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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 autophosphorylation 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 EGFR-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. Immunofluorescence microscopy and coinmunoprecipitation 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; epithelial growth factor receptor; androgen receptor; PI3K; invasion

Prostate Cancer (PC) is one of the most common cancers 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 treatment for a subset of these patients. However, the development of escape mechanisms and metastatic potential of PC cells, characterized by higher invasiveness and metastatic properties, has focused attention on the molecular mechanisms that lead to invasion and metastasis in prostate cancer cells.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 identified4-6 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 our present study, we investigated EGF-activated signaling in PC3-Neo and -AR cells. We report that EGF-mediated EGFR autophosphorylation and PI3K activation is reduced in PC3-AR cells. In addition, we demonstrate colocalization and coinmunoprecipitation 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 (Beverly, MA). Recombinant human epidermal growth factor (EGF) 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-B4 (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). Antiphosphotyrosine PY20 antibody was obtained from ICN (Costa Mesa, CA); antiphosphotyrosine 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 Oncogene (Cambridge, England). Rat MAb anti-B4 (439-9B) 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-B4 (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). Antiphosphotyrosine PY20 antibody was obtained from ICN (Costa Mesa, CA); antiphosphotyrosine 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 (Santa Cruz, CA). Mouse monoclonal anti PI 3-kinase p110 (D-4) and anti EGFR (1005) was 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 (Santa Cruz, CA).

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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 (‘Tissa’) 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 (Baltimore, 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 cell cultures were transfected with human full length androgen receptor construct (pSHbHR5) or vector alone (PC3-NEO cells) by electroporation and selected in the presence of 0.5 mg/ml genetin (G418) as described previously. 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**

Cell surface EGFR expression was evaluated by flow cytometry performed as described. Cells were grown on Petri dish until confluence, detached with PBS, washed in PBS containing 0.1% trypsin-EDTA and resuspended in PBS supplemented with 1 mM CaCl2 and 1 mM MgCl2. After the indicated treatments, cells were incubated for 30 min at 4°C with the monoclonal anti-EGFR antibody Ab1, or non-specific 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 in 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 FACSscan 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. Immunoprecipitation was performed as previously described with few modifications. Briefly, cells were harvested in PBS supplemented with 1 mM Na3VO4, centrifuged and resuspended in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1% NP-40, 1 mM Na2VO3 and 1 mM PMSF). Precleared lysates were then incubated for 1 hr with 50 μl of Protein G-Sepharose for preclaring. Preclarsed 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 EDTA 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 MgCl2 and 10 μCi of [γ-32P]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 (60:47:13:2). 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 (1×106 cells) on the matrix-coated glass slides and allowed to adhere for 2 hr in a humidified atmosphere with 5% CO2 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 used 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. 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
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. 1a β4 panel) and following integrin β4 immunoprecipitation (Fig. 1a, lower panel) confirms the reduced expression of the protein in AR positive PC3 cells (Fig. 1). 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. 1a β4 and lower panels), confirming previous results obtained by FACScan analysis. The expression of EGFR is similar in the 2 cell lines and is not modified by treatment with R1881 (Fig. 1a, 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. 1a (β4 panel) with an anti-phosphotyrosine antibody (PY20) demonstrates a different pattern of tyrosine phosphorylated protein bands in the 2 cell lines (Fig. 1a, 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. 1a, 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 1b (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. 1b; 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 1c. 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, caveolin-1 and PI3K 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. 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 another PC3-AR clone (not shown). No staining for AR was present in PC3-Neo cells (Fig. 2, lower panels). To
representative of 2 similar experiments. 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 cells lysates immunoprecipitated with an anti-AR antibody. Cells lysates were immunoprecipitated (IP) using an anti-AR antibody (N-20), 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. (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 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-EGFR (Ab-1) and blotted (IB) with anti-phosphotyrosine (PY20, upper panel) and anti-EGFR (Ab-2, lower panel) antibody. Representative of 2 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 an anti-EGFR antibody. Cells lysates were immunoprecipitated (IP) using an anti-EGFR antibody (Ab-1), run by SDS-PAGE and immunoblotted (IB) with anti-EGFR (Ab-1) and anti-AR (N-20) (upper 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.

demonstrate that the colocalization between the AR and EGFR observed in PC3-AR cells is due to an association of the 2 molecules, communoprecipitation studies using both anti-EGFR and anti-AR antibodies were conducted. As shown in Figure 3a after immunoprecipitation with an antibody against EGFR, a band at 110 kDa was detected by immunoblotting with an anti-AR antibody in PC3-AR cells. A tyrosine phosphorylated band corresponding to EGFR was also detected (Fig. 3a). Similarly, following immunoprecipitation of AR in PC3-AR cells, EGFR was detected by immunoblotting (Fig. 3b). Interestingly, EGF induced tyrosine phosphorylation of EGFR was reduced in PC3-AR cells in both experimental conditions (Fig. 3a,b), in particular in the presence of R1881 (see also Fig. 1b). Coimmunoprecipitation between the EGFR and AR was also detected in the cell line LNCaP, which physiologically express the AR (Fig. 3c). 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 autophosphorylation also in this cell line (Fig. 3c), in agreement with results obtained in PC3-AR cells (Fig. 3a,b). In the lanes treated with EGF a decrease in co-immunoprecipitated AR protein was observed (Fig. 3c). 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. 

**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 (10⁵), 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), °p<0.05 vs. EGF
inhibitor selective for EGFR\textsuperscript{19} (Fig. 4). On the contrary, we found that PD098059 (50 \textmu M, 30 min) an inhibitor of MAPK signaling cascade\textsuperscript{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-\alpha-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\textsuperscript{21} or following androgen deprivation,\textsuperscript{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.\textsuperscript{23} PI3K is activated by the interaction of the p85 subunit with phosphorylated tyrosine residues on activated growth factor receptors including the EGFR.\textsuperscript{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 immunoprecipitated PI3K from PC3-Neo and -AR cells using an anti-p110 PI3K antibody and evaluated coimmunoprecipitation with EGF in both cell lines. As shown in Figure 6a, although p110 PI3K expression was similar in the 2 cell lines, the fraction of EGF 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 EGF, was detected only in PC3-Neo cells (Fig. 6a). However, treatment with EGF does not appear to modify either tyrosine phosphorylation of this band or EGFR-PI3K interaction (Fig. 6a). 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.
shown in Figure 6b (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. 6b 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. 6b 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. 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 FACS analysis of surface EGFR expression following treatment with EGF (50 ng/ml, 15 min incubation) in the presence or absence of R1881 (1 nM). 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 of cells expressing EGFR on the surface respect to control in PC3-Neo and PC3-AR cells in the different experimental conditions.

**DISCUSSION**

Increasing evidence demonstrates that androgen-responsive prostate cancer cells are characterized by reduced growth, adhesion, invasion, migration and colony-forming abilities, 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. 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 li-
EGFR and are involved EGF-stimulated signaling.36–38 It is thus cbers14 and caveolin-115 in PC cell lines. More recently, an asso-

the AR has been shown to associate with src kinase family mem-

evaluate the site and the mechanisms that determine the associa-

tate 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 associa-

tion between AR and EGFR in prostate carcinoma cells. Recently, the AR has been shown to associate with src kinase family mem-
bbers14 and caveolin-115 in PC cell lines. More recently, an associ-

aion 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 NH2-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 caveo-

lin-1 and EGFR results in inhibition of ERK activation, lamelli-
podia formation and cell migration of mammary carcinoma cells39 and caveolin-1 expression inhibit invasiveness in the same cells.40

The occurrence of a possible interaction between AR and caveo-

lin-1 and the formation of a complex among AR, caveolin and EGFR is currently under investigation in our Lab. We prospect a sensiti-

vity interaction between AR and EGFR that may be responsible for the reduction of internalization and downregulation of the receptor.42 We also show here that EGFR-AR interaction is mediated by these proteins, such as eps8, a protein involved in cytoskeletal reorganization and actin remodeling.46 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 EGFR as demonstrated previously,42 and by interfering with EGFR-ERK42 interaction and signaling leading to invasion through an interaction between AR and EGFR.

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REFERENCES
