EGF receptor (EGFR) signaling promoting invasion is disrupted in androgen-sensitive prostate cancer cells by an interaction between

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EGF RECEPTOR (EGFR) SIGNALING PROMOTING INVASION IS DISRUPTED IN ANDROGEN-SENSITIVE PROSTATE CANCER CELLS BY AN INTERACTION BETWEEN EGFR AND ANDROGEN RECEPTOR (AR)

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We previously demonstrated that expression of androgen receptor (AR) by transfection of the androgen-independent prostate cancer cell line PC3 decreases invasion and adhesion of these cells (PC3-AR) through modulation of α6β4 integrin expression. The treatment with androgens further reduced invasion of the cells without modifying α6β4 expression, suggesting an interference with the invasion process by androgens. Here, we investigated EGF-mediated signal transduction processes that lead to invasion in PC3-AR cells. We show that EGF-induced EGFR autotransphosphorylation is reduced in PC3-AR cells compared to PC3 cells transfected only with the vector (PC3-Neo). EGF-stimulated PI3K activity, a key signaling pathway for invasion of these cells, and EGF-PI3K interaction are also decreased in PC3-AR cells and further reduced by treatment with androgen. Finally, we show that EGFR internalization process was reduced in PC3-AR and LNCaP cells compared to PC3-Neo. Investigations on the location of AR in PC3-AR transfected cells were also conducted. Immunoconfocal microscopy and coimmino-precipitation studies demonstrated the presence of an interaction between EGFR and AR at membrane level in PC3-AR and LNCaP cells. In conclusion, our results suggest that the expression of AR by transfection in PC3 cells confers a less-malignant phenotype by interfering with EGFR signaling leading to invasion through a mechanism involving an interaction between AR and EGFR.

Key words: *prostate cancer; epidermal growth factor receptor; androgen receptor; PI3K; invasion*

Prostate Cancer (PC) is one of the most common cancer and the second leading cause of death in American men.[1] Since prostate cancer cell growth is enhanced by androgens, in the advanced stages of the disease, androgen ablation therapy represents a valuable tool for the treatment of these patients. However the development in most patients after few years of treatment of androgen-independent clones, characterized by higher invasiveness and metastatic properties, has focused attention on the molecular mechanisms that lead to loss of androgen-dependence as well as on the pathways that are regulated by androgens in these cells besides proliferation. Indeed, although androgens are the major stimulus for proliferation of prostate cancer cells, maintenance of androgen-sensitivity appears to keep a more differentiated and less malignant phenotype of these cells. The ability to produce tumors in nude mice, for instance, is higher in androgen-insensitive cell lines (such as PC3 and DU145) with respect to androgen sensitive (LNCaP).[2] In this light, the role of androgens in the regulation of the pathways involved in invasion and metastasis represents a major task in studies on prostate cancer biology.[3] As a result, some androgen-regulated genes involved in signaling pathways that lead to invasion have been recently identified[4–6] and their role in decreasing invasion ability of androgen-sensitive prostate cancer cells indicated. Migration and invasion of cancer cells is regulated by multiple pathways that employ various growth factor and their receptors, integrins and cytoskeletal elements. A key role is played by the EGF receptor (EGFR), which, following interaction with the integrin α6β4, promotes cell migration through activation of PI3K and other downstream pathways.[7,8]

In a previous study, we demonstrated that the expression of androgen receptor in PC3 cells by transfection with a full-length human androgen receptor expression vector (PC3-AR) determined a decrease in the expression of the integrin α6β4 and in the ability of these cells to invade Matrigel in response to EGF.[6] The treatment with the synthetic androgen R1881 determined a further decrease of the invasion ability of these cells, without however modifying the surface expression of α6β4[6] and prospecting an effect of the androgen on EGF-mediated signaling related to invasion.

In our present study, we investigated EGF-activated signaling in PC3-Neo and -AR cells. We report that EGF-mediated EGFR autotransphosphorylation and PI3K activation is reduced in PC3-AR cells. In addition, we demonstrate colocalization and coimmunoprecipitation of AR and EGFR in PC3-AR and LNCaP cells, indicating an interaction between the 2 receptors.

MATERIAL AND METHODS

Antibodies and chemicals

Rabbit polyclonal anti-androgen receptor antibody (N-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal anti-androgen receptor antibody (Pa1-110) was obtained from Affinity BioReagents, Inc. (Golden, CO). Rabbit Ab (β4 cyto) was generously provided by Prof. F. Giancotti (Memorial Sloan-Kettering Cancer Center, New York, NY). Mouse MAb Ab2 (anti EGFR) and mouse MAb Ab1 (anti EGFR) were from Oncogene (Cambridge, England). Rat MAb anti-β4 (439-9B) was provided by Dr. R. Falcioni (Molecular Oncogenesis Laboratory, Regina Elena Cancer Institute, Rome, Italy) and mouse anti-human integrin β4 monoclonal antibody was obtained from Chemicon International, Inc. (Temecula, CA). Antiphosphtyrosine PY20 antibody was obtained from ICN (Costa Mesa, CA); antiphosphtyrosine PY99 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti PI 3-kinase p110 (D-4) and anti EGFR (1005) was from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-AKT (Ser473) antibody and mouse monoclonal anti-AKT antibodies were from Cell Signaling Technology (Beverly, MA). Recombinant human epidermal

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growth factor (EGF) was obtained from Pepro Tech EC (London, England). Matrigel was from Collaborative Biomedical Products (Bedford, MA). The tyrosine kinase inhibitor ZD1839 ("Iressa") was a generous gift from Astrazeneca (London, England). The antibiotic Geneticin (G418), PD 098059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one] and LY294002 were obtained from Calbiochem (San Diego, CA). Laminin-1 and other not specified reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell culture and transfection

PC3 cell line was obtained from American Tissue Culture Collection (Bethesda, MD) and maintained in HAM-F12 Coon supplemented with 10% FBS, 1% penicillin/streptomycin and 1% glutamine. Before stimulation with androgens, cells were kept for 24 hr 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 and selected in the presence of 0.5 mg/ml geneticin (G418) as described previously.[6] 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.[6]

Flow cytometry analysis

Cell surface EGFR expression was evaluated by flow cytometry performed as described.[6,9] 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 $CaCl_2$ and 1 mM $MgCl_2$. After the indicated treatments, cells were incubated for 30 min at 4°C with the monoclonal anti-EGFR antibody Ab1, or nonspecific IgG as control, washed 3 times with PBS and further incubated with FITC-conjugated goat anti-mouse secondary antibody (1:200) for 30 min. In some experiments, cells were labeled with FITC-conjugated EGF (Molecular Probes, Eugene, OR). After washing 3 times, cells were fixed with 3% paraformaldehyde in PBS. FITC green fluorescence was detected at 515–555 nm using a FL-1 detector of a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 mW argon-ion laser for excitation. Debris were gated out by establishing a region around the population of interest on the Forward Scatter *vs.* Side Scatter dot plot. For each sample, 10,000 events in the region of interest were recorded at a flow rate of 200–300 cells/sec. Data were processed with analysis software LYSYS II (Becton Dickinson).

Immunoprecipitation and western blot analysis

Protein extraction and Western blot analysis were performed as previously described.[6,10] Immunoprecipitation was performed as previously described[10] with few modifications. Briefly, cells were scraped in PBS supplemented with 1 mM Na_3VO_4, centrifuged and resuspended in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.25% NP-40, 0.2% Triton-X and 1 mM Na_3VO_4, 1 mM PMSF). After protein measurement, aliquots of cell lysates containing equal amount of proteins (500 μg) were incubated for 1 hr with 30 μl of Protein A (or Protein G)-Sepharose for preclearing. Precleared lysates were then incubated for 1 hr using 5 μg of specific antibodies on ice followed by overnight incubation at 4°C with 30 μl of Protein A (or Protein G)-Sepharose. The immunobeads were washed 3 times in lysis buffer and then resuspended in 10 μl of 2× reducing sample buffer, boiled and loaded onto 8% polyacrylamide-bisacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C in 5% BSA-TTBS (0.1% Tween-20, 20 mM Tris and 150 mM NaCl). After washing in TTBS, the membranes were incubated for 2 hr with the different primary antibodies, followed by incubation with peroxidase-conjugated relative secondary antibodies. Finally, probed proteins were revealed by enhanced-chemiluminescence system (BM, Roche). After the first blotting with peroxidase-conjugated secondary antibodies, nitrocellulose membranes were stripped at 50°C for 30 min in stripping buffer (100 mM 2β-mercaptoethanol, 2% sodium dodecyl sulphate and 62.5 mM Tris-HCl, pH 6.7) and reprobed with specific primary antibodies.

Invasion assay

Invasion assays were performed as described previously[6,9] according to Albini *et al.*[11] using the Boyden chambers equipped with 8 μm porosity polyvinylpyrrolidone-free polycarbonate filters. A thin layer of Matrigel solution (50 μg/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 hr at 37°C. Migrated cells were quantitated by counting cells with a Zeiss microscope (Oberkochen, Germany) equipped with brightfield optics (×40 magnification). Results are expressed as the number of migrated cells per high-power field.

PI3 kinase assay

Cells were pretreated for 24 hr with R1881 (1 nM) or vehicle alone (DMSO), detached with 0.05% trypsin-EDTA, blocked with trypsin-inhibitor and plated on laminin-1 (20 μg/ml) coated dishes for 2 hours. Cells were then stimulated with EGF (50 ng/ml 15 min), scraped in PBS supplemented with 1 mM Na_3VO_4, centrifuged and extracted with lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 1 mM Na_3VO_4 and 1 mM PMSF). PI3K activity was measured as described previously.[12,13] After measurement of proteins, the aliquots of cell extracts containing equivalent amount of proteins (500 μg) were incubated for 1 hr with 50 μl of Protein G-Sepharose for preclearing. Precleared lysates were then incubated with an anti-phosphotyrosine MAb (PY99) from Santa Cruz Biotechnology (Santa Cruz, CA) overnight at 4°C with 50 μl of Protein G-Sepharose as described above. The Sepharose beads were washed 2 times with lysis buffer and twice with a 10 mM Tris-HCl (pH 7.4) containing 0.1 mM EGTA and 5 Mm LiCl. After removal of the last wash, the beads were suspended in kinase buffer (10 mM Tris-HCl, 150 mM NaCl and 5 mM EDTA) containing 20 μg of L-α-phosphatidylinositol (Sigma Chemical Co.) 25 mM $MgCl_2$ and 10 μCi of [γ^{32}P]ATP and incubated for 20 min at room temperature. The reaction was stopped by the addition of 60 μl of 6 M HCl and 160 μl of a mixture of chloroform and methanol (1:1). Lipids were then resolved by thin layer chromatography plates (TLC silica gel 60) (Merck, Darmstadt, Germany) in chloroform, methanol, water and ammonium hydroxide (60:47:11.3:2). Dried TLC sheets were developed by autoradiography. Quantifications of the bands was performed using a Kodak image analysis system.

Indirect immunofluorescence microscopy

Glass slides were coated with 20 μg/ml of laminin-1 for 2 hr at 37°C and were then blocked with PBS containing 1% heat-inactivated bovine serum albumin (BSA) at 4°C overnight. Cells were plated (1×10^6 cells) on the matrix-coated glass slides and allowed to adhere for 2 hr in a humidified atmosphere with 5% CO_2 at 37°C. The cells were then stimulated with EGF (50 ng/ml 15 min) and subsequently fixed with 2% paraformaldehyde. In case of staining with anti-AR antibodies the cells were permeabilized with 0.1% triton-X in PBS. After fixation, the cells were rinsed in PBS and incubated in a blocking solution containing 1% albumin and 5% goat serum in PBS for 30 min. Primary antibodies in blocking solution were added in combination to the fixed cells and incubated at room temperature for 30 min. After washing in PBS, the immunoreactivity was revealed using FITC-conjugated or Texas Red- or Rhodamine-conjugated anti rat, anti-mouse, or anti-rabbit secondary antibodies (minimal cross reaction inter-species) in blocking buffer (1:50), used separately or in combination to stain the cells for 30 min. Negative controls were performed by substituting the primary antibodies with the blocking buffer. The immunostained cells were rinsed with PBS and mounted in a mixture (8:2) of glycerol and PBS (pH 8.5). The cells were observed under a laser scanning confocal microscopy (Bio-Rad MRC 1024 ES, Hercules, CA) equipped with a Krypton/Argon laser source 15 nm. A series of optical sections (512×512 pixels) were taken through the depth of the cells with a thickness of 1 μM at intervals of 0.8 μM by using a Nikon 60× 1.4 oil immersion objective. Each

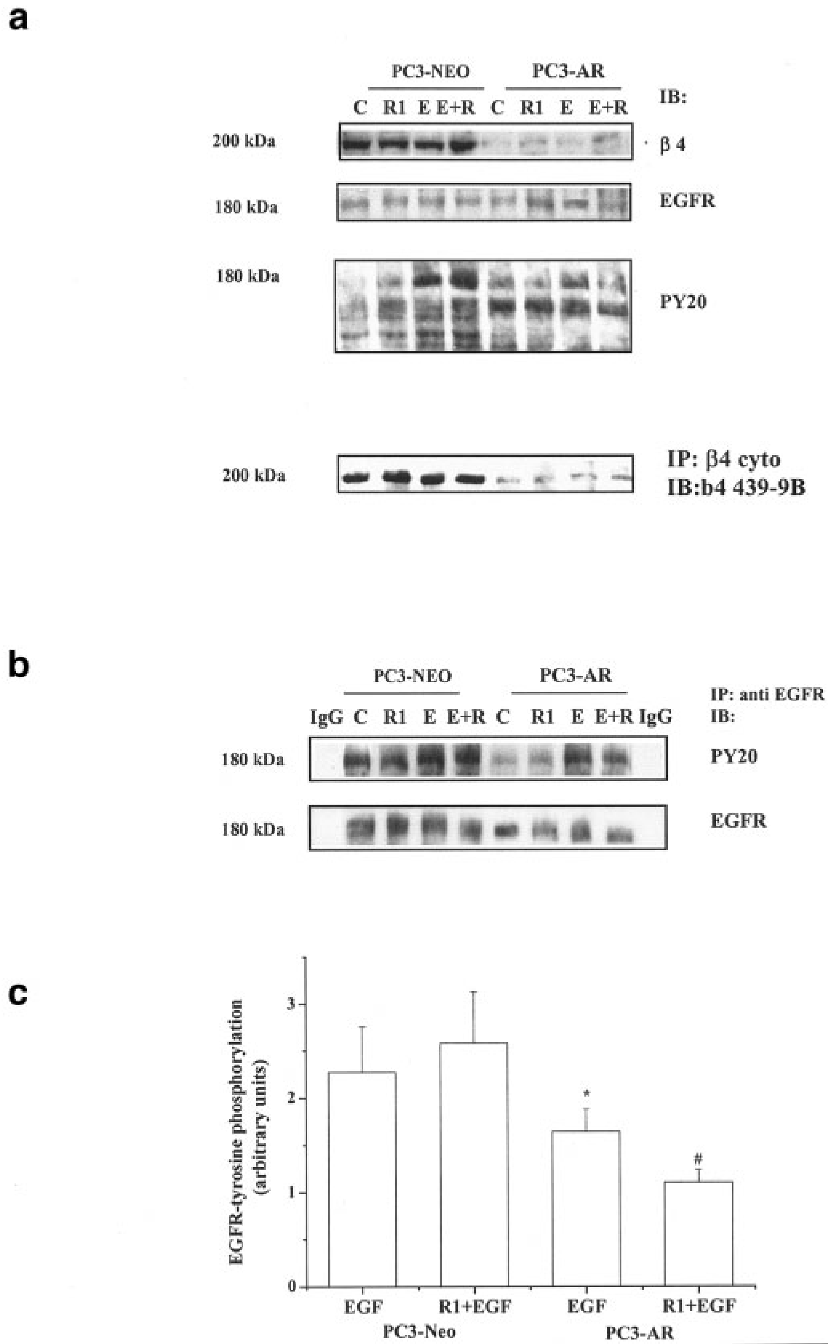

FIGURE 1 – (*a*) Western blot analysis of integrin β4 and EGFR expression in total lysates of PC3-Neo and PC3-AR cells in basal conditions (*c*) and following stimulation with the synthetic androgen R1881 (R1, 1 nM, 3 days), EGF (50 ng/ml, 5 min) or the 2 agonists together (E+R). The blot was first probed for β4 integrin, stripped and reprobed with anti-EGFR and finally with anti-phosphotyrosine PY20 antibody. In the lower panel, Western blot analysis of integrin β4 following immunoprecipitation of the integrin from the lysates of the 2 cell lines in the same conditions as indicated for (*a*) is shown. Both blots are representative of at least 3 different experiments. (*b*) Western blot analysis of EGFR tyrosine phosphorylation following treatments as described for (*a*), in PC3-Neo and PC3-AR cell lysates immunoprecipitated with anti-EGFR antibody. Cells lysates were immunoprecipitated (IP) using an anti-EGFR antibody (Ab-1), run by SDS-PAGE and immunoblotted (IB) with anti-phosphotyrosine (PY20, upper panel), and anti-EGFR (Ab-2, lower panel) antibodies. IgG: negative controls (immunoprecipitated with control IgG). C = control conditions. Representative of 3 similar experiments. (*c*) Densitometric analysis of tyrosine phosphorylation of EGFR (reported as the ratio between EGFR tyrosine phosphorylated band/EGFR band) in response to EGF in presence or absence of R1881 of 4 different immunoprecipitation experiments in PC3-Neo and PC3-AR cells. $*p<0.005$ *vs*. the same treatment in PC3-Neo cells. $\#p<0.05$ *vs*. EGF in PC3-AR cells

section was signal-averaged during acquisition to improve image quality, using the Kalman averaging option (5 scan), and the entire series was projected as a single composite image by superimposition. To reduce bleed-through effects dual channel scanning of red and green signals were recorded separately and saved in 2 different files.

Statistical analysis

Statistical analysis was performed with ANOVA and Student's *t*-test for unpaired and, when applicable, for paired data.

RESULTS

EGFR autotransphosphorylation in response to EGF is reduced in PC3-AR cells

Western blot analysis of integrin β4 in PC3-Neo and -AR total cell lysates (Fig. 1*a* β4 panel) and following integrin β4 immunoprecipitation (Fig. 1*a*, lower panel) confirms the reduced expression of the protein in AR positive PC3 cells (Fig. 1).[6] In addition, both experiments demonstrate that β4 protein expression is not regulated by androgen treatment (R1881, 1 nM, 3 days of treatment) in PC3-AR cells (Fig. 1*a* β4 and lower panels), confirming previous results obtained by FACScan analysis.[6] The expression of EGFR is similar in the 2 cell lines and is not modified by treatment with R1881 (Fig. 1*a*, EGFR panel) as also confirmed by FACScan analysis of surface expression of the protein in PC3-AR cells compared to PC3-Neo (not shown; see also Fig. 7). Stripping and reprobing of the blot in Fig. 1*a* (β4 panel) with an anti-phosphotyrosine antibody (PY20) demonstrates a different pattern of tyrosine phosphorylated protein bands in the 2 cell lines (Fig. 1*a*, PY20 panel). In particular, although EGF treatment (50 nM, 5 min) induces an increase in tyrosine phosphorylation in a protein band of about 180 kDa, comigrating with EGFR, in both cell lines (Fig. 1*a*, PY20 panel), such increase appears to be reduced in PC3-AR cells, in particular in the presence of R1881. To confirm that the observed increase of tyrosine phosphorylation in the 180 kDa protein band in response to EGF corresponds to autotransphosphorylation of the EGFR, the latter was immunoprecipitated in both cell lines. As shown in Figure 1*b* (PY20 panel), EGF induces autotransphosphorylation of EGFR is reduced in PC3-AR cells compared to PC3-Neo and treatment with R1881 (10 nM, 3 days) further reduces it (Fig. 1*b*; see also Fig. 3). Densitometric analysis of the ratio between EGFR tyrosine phosphorylated band and EGFR band from 4 different experiments performed by immunoprecipitation in the 2 cell lines is reported in Figure 1*c*. These results confirm reduced EGFR autotransphosphorylation in PC3-AR cells in the presence of R1881 and respect to PC3-Neo. Overall, these data suggest that the presence of an active androgen receptor interferes with EGFR signaling in PC3 cells.

The androgen receptor co-localizes and co-immunoprecipitates with the EGFR in PC3-AR cells

Emerging evidence indicates that besides its classical location at the nuclear level, the AR may be targeted at the membrane, where interactions with proteins involved in growth factor signaling, such as src kinase family members,[14] caveolin-1[15] and PI3K[16] have been demonstrated. We investigated the localization of AR in our cell line by confocal laser microscopy in PC3-AR cells stained for both AR and EGFR. As shown in Figure 2 the AR (in red, middle panels) localizes both to the nucleus and the cytoplasm of PC3-AR cells, although after stimulation with R1881, increased location to the nuclei was evident. The presence of some nuclear staining in PC3-AR also in control conditions is in agreement with results obtained by other authors in PC3 cells transfected with AR.[17] Interestingly, a striking colocalization (in yellow, right panels) of the AR with the EGFR (in green, left panels) at plasma membrane level was present. In response to EGF, colocalization between the EGFR and the AR was also evident at the level of intracellular granules, possibly reflecting internalization vesicles of the EGFR-AR complex. When cells are stimulated with EGF in the presence of R1881, the pattern of colocalization between the 2 proteins appears to be different compared to EGF alone since much less vesicles are present (Fig. 2). Similar results were obtained in the another PC3-AR clone (not shown). No staining for AR was present in PC3-Neo cells (Fig. 2, lower panels). To

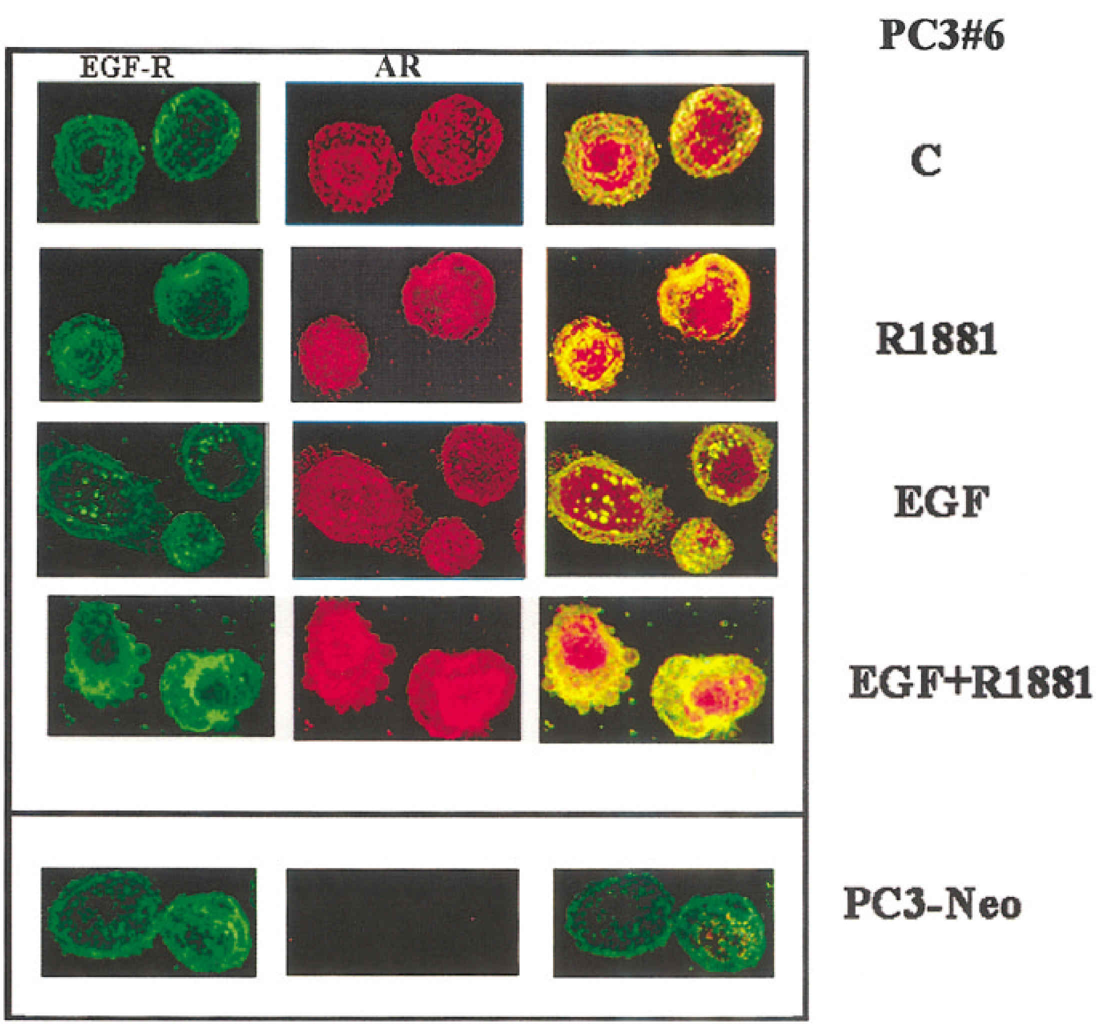

FIGURE 2 – Confocal microscopy analysis of localization of the androgen receptor (AR) and EGFR in PC3-AR cells. PC3-AR cells were pretreated or not with the synthetic androgen R1881 (1 nM, 3 days), plated on laminin-1 for 2 hr and then stimulated with EGF (50 ng/ml) or vehicle (C) for 15 min. Cells were permeabilized and processed for double staining with rabbit polyclonal anti-AR (N-20) (red) and anti-EGFR (Ab-1) (green) antibodies. Yellow depicts colocalization of the 2 antibodies. Lower panel, PC3-Neo cells were plated on laminin-1 for 2 hr and then were processed for double staining with anti-AR (N-20) (red) and anti-EGFR (Ab-1) (green) antibodies. Representative of 3 similar experiments.

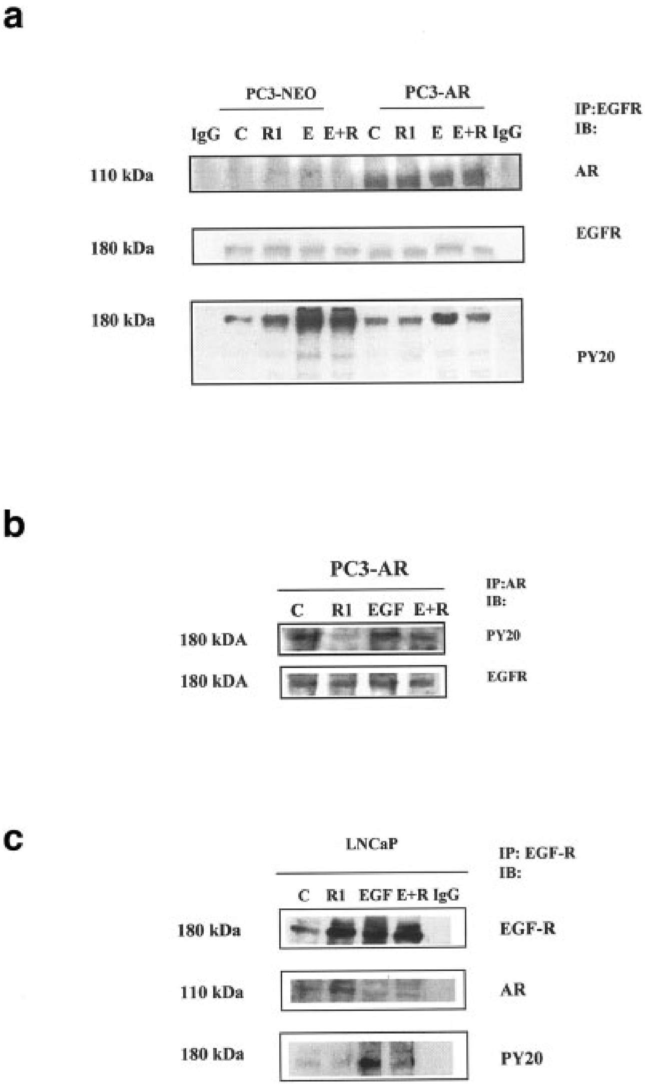

FIGURE 3 – (*a*) Western blot analysis of EGFR and AR, EGFR tyrosine phosphorylation in response to EGF (50 ng/ml, 5 min) in the presence or absence of the synthetic androgen R1881 (R1, 1 nM, 3 days) in PC3-Neo and PC3-AR cell lysates immunoprecipitated with anti-EGFR antibody. Cells lysates were immunoprecipitated (IP) using an anti-EGFR antibody (Ab-1), run by SDS-PAGE and immunoblotted (IB) with anti-AR (N-20) (upper panel), anti-EGFR (Ab-2) (middle panel), anti-phosphotyrosine antibody (PY20, lower panel) antibodies. IgG: negative controls (immunoprecipitated with control IgG). C= control conditions. Representative of three similar experiments. (*b*) Western blot analysis of EGFR and AR, EGFR tyrosine phosphorylation after treatments as described for (*a*) in PC3-AR cell lysates immunoprecipitated with anti-AR antibody. PC3-AR cells lysates were immunoprecipitated (IP) using an anti-AR antibody (Pa1-110) and blotted (IB) with an anti-phosphotyrosine (PY20, upper panel) and anti-EGFR (Ab-2, lower panel) antibody. Representative of 2 similar experiments. (*c*) Western blot analysis of EGFR and AR, EGFR tyrosine phosphorylation in response to EGF (50 ng/ml, 5 min) in the presence or absence of the synthetic androgen R1881 (R1, 1 nM, 3 days) in LNCaP cell lysates immunoprecipitated with anti-EGFR antibody. LNCaP cells lysates were immunoprecipitated (IP) using an anti-EGFR antibody (Ab-1) and blotted (IB) with an anti-EGFR (Ab-2, upper panel), anti-phosphotyrosine (PY20, middle panel) and anti-AR (N-20) (lower panel). Representative of 2 similar experiments.

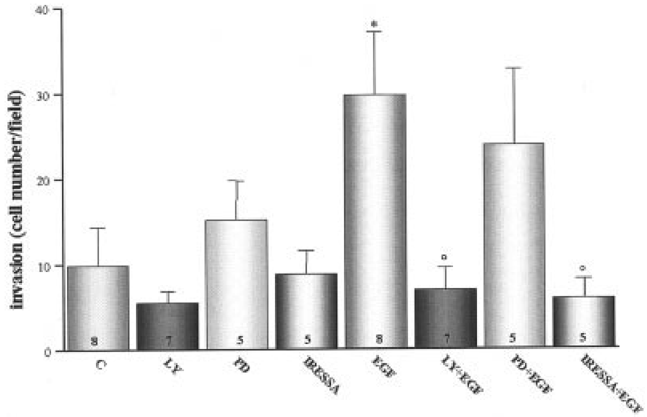

FIGURE 4 – Effect of different kinase inhibitors on Matrigel invasion of PC3-Neo cells. Matrigel was diluted in DMEM (50 mg/ml) and overlaid on the upper surface of the polycarbonate filter. EGF (100 ng/ml) or DMEM were added to the bottom wells of the boyden chambers. Cells (10^5), untreated or treated with the PI3K inhibitor LY294002 (80 mM, LY), the MAPK cascade inhibitor PD098059 (50 mM, PD) and the EGFR tyrosine kinase inhibitor ZD1839 ("Iressa") (10 mM) were added to the top of the wells of the chambers and then incubated for 24 hr at 37°C. The cells that reached the lower surface were quantitated by light microscopy. Values are expressed as mean±SEM of the indicated number of experiments. $^{**}p<0.05$ *vs.* control (C), $^{\circ}p<0.05$ *vs.* EGF

demonstrate that the colocalization between the AR and EGFR observed in PC3-AR cells is due to an association of the 2 molecules, coimmunoprecipitation studies using both anti-EGFR and anti-AR antibodies were conducted. As shown in Figre 3*a* after immunoprecipitation with an antibody against EGFR, a band at 110 kDa was detected by immunoblot analysis with an anti-AR antibody in PC3-AR cells. A tyrosine phosphorylated band corresponding to EGFR was also detected (Fig. 3*a*). Similarly, following immunoprecipitation of AR in PC3-AR cells, EGFR was detected by immunoblotting (Fig. 3*b*). Interestingly, EGF induced tyrosine phosphorylation of EGFR was reduced in PC3-AR cells in both experimental conditions (Fig. 3*a*,*b*), in particular in the presence of R1881 (see also Fig. 1*b*). Coimmunoprecipitation between the EGFR and AR was also detected in the cell line LNCaP, which physiologically express the AR (Fig. 3*c*). As shown in the figure, following immunoprecipitation of EGFR, a band corresponding to AR was found. Stripping and re-probing of the blot with an anti-phosphotyrosine antibody demonstrated that the treatment with R1881 determined a decrease in EGFR autotransphosphorylation also in this cell line (Fig. 3*c*), in agreement with results obtained in PC3-AR cells (Fig. 3*a*,*b*). In the lanes treated with EGF a decrease in co-immunoprecipitated AR protein was observed (Fig. 3*c*). This results, consistently found in LNCaP cells, is difficult to explain. One possibility is that in this cell line the receptor may somehow escape from interaction with AR following treatment with EGF. Overall, results shown in Figures 2 and 3 indicate that an interaction between EGFR and AR occurs in androgen-sensitive prostate carcinoma cells and that this interaction may be involved in determining a decreased signaling ability of EGFR (this manuscript) and the decreased invasive ability of these cells.[6,18]

EGF-induced activation of PI3K is reduced in PC3-AR cells

As mentioned above, EGF promotes the activation of distinct signaling pathways in cancer cells, including PI3K,[7] which finally leads to cell migration and invasion. To evaluate whether PI3K activation is involved in Matrigel invasion of PC3-Neo cells, invasion assays were performed in the absence or presence of the PI3K inhibitor LY294002 (80 μM). As shown in Figure 4, EGF-induced invasion through Matrigel was inhibited by pretreatment (30 min) with LY294002, indicating that the activation of PI3K pathway is essential for EGF-dependent invasion. Matrigel invasion in response to EGF was also suppressed by ZD1839 (also known as "gefitinib" and "Iressa", 10 μM), a tyrosine kinase inhibitor selective for EGFR[19] (Fig. 4). On the contrary, we found that PD098059 (50 μM, 30 min) an inhibitor of MAPK signaling cascade[20] did not affect Matrigel invasion in response to EGF (Fig. 4), indicating that this pathway is not involved in EGF-mediated invasion in PC3 cells. To investigate whether PI3K activity was altered in PC3-AR cells, cell lysates from PC3-Neo and PC3-AR cells, stimulated or not with EGF (50 ng/ml), were immunoprecipitated with an anti-phosphotyrosine antibody to recruit the activated fraction of PI3K and assayed for their ability to phosphorylate L-α-phosphatidylinositol. As shown in Figure 5, both basal and EGF-stimulated PI3K activity are reduced in PC3-AR cells with respect to PC3-Neo. Pretreatment with R1881 determined a decrease of EGF-stimulated PI3K activity in PC3-AR cells, whereas it was ineffective in PC3-Neo cells. Figure 5, lower panel, reports mean±SEM values of PI3K activity (measured by quantification of the bands) of 4 different experiments. This result suggests that in the presence of an AR, PI3K activity of PC3 cells is reduced, in line with recent results that demonstrate that losing dependence or sensitivity from androgens, such as in LNCaP cells in high passages[21] or following androgen deprivation,[22] leads to higher activity of PI3K respect to cells expressing a more differentiated phenotype (LNCaP at early passages or androgen-sensitive).

In carcinoma cells, constitutive activation of PI3K is often found.[23] PI3K is activated by the interaction of the p85 subunit with phosphorylated tyrosine residues on activated growth factor receptors including the EGFR.[24] To study whether the reduced basal and EGF-stimulated PI3K activity in PC3-AR cells is due to an impairment of PI3K-EGFR interaction, we immunpreciptated PI3K from PC3-Neo and -AR cells using an anti-p110 PI3K antibody and evaluated coimmunoprecipitation with EGFR in both cell lines. As shown in Figure 6*a*, although p110 PI3K expression was similar in the 2 cell lines, the fraction of EGFR coimmunoprecipitating with PI3K was much higher in PC3-Neo cells, suggesting an impairment of EGFR-PI3K interaction in the presence of the AR. In addition, when the blot was probed using an anti-phosphotyrosine antibody, a phosphorylated protein band at 180 kDa molecular weight, comigrating with EGFR, was detected only in PC3-Neo cells (Fig. 6*a*). However, treatment with EGF does not appear to modify either tyrosine phosphorylation of this band or EGFR-PI3K interaction (Fig. 6*a*). This result was constantly reproduced in 3 different experiments.

To confirm that PI3K activation is reduced in PC3-AR cells, we performed Western blot analysis of total lysates from PC3-Neo and PC3-AR cells using anti-phosphoserine AKT antibodies. As

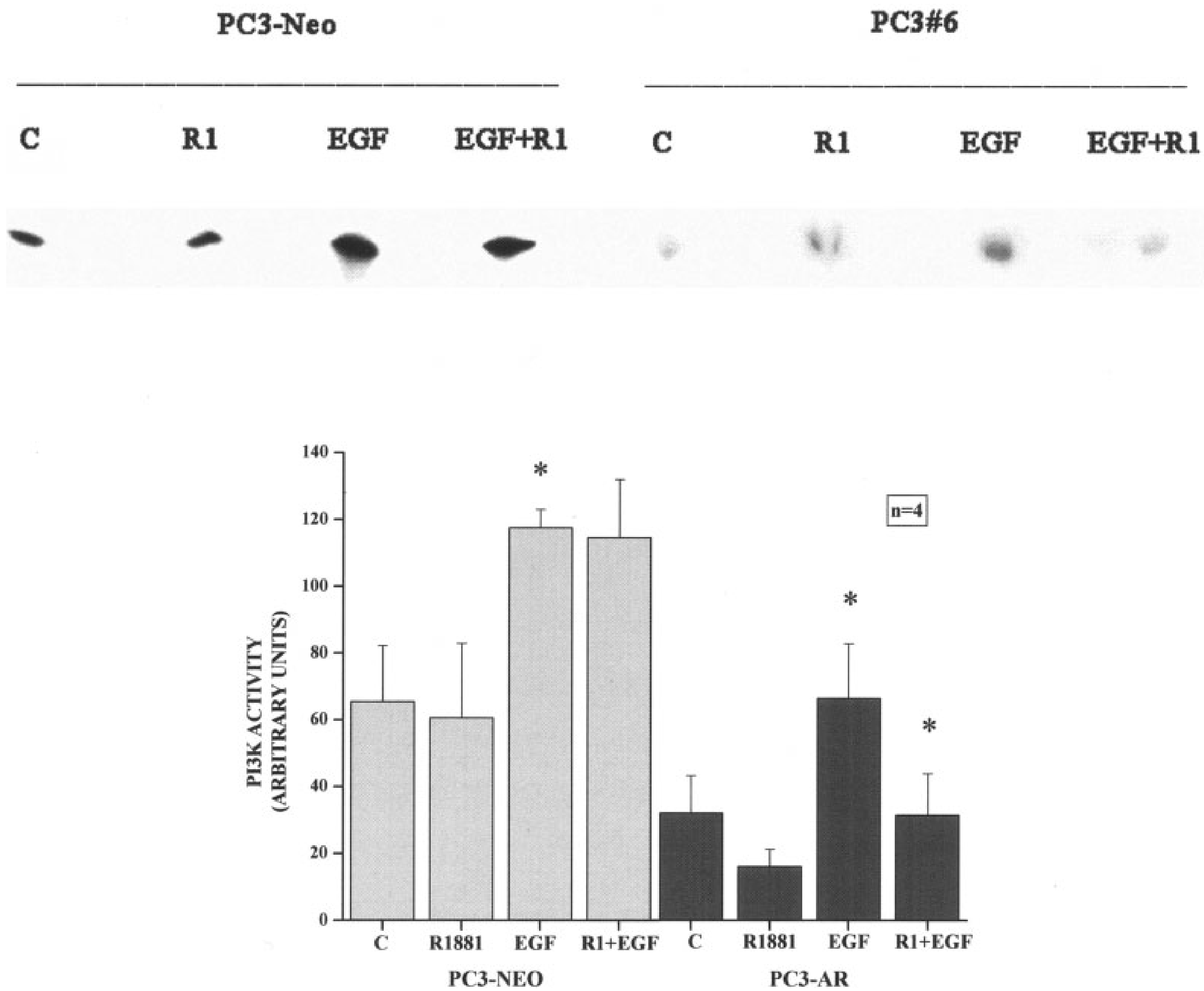

FIGURE 5 – Phosphatidylinositol-3 kinase (PI3K) activity in response to EGF and R1881 in PC3-Neo and PC3-AR cells. (upper panel) PC3-Neo and PC3-AR cells, pretreated or not with R1881 (R1, 1 nM, 24 hr), were plated on laminin-1 for 2 hr and then stimulated with EGF (50 ng/ml). Cell lysates were immunoprecipitated with an anti-phosphotyrosine antibody (PY99) and PI3K activity measured as described in Material and Methods. (lower panel) Mean±SEM values of PI3K activity (arbitrary units) in 3 different experiments.

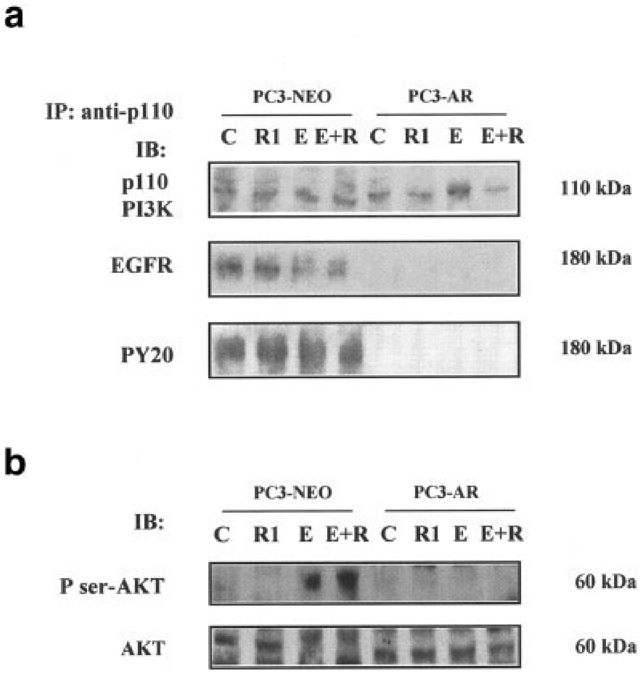

FIGURE 6 – (*a*) Western blot analysis of p110 PI3K subunit, EGFR and tyrosine phosphorylation in response to EGF (50 ng/ml, 5 min) in the presence or absence of the synthetic androgen R1881 (R1, 1 nM, 3 days) in PC3-Neo and PC3-AR cell lysates immunoprecipitated with anti-p110 PI3K antibody. Cells lysates were immunoprecipitated (IP) using an anti-p110 antibody, run by SDS-PAGE and immunoblotted (IB) with anti-p110 PI3K (upper panel), anti-EGFR (Ab-2) (middle panel), anti-phosphotyrosine antibody (PY20, lower panel) antibodies. Representative of three similar experiments. (*b*) Western blot analysis of AKT phosphorylation, in response to EGF (50 ng/ml, 5 min) in the presence or absence of the synthetic androgen R1881 (R1, 1 nM, 3 days) in PC3-Neo and PC3-AR cell total lysates. The blot was first probed with anti p-ser AKT antibody followed by stripping and reprobing with anti-AKT antibody. C= control. Representative of 3 similar experiments.

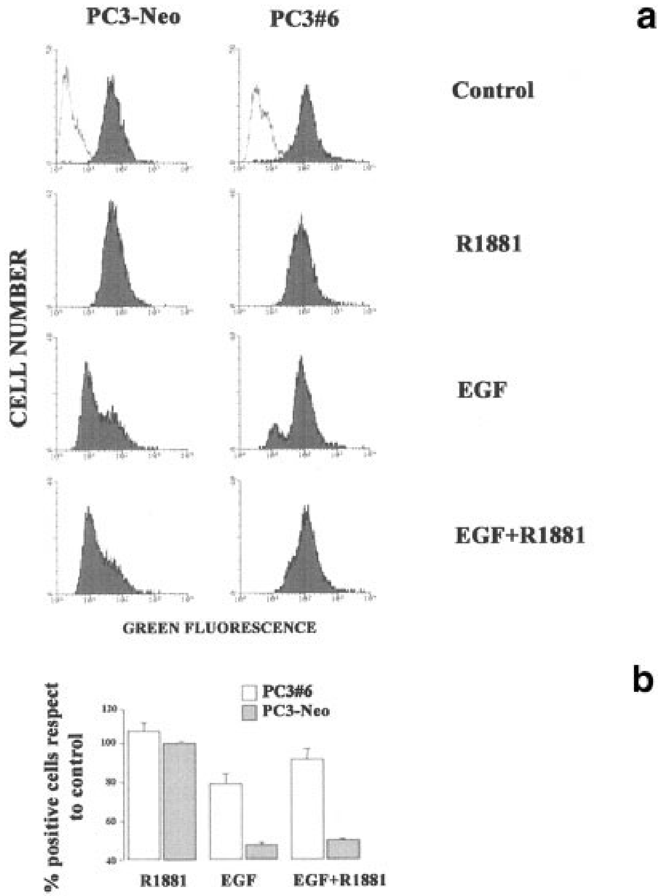

FIGURE 7 – Effect of R1881 on internalization of EGFR in PC3-Neo and PC3-AR6 cells. (*a*) PC3-Neo and PC3-AR cells were left untreated (C) or incubated with R1881 (1 nM, 3 days). Cells were then incubated with EGF (50 ng/ml) or vehicle for 20 min at 37°C to allow internalization of the EGFR, placed on ice and processed for detection of EGFR expression (solid histograms) by flow cytometry analysis as described in Material and Methods. Nonspecific fluorescence (open histograms shown in the control panels) was assessed by omitting incubation with the primary antibody. (*b*) Average (n=2) percentage cells expressing EGFR on the surface respect to control in PC3-Neo and PC3-ARcells in the different experimental conditions.

shown in Figure 6*b* (upper panel) treatment with EGF induces AKT phosphorylation in PC3-Neo but not in PC3-AR cells, whereas AKT expression was similar in both cell lines (Fig. 6*b* lower panel), confirming reduced activation of the PI3K/AKT pathway in PC3-AR cells. The lower mobility of AKT band in the lanes treated with EGF in PC3-Neo cells may reflect a shift due to increased phosphorylation (Fig. 6*b* upper panel).

EGFR internalization is altered in PC3-AR cells

Following EGF treatment, EGFR is rapidly downregulated from the cell surface undergoing a process of internalization/endocytosis.[25] As shown in Figure 4, in PC3-AR cells, EGFR is located at the level of discrete vesicles, probably reflecting internalization of the receptor. However, the presence of these vesicles was reduced when treatment with EGF was performed in the presence of R1881 (Fig. 2), suggesting an impairment of the process of EGFR internalization. To further investigate EGFR internalization process in our cell model, we performed FASCan analysis of surface EGFR expression following treatment with EGF (50 ng/ml, 15 min incubation) in the presence or absence of R1881 (1 nM) in PC3-Neo and -AR cells. As shown in Figure 7*a*, when PC3-Neo cells were incubated with EGF at 37°C, a significant decrease in cell surface expression of the receptor was detected, as evidenced by the shift of the positive peak to the left. In PC3-AR cells the shift of the peak to the left was dramatically reduced, indicating decreased internalization. Treatment with R1881 (1 nM) further reduced EGFR internalization induced by EGF in PC3-AR cells but did not affect receptor internalization in PC3-Neo cells (Fig. 7*a*). Mean (n=2) percentage of cells expressing EGFR on the surface respect to control in the 2 cell lines in the different conditions are reported in Figure 7*b*. Similar results were obtained by using FITC-conjugated EGF to label surface EGFR (data not shown). In analogy with results obtained in PC3-AR cells, EGFR internalization was found to be reduced also in LNCaP cells when compared to PC3-Neo cells. Indeed, following EGF treatment, surface EGFR expression in LNCaP cells was reduced only of 25% (not shown), a value similar to the 22% decrease observed in PC3-AR cell (Fig. 7*b*).

DISCUSSION

Increasing evidence demonstrates that androgen-responsive prostate cancer cells are characterized by reduced growth, adhesion, invasion, migration and colony-forming abilities,[2,6,17,26] suggesting that maintenance of an AR-sensitive phenotype reduces the malignant potential of these cells. In addition, the presence of a functional AR in PC3 cells confers sensitization to anticancer therapy.[26] Similarly, in another hormone-dependent cancer, such as breast cancer, the presence of estrogen receptors maintains a less invasive phenotype of the cells even in the absence of ligands.[27–29] Previous results from our[6] and other groups[30] demonstrated that, at least in part, decreased *in vitro* Matrigel invasion of androgen-sensitive PC cells[18] is due to decreased expression of surface integrins. Other studies performed in androgen-independent and -dependent PC cell models *in vitro*, demonstrated regulation, by androgens, of several other genes involved in modulating invasion ability of these cells, including prostin-1,[31] urokinase-type palsminogen activator[32] and neutral endopeptidase (NEP).[5] Here we show that EGF-mediated autotransphosphorylation of EGFR and downstream PI3K/AKT pathway activation are also reduced in androgen-sensitive cell lines. In addition, we demonstrate the occurrence of an interaction between the AR and the EGFR at the membrane level in the same cell lines. The reduced autotransphosphorylation of EGFR in androgen-responsive PC3-AR cells respect to unresponsive cells is in line with previously published results that show an inverse correlation between phosphotyrosine levels of ErbB-2, an EGFR mutant, and androgen responsiveness of prostate cancer cells.[33]

Recent data demonstrate that the AR may interact with membrane proteins that activate signaling pathways such as ERKs and PI3K.[14,16,34] These mechanisms of androgen receptor signaling are believed to represent alternative pathways for stimulation of prostate carcinoma cell growth. Our data demonstrate that in PC3-AR cells the AR colocalizes with the EGFR at the membrane level through an interaction of the 2 proteins as demonstrated by coimmunoprecipitation studies performed in the 2 androgen-sensitive cell lines PC3-AR and LNCaP. Further studies are needed to evaluate the site and the mechanisms that determine the association between AR and EGFR in prostate carcinoma cells. Recently, the AR has been shown to associate with src kinase family members[14] and caveolin-1[15] in PC cell lines. More recently, an association with the signaling protein calmodulin has been also shown in LNCaP cells.[35] In the case of src, the interaction is due to a proline-rich region in the AR,[14] whereas in the case of caveolin-1 both the NH_2-terminal and ligand-binding domains of the AR are involved.[15] Both src and caveolin-1 are capable of interacting with EGFR and are involved EGF-stimulated signaling.[36–38] It is thus possible that EGFR-AR interaction detected in our study is mediated by these proteins. Of interest, the interaction between caveolin-1 and EGFR results in inhibition of ERK activation, lamellipodia formation and cell migration of mammary carcinoma cells[39] and caveolin-1 expression inhibit invasiveness in the same cells.[40] The occurrence of a possible interaction between AR and caveolin-1 and the formation of a complex among AR, caveolin and EGFR is currently under investigation in our Lab. We prospect a scenario where interaction between AR and EGFR provokes a disruption of the mechanisms leading to autotransphosphorylation of the receptor and PI3K activation in response to EGF leading to decreased invasion of these cells. It is possible that the interaction between the 2 proteins may occur at sites that are fundamental for interaction with other downstream signaling molecules such as PI3K. On the other hand, our coimmunoprecipitation studies with anti-PI3K antibodies suggest that EGFR-PI3K interaction is disrupted in PC3-AR cells, possibly explaining the lower activity of the enzyme in PC3-AR cells. Decreased PI3K activation may be thus responsible for the described lower aggressive behavior of PC3-AR, LNCaP and other androgen-sensitive cell lines.[2,6,17,25,41] In agreement with our data that shows reduced PI3K/AKT activation in androgen-sensitive PC3 cells, recent results from different groups demonstrate that losing dependence or sensitivity from androgens, such as in LNCaP cells in high passages[21] or following androgen deprivation[22] leads to higher activation of PI3K/AKT respect to cells expressing a more differentiated phenotype (LNCaP at early passages or androgen-sensitive).

Our data on EGFR internalization demonstrate that a considerable amount of EGFR remains on the surface after treatment with EGF in PC3-AR cells compared to PC3-Neo, indicating a disruption of the endocytotic process in our cells. The internalization of EGFR is further decreased by treatment with R1881. The molecular mechanism responsible for reduced internalization of EGFR in androgen-sensitive cells is obscure. Recently it has been shown that EGFR signaling intensity determines the rate of internalization of the receptor.[42] In particular, Schmidt *et al*.[42] showed that if autotransphosphorylation of EGFR is reduced below a threshold, a reduction of internalization and downregulation of the receptor occurs. We show here that EGFR autotransphosphorylation is reduced in PC3-AR cells and therefore it is possible that also internalization is consequently reduced. Although internalization by endocytosis has been assumed to be a mechanism to attenuate the signaling in response to the growth factor, increasing evidence demonstrates that a correct endocytotic pathway is important for EGFR signaling by controlling the specificity of the response.[36,37] Several studies have shown that internalized EGFR are enzymatically active, are still phosphorylated and maintain association with many adaptor proteins.[43–45] In addition, it has been recently shown that interaction with some adaptor proteins, such as eps8, a protein involved in cytoskeletal reorganization and actin remodeling,[46] occurs only at endosomal level.[46] Furthermore, blocking of EGFR endocytosis by using a dynamin mutant results in downregulation of ERK and PI3K activation in response to EGF[47] and insulin.[48] Whether EGFR-AR interaction may be responsible for decreased EGFR signaling and internalization remains to be addressed; however, it is possible that such interaction disrupts the ability of the receptor to autophosphorylate by attenuating its intrinsic tyrosine kinase activity, determining as a consequence a reduction of internalization and PI3K activation.

It must be mentioned that AR expression is found in a high percentage androgen-independent PC tumors, although increased DNA methylation of the AR promoter is found in the latter respect to androgen-dependent tumors (for review, see reference 49). However, although these results indicate that expression of AR protein is not frequently lost, it is possible that the function of the receptor or the pathways that are regulated by it are altered in androgen-independent PC. On the other hand, bypassing the AR *via* other cellular pathways has been indicated as one of the possible mechanisms of transition to androgen-independence.[50]

In conclusion, androgens and AR contribute to confer a less malignant phenotype of androgen-sensitive prostate tumors, by reducing the expression of $\alpha6\beta4$ as demonstrated previously,[6] and by interfering with EGFR-$\alpha6\beta4$ interaction and signaling leading to invasion through an interaction between AR and EGFR.

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