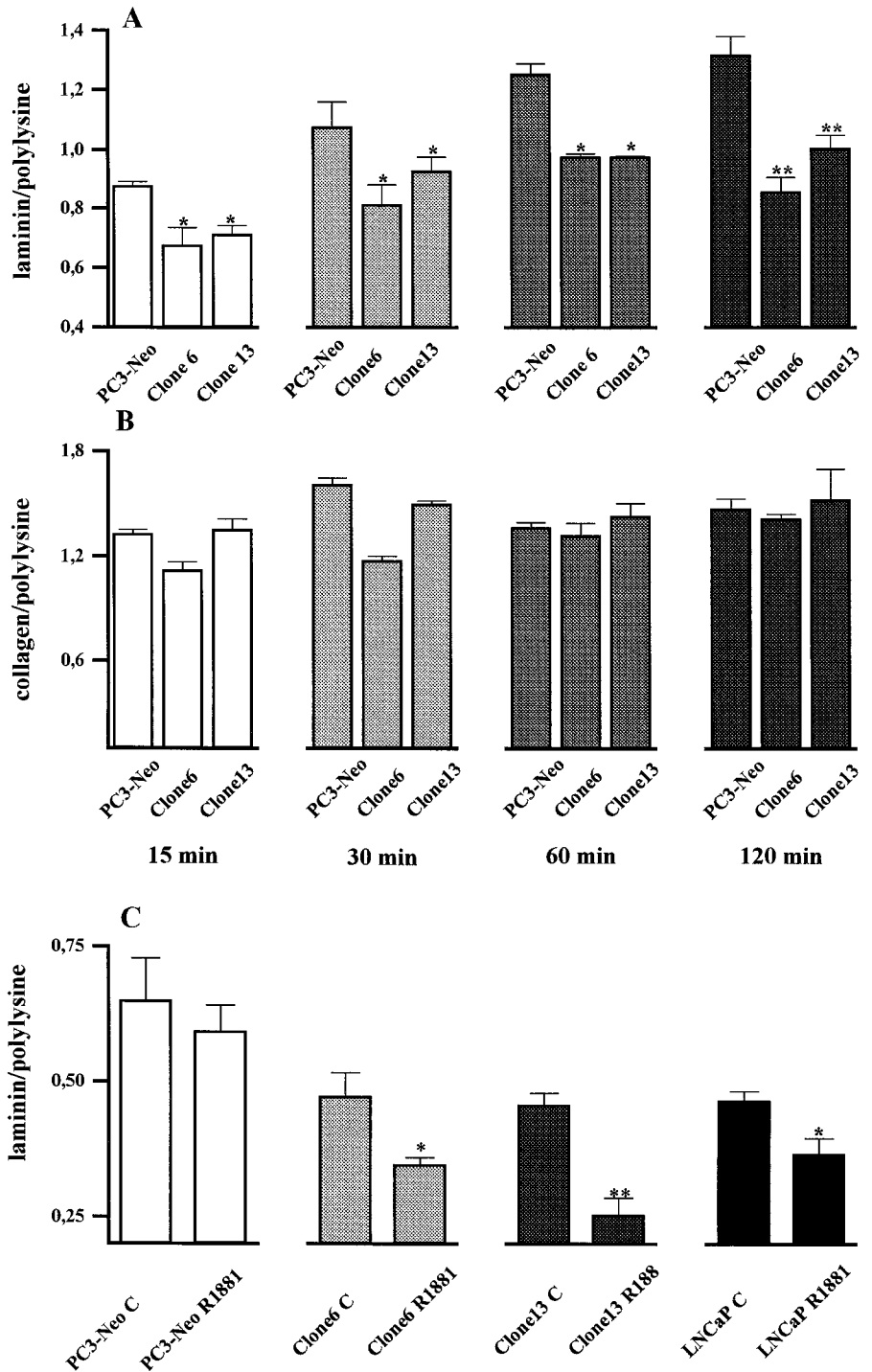


FIG. 8. Time course of adhesion of PC3-transfected cells to laminin and collagen I. Cells were assayed for their ability to adhere to 10  $\mu\text{g/ml}$  laminin (A) and 10  $\mu\text{g/ml}$  collagen I (B) for the indicated times. C, Adhesion to laminin of PC3-Neo, clone 6 and 13 and LNCaP cells at 15 min with or without treatment with R1881 (1 nM, 3 days). Values shown are expressed as the percentage of cells bound to poly-L-lysine (mean  $\pm$  SEM of triplicates of a representative out of three similar experiments). \*  $P < 0.05$  vs. respective controls; \*\*  $P < 0.01$  vs. respective controls.

as PC3 and DU145 and is highly expressed in LNCaP (3). Our study documents that loss of androgen regulation may result in increased  $\alpha 6\beta 4$  integrin expression and consequent more aggressive, metastatic phenotype. Indeed,  $\alpha 6\beta 4$  expression is high in the androgen-independent prostatic cancer cell line PC3 and is barely detectable in LNCaP cells that retain androgen sensitivity, suggesting a relation between androgen sensitivity and expression of the integrin. In addition, we demonstrate that expression of the integrin is strongly decreased in PC3 clones regaining androgen sensitivity after

transfection with an androgen receptor expression vector. Of importance, androgen receptor transfected-PC3 as well as LNCaP cells exhibit lower invasion on Matrigel and adhesion to laminin, indicating that expression of this receptor affects these two processes. In particular, the lower invasion ability demonstrated by these cells indicates that expression of the androgen-receptor is associated to a lower invasive potential. Our study clearly indicates that such effects of the androgen receptor are due to modulation of  $\alpha 6\beta 4$  expression. Indeed, decreased response to EGF does not appear to be responsible

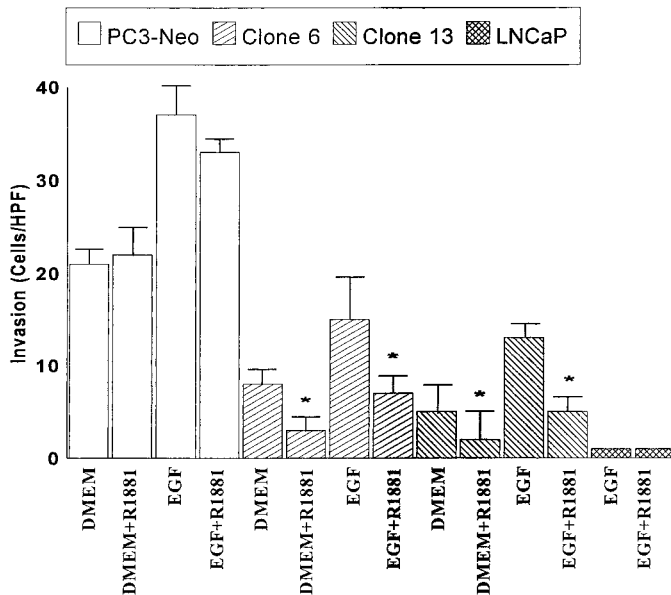


FIG. 9. Matrigel invasion of PC-3-transfected cells. Matrigel was diluted in DMEM (50  $\mu$ g/ml) and overlaid on the upper surface of the polycarbonate filter. EGF (100 ng/ml) or DMEM were added to the bottom wells of the Boyden chambers. Cells ( $10^5$ ), untreated or treated with R1881 (1 nM, 3 days), were added to the top wells of the chambers and then incubated for 24 h at 37 C. The cells that reached the lower surface of the filters were quantitated as described in *Materials and Methods*. Values are expressed as mean  $\pm$  SEM from three different experiments. \*  $P < 0.05$  vs. untreated cells.

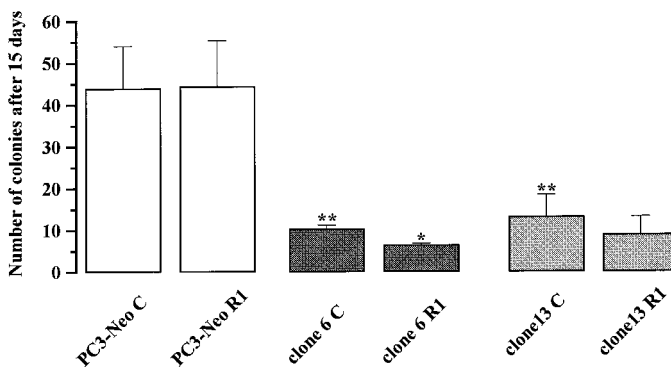


FIG. 10. Anchorage-independent growth of androgen receptor transfected PC3 cells. PC3-Neo, clone 6, and clone 13 cells were plated in soft agar at 2000 cells/well in the presence or absence of 1 nM R1881 and the number of colonies obtained after 15 days carefully counted. Values are expressed as mean  $\pm$  SEM from three different experiments. C, Control; R1, R1881 1 nM. \*\* $P < 0.001$  vs. PC3-Neo; \*  $P < 0.05$  vs. Clone 6C.

for the lower invasion observed in our study because it has been shown that expression of EGF receptor and affinity for its ligand are actually increased in PC3 cells transfected with the androgen receptor (26). Similarly, the presence of EGF receptors has been shown in LNCaP cells (27), which, in our hands, were not able to invade Matrigel. In addition, preliminary data obtained in our laboratory indicate that thymidine incorporation in response to EGF increased similarly both in PC3 transfectants and LNCaP cells (results not shown). Hence, decreased (PC3 transfectants) or absent (LNCaP cells) expression of  $\alpha 6 \beta 4$  are likely responsible for

the lower invasion. These results confirm and extend previous findings indicating a major role for this integrin in invasion and progression of cancer cells (11–14, 28–29). Expression of androgen receptor also abrogated anchorage-independent growth of PC3 transfectants, suggesting that  $\alpha 6 \beta 4$  expression is critical to maintaining the transformed phenotype of these cells. A role for integrins in anchorage-independent growth of cancer cells has been already suggested (30–31). In particular, it has been recently shown that knockout of  $\alpha 6 \beta 1$  in hepatocarcinoma cells by transfection with a  $\beta 4$  deleted subunit resulted in a decrease of colony formation in soft agar (20), and function blocking  $\beta 1$  antibodies abrogated colony formation of breast cancer cells in a 3-dimensional basement membrane (32). It is of interest that the decrease of expression of  $\alpha 6 \beta 4$  integrin in PC3 transfectants is obtained without stimulation of the androgen-receptor. It is possible that androgen present in serum (10% FBS) contained in the culture medium is sufficient to stimulate the androgen receptor in the cells. Alternatively and in addition, other factors present in serum may act as stimuli for the androgen receptor. In this respect, it has been demonstrated that steroid receptor in general (33) and androgen receptor in particular may be activated by nonsteroidal molecules such as interleukin 6 (34), insulin-like growth factor I, keratinocyte growth factor, and epidermal growth factor (35, 36).

Stimulation of transfected PC3 cells and LNCaP cells with the synthetic androgen analog R1881 determined a further decrease of  $\alpha 6 \beta 4$  mRNA expression, adhesion on laminin, and invasion, indicating that the androgen may have direct effect in the regulation of the invasive phenotype of these cells. However, despite the effects on steady-state mRNA levels and function of the integrin, by FACScan analysis, we were unable to demonstrate any significant effect of treatment with R1881 on surface expression of  $\alpha 6$  and  $\beta 4$  subunits in androgen receptor transfected PC3 cells. The reason for this discrepancy is unknown and is at present under investigation in our lab. One possible explanation is that stimulation with androgens leads to functional modifications of  $\alpha 6 \beta 4$  also through posttranscriptional regulation as it has been shown for the androgen receptor in LNCaP cells (37, 38). In these cells, androgens induce a decrease of mRNA expression and function of androgen receptor without observed quantitative alterations (37) or even an increase (38) of protein expression. Moreover, a possible interference of androgens with the signal transduction mechanisms of  $\alpha 6 \beta 4$  determining a functional alteration of its affinity state cannot be excluded. Suppression of integrin activation through modulation of affinity state by ras/MAPK pathway has been demonstrated (39). Such a pathway may be activated by the androgen receptor (40). On the other hand, it has been recently shown that retinoids inhibit adhesion to laminin of human pancreatic carcinoma cells by interfering with  $\alpha 6 \beta 1$  function without altering surface expression of the integrin (41) and that effects of dexamethasone on adhesion to laminin of human melanoma cell lines occur without alterations of laminin receptor expression (42), indicating a possible interference of molecules with steroid configuration with the signal transduction pathways and/or affinity state of the integrin.

A steroid receptor binding homologue is present in the human  $\alpha 6$  gene promoter (43, 44), suggesting that the protein can be regulated by steroids. However, this element does not appear to be required for basal promoter activity (44). Other possible regulatory elements of  $\alpha 6$  gene promoter are represented by Sp1, AP2, Ker1, NF- $\kappa$ B, and *c-myc* binding elements, located upstream of the transcription start site (43, 44). Cell-type specific negative regulatory elements, not yet identified, have been hypothesized in the upstream region of the promoter (43, 44). The promoter of  $\beta 4$  has been characterized recently (45). Gel retardation assays and mutational analysis have disclosed that a cooperation between AP1 and Ets regulates the promoter activity of  $\beta 4$  (45). It has been shown that androgen receptor-mediated negative regulation of gene expression occurs mainly by physical interaction of the androgen receptor with Ets proteins (40), and most of the genes that are transcriptionally down-regulated by the androgen receptor have Ets binding sites in their promoter region (40). Interactions of the androgen receptor with AP-1/*c-Jun* (46) and NF- $\kappa$ B (40) have been also demonstrated. In this light, it is conceivable that interaction of the androgen receptor with  $\alpha 6$  or  $\beta 4$  transcription factors might represent a pathway of regulation of these genes. It has been recently reported that expression of  $\alpha 6$  and  $\beta 4$  in several human breast cancer cell lines is inversely related to the expression of the estrogen receptor in the same cells (47). This report, together with results described in the present study, strongly suggests a role for steroid receptors in the modulation of  $\alpha 6\beta 4$  integrin.

In conclusion, we show consistent evidence that adhesion and invasion of prostate carcinoma cells is suppressed by expression of androgen receptor through regulation of  $\alpha 6\beta 4$  integrin expression. These results imply that androgens have negative effects on genes involved in the development of invasive phenotype of this tumor and that antagonistic hormonal treatment may have significant clinical implications because androgen withdrawal may result in  $\alpha 6\beta 4$  up-regulation and higher invasive phenotype.

### Acknowledgments

We thank Dr. Arthur M. Mercurio for providing the plasmids.

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