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Abstract

Although androgens and the androgen receptor (AR) are involved in tumorigenesis of prostate cancer (PC) in initial phases, less clear is the role played in advanced androgen-independent (AI) stages of the disease. Several recent reports indicated that re-expression of AR in PC-derived cell lines determines a less aggressive phenotype of the cells. We have previously demonstrated that re-expression of AR decreases the invasion ability of PC3 cells *in vitro* by affecting signalling and internalization processes of epidermal growth factor receptor (EGFR). Here, we show that reduced EGFR internalization is also a characteristic of AR positive PC cell lines LNCaP and 22Rv1. Reduced internalization in PC3-AR cells is associated to a defective interaction between the EGFR and two adaptor proteins which mediate the endocytotic process, Grb2 and c-Cbl. As a consequence of such reduced interaction, ubiquitination of the receptor, which is mainly mediated by c-Cbl, is also altered. In addition, we show that internalized EGFR co-localizes with early endosome antigen-1, a marker of clathrin-mediated endocytosis, in PC3-Neo cells but not in AR positive cell lines. Conversely, EGFR maintains co-localization with caveolin-1 after EGF stimulation in PC3-AR cells. These data suggest that expression of AR affects clathrin-mediated endocytosis pathway of EGFR, which, according to recent findings, plays an essential role in the completeness of signalling of the receptor. Taken together, these data emphasize the role of AR in the regulation of EGFR endocytotic trafficking and active signalling in PC cells. In view of the role of EGFR signalling in invasion of carcinoma cells, our data may explain the lower invasive phenotype observed in AR-positive cell lines.

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Introduction

Trafficking of growth factor receptors is an important process in the pathogenesis of cancer (Polo *et al.* 2004). Although until a few years ago, ligand-induced receptor internalization was considered a mechanism exclusively devoted to down-regulate growth factor signalling, it is now evident that signalling of tyrosine kinase receptors proceeds at endosomal level (Sorkin 2001) and recruitment of certain adaptor proteins occurs after internalization (Burke *et al.* 2001) implicating endocytosis in the propagation of intracellular signals, assuring compartmentalization and transporting signalling complexes to specific subcellular locations (McPherson *et al.* 2001). As an example, normal endocytotic trafficking of activated epidermal growth factor receptor (EGFR) is required to achieve full activation of epidermal growth factor (EGF) signalling (Vieira *et al.* 1996). In addition, it has been recently shown that Huntingtin-interacting protein 1 causes transformation of cells by prolonging activation and signalling of growth factor receptors after endocytosis (Hyun & Ross 2004). EGFR endocytosis may proceed through two different routes, named respectively clathrin-dependent and -independent (also known as lipid raft-dependent). While the clathrin-dependent pathway leads to signalling, the clathrin-independent leads mainly to lysosomal degradation (Aguilar & Wendland 2005).

The EGFR and its ligand, the EGF, play a key role in the pathogenesis of different tumours, including prostate cancer (PC; Russell *et al.* 1998) where both EGF and EGFR are often up-regulated in particular in advanced stages (Di Lorenzo *et al.* 2002). In addition to proliferation, EGFR plays a key role in invasion of cancer cells (Wells *et al.* 2002) participating, in association with specific integrins, in the formation of the plasma membrane structures (lamellipodia) that mediate migration through the basal membrane (Rabinovitz *et al.* 2001). Recently, it has been shown that increased EGFR expression enhances tumour invasiveness of mammary adenocarcinomas by increasing cell motility *in vivo* without affecting the growth of the tumour (Xue *et al.* 2006), pointing out the key role exerted by the EGF/EGFR system in invasion and metastasis. Inhibition of EGFR with monoclonal antibodies or with tyrosine kinase inhibitors inhibits either growth and invasion of

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androgen-dependent (AD) and -independent PC cells *in vitro* (Bonaccorsi *et al.* 2004*b*, Festuccia *et al.* 2005).

We previously demonstrated that re-expression of AR in the androgen-independent (AI) prostate cancer cell line PC3 by transfection (PC3-AR cells) determined a decrease in their ability to invade Matrigel in response to EGF (Bonaccorsi *et al.* 2000). This effect is determined either by a decrease in expression levels of the integrin α6β4 (Bonaccorsi *et al.* 2000) or by an alteration of EGF-activated signalling with respect to control cells (Bonaccorsi *et al.* 2004*a*). In particular, EGFR autotransphosphorylation, recruitment of phosphatidyl inositol-3-kinase (PI3K), PI3K/AKT activation and internalization of the receptor in response to the ligand were all reduced in PC3-AR cells and were further reduced by treatment with an androgen (Bonaccorsi *et al.* 2004*a*). We also demonstrated that EGFRs co-localize and -immunoprecipitate with AR in PC3-AR cells and suggested that such interaction interferes with activation of EGFR (Bonaccorsi *et al.* 2004*a*, 2006). Overall, these results indicate that the higher invasive phenotype displayed by AI PC cell lines with respect to AD ones (Baldi *et al.* 2003) might be in part explained by a different sensitivity to EGF.

The present study was undertaken to investigate the possible alterations of EGFR signalling leading to deregulated endocytosis of the receptor in AR-positive PC cell lines. We show that phosphorylation of specific tyrosines of COOH-terminal domain, interaction with the specific adaptor proteins Grb-2 and c-Cbl, ligand-mediated ubiquitination of EGFR and interaction with early endosome antigen-1 (a marker of clathrin-dependent endocytosis) are all reduced in PC3-AR with respect to control cells.

Materials and methods

Antibodies and chemicals

Rabbit polyclonal anti-EGF receptor antibody was obtained from Cell Signaling Technology Inc. (Danvers, MA, USA), rabbit polyclonal anti-phospho-EGF receptor (Tyr845), (Tyr992), (Tyr1045), (Tyr1148), (Tyr1168), (Tyr1173), (Tyr1086) antibodies, rabbit polyclonal anti-phospho-EGF receptor (ser1046/1047) antibody and rabbit polyclonal anti-GRB2 antibody was obtained from Cell Signaling Technology Inc. Mouse mAb Ab2 (anti EGFR) and mouse mAb Ab1 (anti EGFR) were from Oncogene (Cambridge, UK). Mouse monoclonal EGF Receptor Ab12 was from NeoMarkers, Lab Vision Corporation (CA, USA). Mouse anti-human mAb anti-c-Cbl was from BD Biosciences (Pharmingen San Diego, CA, USA). Rabbit polyclonal anti-early endosome marker (EEA-1) antibody was from Abcam Ltd (Cambridge, UK). Antiphosphotyrosine PY20 antibody was obtained from ICN (Costa Mesa, CA, USA), antiphosphotyrosine PY99 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). Mouse monoclonal anti EGFR (1005) was from Santa Cruz Biotechnology. Mouse monoclonal antibody against ubiquitin was from Covance Research Products Inc (Denver, PA, USA). Monoclonal anti-ubiquitin antibody was provided from Medical & Biological Laboratories Co. (Nagoya, Japan). Rabbit anti-ubiquitin antibody was from SIGMA Chemical Co. Anti-caveolin antibody was obtained from BD Farmingen (San Diego, CA, USA). Recombinant human EGF was obtained from Pepro Tech EC (London, UK). Fluorescein-conjugated EGF and Alexa Flour 488 goat anti mouse IgG were obtained from Molecular Probes Inc, Invitrogen. Anti rabbit IgG (goat) Texas Red conjugate was from Calbiochem (CA, USA). The antibiotic Geneticin (G418) was obtained from Calbiochem. Protease inhibitor cocktail and other not specified reagents were from SIGMA Chemical Co. R1881 was from NEN Perkin–Elmer Life and Analytical Sciences (MA, USA).

Cell lines

Androgen receptor (AR)-negative PC3 and AR-positive LNCaP PC cell lines were obtained from American Tissue Culture Collection (Bethesda, MD, USA). AR-positive 22Rv1 PC cells were generously provided by Prof. Culig (Innsbruck Medical University, Austria).

Cell culture and transfection

PC3 cells were maintained in 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 and selected in the presence of 0·5 mg/ml geneticin (G418) as described previously (Bonaccorsi *et al.* 2000). To obtain stable colonies, individual clones were isolated by limiting dilution and tested for the presence of the AR both by northern and western analysis. Two independent clones of PC3-AR transfected cells (# 6 and #13) were used in this study. The growth characteristics in response to androgens of these two clones have been described previously (Bonaccorsi *et al.* 2000). Both clones exhibit similar growth characteristics although they grow slowly with respect to parental or Mock-transfected PC3-Neo cells (basal thymidine incorporation (mean ± S.E.M. cpm/well/4 h): PC3-Neo = 12572 ± 4939; clone#6 = 2355 ± 1384*; clone #13 = 5378 ± 2829†, $^{*}P<0{\cdot}005$ and $^{\dagger}P<0{\cdot}05$ vs PC3-Neo). LNCaP cells were cultured in RPMI medium

supplemented with 10% FBS, 1% penicillin/streptomycin and 1% glutamine. The 22Rv1 cells were grown in MCDB medium supplemented with 10% FBS, 1% penicillin/streptomycin and 1% glutamine.

Internalization assay by flow cytometry analysis

Cell surface EGFR expression was evaluated by flow cytometry performed as described (Bonaccorsi *et al.* 2004*a*). Cells were grown on a Petri dish until confluence, washed with PBS, detached with 0·1% trypsin–EDTA and resuspended in PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ that was supplemented with 4% FBS. After the indicated treatments, cells were incubated 30 min at 4 °C with the monoclonal anti-EGFR antibody Ab1 or non-specific IgG as control, washed twice with PBS and further incubated with FITC-conjugated goat anti-mouse secondary antibody (1:200) for 30 min. After washing twice, cells were fixed with 3% paraformaldehyde in PBS at room temperature for 15 min and washed twice. FITC green fluorescence was detected at 515–555 nm using a FL-1 detector of a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with 15 mW argon–ion laser for excitation. Debris were gated out by establishing a region around the population of interest on the Forward Scatter versus 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/s. Data were processed with analysis software LYSYS II (Becton Dickinson) and are expressed as median value of EGFR expressing cells of the fluorescence histograms normalized to the corresponding negative control obtained by omitting the primary antibody.

Immunoprecipitation and western blot analysis

Protein extraction and western blot analysis were performed as previously described (Bonaccorsi *et al.* 2000). Immunoprecipitation was performed as previously described (Bonaccorsi *et al.* 1997) with few modifications. Briefly, cells were scraped in PBS supplemented with 1mM Na_3VO_4, centrifuged and resuspended in lysis buffer (20 mM Tris, pH 7·4; 150 mM NaCl; 0·5% NP-40; 0·2% Triton-X; 1 mM Na_3VO_4 and 1 mM PMSF). After protein measurement, aliquots of cell lysates containing equal amount of proteins (1000 µg) were incubated for 1 h at 4 °C with 30 µl of Protein A (or Protein G)-Sepharose for preclearing. Precleared lysates were then incubated for 1 h using 5 µg specific antibodies on ice followed by overnight incubation at 4 °C with 30 µl Protein A (or Protein G)-Sepharose. The immunobeads were washed thrice 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 2 h at room temperature or overnight at 4 °C 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% SDS and 62·5 mM Tris–HCl, pH 6·7) and re-probed with specific primary antibodies.

Confocal microscopy analysis

Immunofluorescence was performed as described previously (Bonaccorsi *et al.* 2004*a*). Briefly, cells were plated (1×10^6 cells) on glass coverslips and allowed to adhere in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were then stimulated with EGF (10 ng/ml for 15 min) and subsequently fixed with 2% paraformaldehyde in PBS. Cells were permeabilized 15 min at RT with 0·1% Triton-X in PBS. After fixation, the cells were rinsed in PBS and incubated in a blocking solution containing 1% BSA 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 4 °C for 30 min. After washing in PBS, the immunoreactivity was revealed using Alexa Fluor 488 goat anti-mouse IgG, FITC-conjugated or Texas Red-conjugated anti-mouse, or anti-rabbit secondary antibodies (minimal inter-species cross reaction) 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). Internalization of fluorescein-conjugated EGF was performed following the protocol described by Sigismund *et al* (2005). Briefly, FITC-conjugated EGF (1 µg/ml) was added for 1 h at 0 °C followed by wash and shift at 37 °C for 15 min to allow internalization. After fixation, the samples were analysed under a laser scanning confocal microscope (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 60X 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 two different files.

Statistical analysis

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

Results

EGFR internalization is altered in androgen-sensitive PC cells

In Fig. 1A western analysis of EGFR and AR expression in the four cell lines is shown. The 22Rv1 cells express the highest level of EGFR with respect to the other cell lines, whereas EGFR expression is similar in PC3-Neo and PC3-AR cells as previously demonstrated (Bonaccorsi *et al.* 2004*a*). In agreement with previous published data (Mendoza *et al.* 2002, Oosterhoff *et al.* 2005), LNCaP cells express lower levels of EGFR when compared with PC3 cells. Concerning AR expression, as expected, LNCaP cells are characterized by high levels of the receptor when compared with PC3-AR (clone 6) and 22RV1 cells (Fig. 1A).

We have shown previously that EGFR internalization is altered in PC3-AR cells with respect to the parent cell line transfected only with the vector (Bonaccorsi *et al.* 2004*a*). Here, we confirm such alteration by confocal immunofluorescence. EGFR was visualized in the absence (C) and presence of EGF (10 ng/ml, 10 min) by staining with anti-EGFR antibody. Before stimulation, EGFR was clearly localized and homogenously distributed to the membrane in all the cell lines studied (Fig. 1B and C; C micrographs), as denoted by almost complete absence of vesicular structures. After 10 min stimulation with EGF, vesicular structures containing EGFR were present at high density in PC3-Neo cells (Fig. 1B, EGF), whereas only a few vesicles were present in the cytoplasm of PC3-AR (Fig. 1B, EGF), LNCaP and 22Rv1 (Fig. 1C, EGF) cells. These results were confirmed by the use of FITC-labelled EGF (1 μg/ml) to stimulate the cells in PC3-Neo and PC3-AR cells. As shown in Fig. 1B (lower panels, EGF-FITC), the probe localized in discrete vesicles in PC3-Neo cells, whereas fewer vesicles were observed in PC3-AR. Following EGF treatment, PC3-Neo cells appear rounder with respect to control. It is possible that such behaviour is due to the fact that cells are induced to proliferate and to migrate by the growth factor. Normalized median value of EGFR before and after EGF stimulation for 10 min in the four cell lines, quantified by flow cytometry analysis, is shown in Fig. 1D. As shown, a high amount of EGFR is internalized in PC3-Neo cells but not in AR-positive cell lines PC3-AR, LNCaP and 22RV1.

To evaluate whether AR needs to be activated in order to affect EGFR internalization, we have used the anti-androgen 5-hydroxyflutamide (HF). PC3-Neo and PC3-AR cells were treated with EGF (10 ng/ml, 10 min) in the presence or absence of HF (1 μM) and EGFR internalization was evaluated by FACScan analysis. As shown in Fig. 2, in PC3-Neo cells, EGFR is almost completely internalized in EGF-treated with respect to control, as demonstrated by the shift of the positive peak to the left, but not in PC3-AR cells, where the shift of the peak was absent. Treatment with HF is not able to restore EGFR internalization in PC3-AR cells, indicating that AR does not need to be activated in order to affect the process. However, it appears that the treatment with HF affects the basal surface expression of EGFR in both cell lines (as demonstrated by the decrease of the positive peaks in untreated cells). This effect appears to be independent of the AR antagonist activity of HF as obtained also in PC3-Neo cells which lack AR.

Phosphorylation of tyrosine and serine residues at regulatory COOH-terminal domain of EGFR in PC3-AR and in PC3-Neo cells

We demonstrated previously that EGF-mediated total tyrosine phosphorylation of EGFR is reduced in PC3-AR cells when compared with control cells (Bonaccorsi *et al.* 2004*a*). The EGFR autotransphosphorylation occurs in several tyrosine residues. In particular, autotrans-phosphorylation in tyrosine residues 845, 992, 1045, 1068, 1086, 1148 and 1173 in the carboxy-terminal region of the EGFR has been shown to play a role in rapid internalization of the receptor and recruitment of adaptor proteins (Helin & Beguinot 1991, Sorkin *et al.* 1992). To investigate which tyrosine residues show reduced phosphorylation in PC3-AR cells, we evaluated EGF-induced phosphorylation of the above mentioned tyrosine residues with respect to control. To this aim, we performed western blot analysis of immunoprecipitated EGFR from PC3-AR and PC3-Neo cells by using phospho-specific antibodies directed against the phosphorylated Tyr residues. We found that, with the exception of tyr 845 and 1068, where EGF induces an increase of phosphorylation albeit much lower with respect to that elicited in PC3-Neo cells (Fig. 3A and D), the growth factor does not stimulate phosphorylation in any of the other analyzed tyrosine residues in PC3-AR cells, whereas it does so efficiently in PC3-Neo (Fig. 3 A–G). As can be observed in Fig. 3B, Y992 phosphorylation is high in basal conditions in both cell lines. Although we do not have a clear explanation for this finding, it has recently been shown that Y992 is more phosphorylated than other sites in

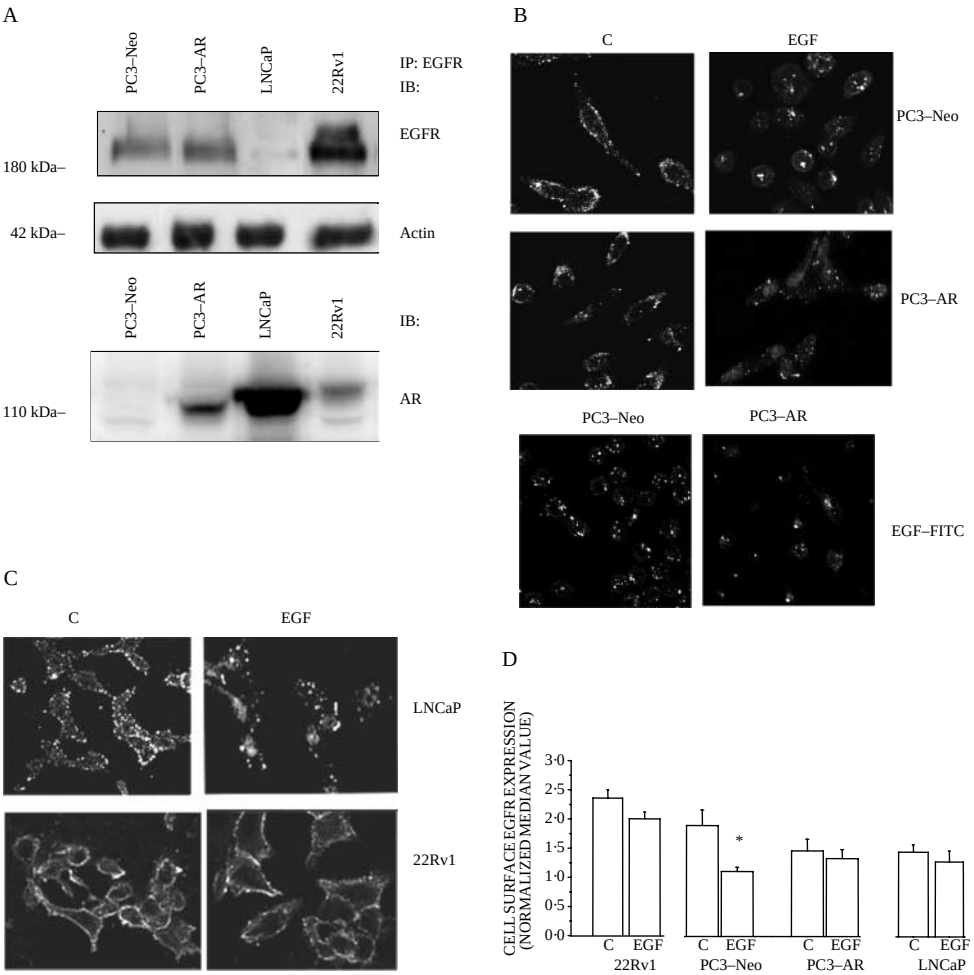
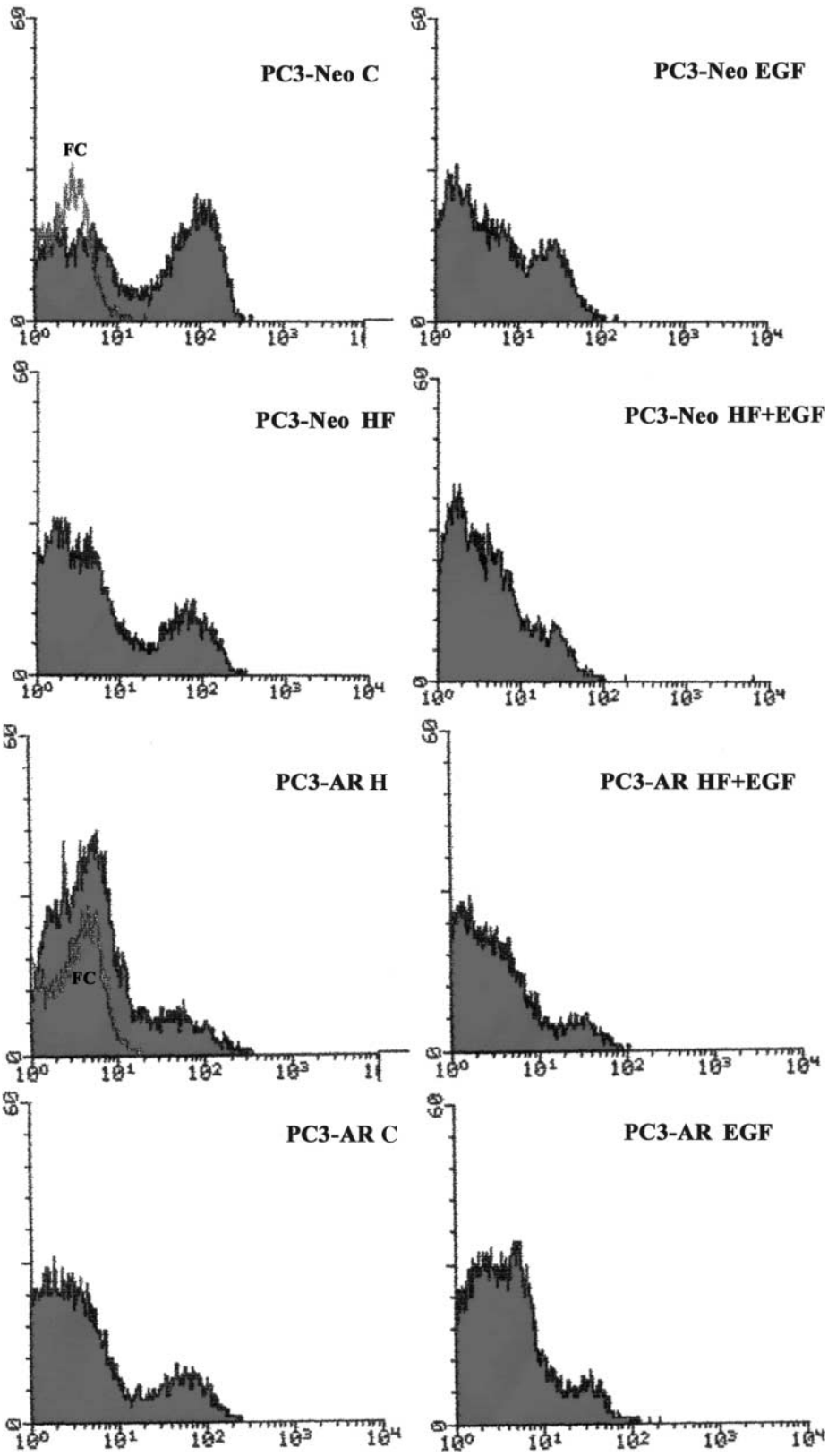


Figure 1 EGFR internalization in different prostate cancer cell lines. Panel A. Expression of EGFR and AR in the different PC cell lines used in the present study. Cell total lysates were obtained from cells maintained in serum-free and phenol red-free conditions for 24 h. For EGFR expression, cell lysates were immunoprecipitated with anti-EGFR Ab1 antibody, run onto SDS-PAGE, blotted and revealed first with a polyclonal anti-EGFR antibody and, after stripping, against actin. For AR expression, total lysates were run onto SDS-PAGE, blotted and revealed with a polyclonal anti-AR antibody. Panels B and C. Confocal immunofluorescence of EGFR expression in basal conditions (C) and after 10 min treatment with EGF (10 ng/ml) in PC3-Neo and PC3-AR cells (upper micrographs), LNCaP cells (Panel C, upper micrographs) and 22Rv1 cells (Panel C, lower micrographs). After treatment, cells were fixed, permeabilized and stained with the monoclonal anti-EGFR antibody (Ab1) (for details see Materials and methods) and analysis was performed by confocal microscopy. In Panel B (lower micrographs) cells were treated with FITC-conjugated EGF (1 μg/ml) for 10 min and then analyzed. Representative of at least three similar experiments. Panel D. EGFR internalization in the four different cell lines has been analyzed by FACScan analysis (for details see Materials and methods). Results are expressed as normalized median value of EGFR surface expression of three different experiments. *$P<0{\cdot}05$ compared with control (C) of PC3-Neo cells.

EGFR in A431 cells and that it is maximally stimulated by low concentrations of the agonist (Guo *et al.* 2003). In our previous paper, we demonstrated that the treatment with androgen reduces both tyrosine phosphorylation and internalization of EGFR further with respect to control (Bonaccorsi *et al.* 2004*a*). We show here that treatment with the synthetic androgen R1881 (0·1 nM for 3 days) does not substantially modify EGFR phosphorylation in the different tyrosine residues with the exception of those where EGF induces an increase (respectively Tyr 845 and 1068, Fig. 3A and D). Therefore, we conclude that the previously observed inhibitory effect on total phosphorylation (Bonaccorsi *et al.* 2004*a*) occurs mainly at the level of these two


PC3-Neo C
FC
PC3-Neo EGF
PC3-Neo HF
PC3-Neo HF+EGF
PC3-AR H
FC
PC3-AR HF+EGF
PC3-AR C
PC3-AR EGF

tyrosine sites. Altogether these results indicate that reduced internalization in PC3-AR cells is associated with lack of autotransphosphorylation in several tyrosine residues.

Recruitment of adaptor proteins Grb2 and c-Cbl and ubiquitination of EGFR are altered in PC3-AR cells

Grb2 and c-Cbl are adaptor molecules that couple EGFR to intracellular signalling and have been demonstrated to play a key role during early stage of internalization pathway of activated EGFR (Yamazaki *et al.* 2002, Jiang & Sorkin 2003, Ravid *et al.* 2004). In particular, c-Cbl mediates ubiquitination of EGFR and phosphorylation of tyr1045 appears to be essential for this function of the adaptor protein (Ravid *et al.* 2004). On the other hand, c-Cbl can also bind to EGFR through binding to Grb2 (Levkowitz *et al.* 1999), which, in turn, binds to Tyr 1068 and Tyr 1086 of EGFR. In view of the absent/reduced phosphorylation in response to EGF in Tyr1045 (Fig. 3C), 1068 (Fig. 3D) and 1086 (Fig. 3E) in PC3-AR cells, we evaluated whether the interaction of EGFR with Grb2 and c-Cbl was also affected by immunoprecipitating EGFR and performing subsequent western analysis using anti-Grb2 and anti-cCbl antibodies. As shown in Fig. 4A, association of both Grb2 and c-Cbl with EGFR is strongly reduced in PC3-AR cells when compared with the parental cells.

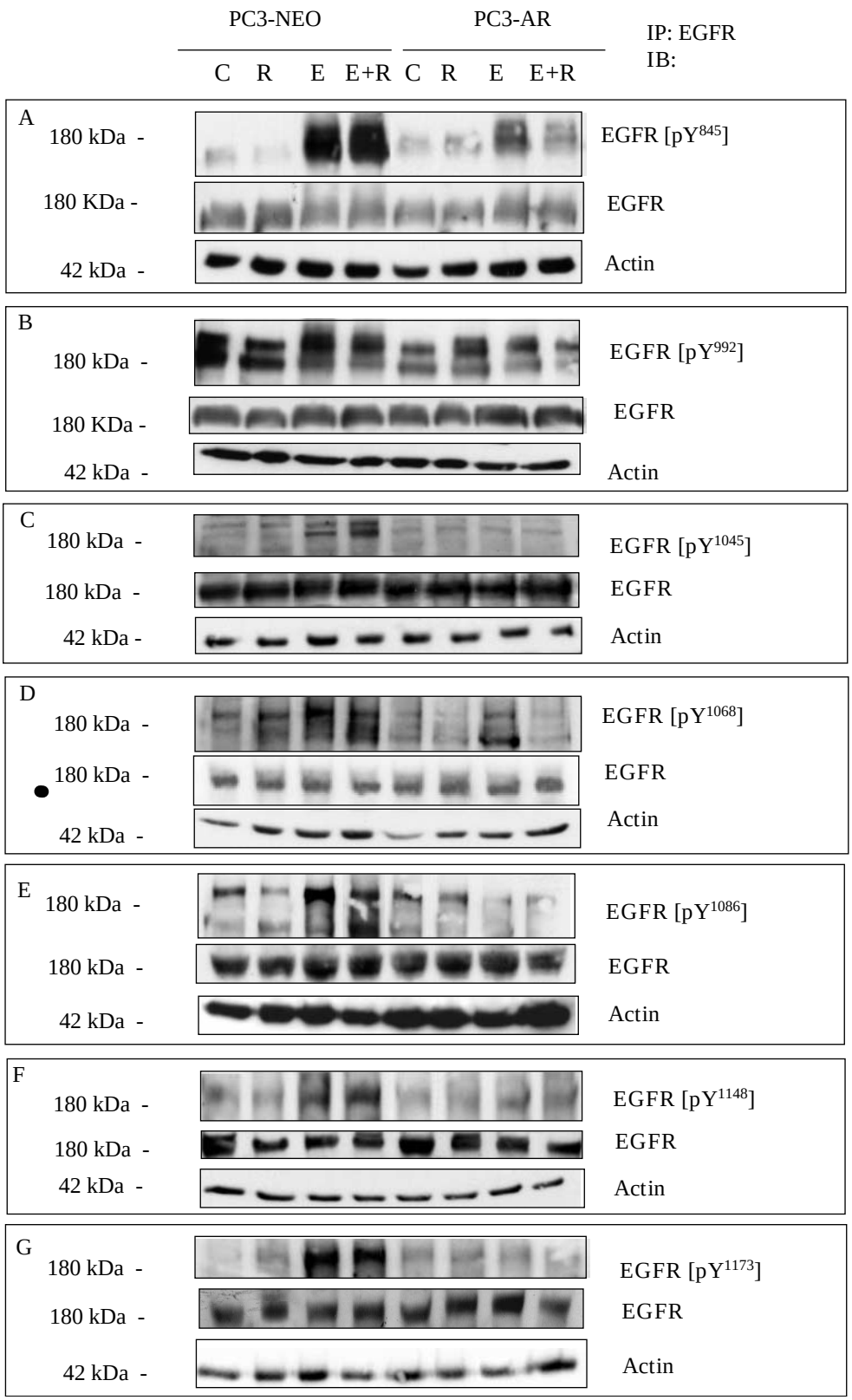
As mentioned above, EGFR–c-Cbl interaction mediates ubiquitination of the receptor. Indeed, EGFR mutants in tyr1045, where interaction of c-Cbl through this tyrosine residue is disrupted, do not ubiquitinate (Ravid *et al.* 2004). Moreover, de Melker *et al.* (2004) have reported evidence that EGFR is ubiquitinated when c-Cbl binds directly to the activated receptor via its phosphotyrosine binding domain but not when it binds through an adaptor such as Grb2. In view of these data, we evaluated EGFR ubiquitination following exposure to EGF. To this aim, cell lysates were immunoprecipitated with anti-ubiquitin antibodies and western analysis was performed first with anti-EGFR and, after stripping, with PY20 antibody. As shown in Fig. 4B (upper panel), a fraction of EGFR co-immunoprecipitates with ubiquitin in PC3-Neo cells following EGF stimulation, whereas EGFR-ubiquitin co-immunoprecipitation is almost absent in PC3-AR cells. Indeed, only after long exposure of the membrane, does a band corresponding to EGFR become evident in PC3-AR cells (not shown). Moreover, the fraction of ubiquitinated EGFR in PC3-Neo cells is tyrosine phosphorylated in response to EGF (Fig. 4B middle panel). An unidentified ubiquitinated band at 85 kDa is found to be tyrosine phosphorylated at the same level in both cell lines and was not modified by EGF treatment (Fig. 4B lower panel), indicating equal amount of ubiquitinated proteins after immunoprecipitation. Similar results were obtained after immunoprecipitation of EGFR and blotting with an anti-ubiquitin antibody in the two cell lines (Fig. 4C). A smeared band corresponding to ubiquitinated EGFR is present in EGF (10 ng/ml, 10 min)-treated PC3-Neo cells, whereas EGFR ubiquitination is much less evident in EGF-treated PC3-AR cells.

It has previously been postulated that the Ser1046/1047 and 1057/1142 are important in the regulation of EGFR tyrosine autotransphosphorylation by promoting an interaction between the cytoplasmic tail and the kinase domain (Feinmesser *et al.* 1999). Recent data have demonstrated that these residues are required for ligand-induced ubiquitination, internalization and degradation of the receptor, since EGFR mutants in these serine residues do not autophosphorylate, ubiquitinate and internalize in response to EGF (Oksvold *et al.* 2003). To establish whether restoration of androgen sensitivity also affects EGF-induced phosphorylation at Ser1046/1047, we performed western analysis of immunoprecipitated EGFR in PC3-AR and PC3-Neo cells using anti-EGFR pSer1046/1047 antibody. As shown in Fig. 4D, serine phosphorylation does not increase in response to EGF in PC3-AR cells, whereas the two sites are phosphorylated in PC3-Neo. Overall, this result confirms reduced EGF signalling in PC3-AR cells and postulates that reduced ubiquitination and internalization of EGFR might also be due to reduced phosphorylation in serine residues 1046/1047.

Clathrin-mediated endocytosis is altered in AR-positive PC cell lines

It has been demonstrated that two pathways exist for endocytosis of membrane receptors, one leading to degradation and the other to signalling and recycling to the surface (Le Roy & Wrana 2005). In particular, the clathrin-dependent pathway of internalization mediates recycling, while the clathrin-independent ends with receptor degradation (Le Roy & Wrana 2005). Recently, it has been shown that EGFR also may follow the two different endocytotic routes (Aguilar & Wendland 2005). We reasoned that in PC3-AR cells, the reduced

Figure 2 Effect of the anti-androgen 5-hydroxyflutamide (HF) on EGFR internalization in PC3-Neo and PC3-AR cells. PC3-Neo (upper panels) and PC3-AR (lower panels) cells were treated for 10 min with EGF (10 ng/ml) in the presence or absence of HF (1 μM). EGFR internalization was evaluated by FACScan analysis (see Materials and methods for details). Left panels, control; right panels, EGF-treated cells. Representative of two similar experiments.


PC3-NEO
PC3-AR
IP: EGFR
IB:
C R E E+R C R E E+R
A
180 kDa -
EGFR [pY845]
180 KDa -
EGFR
42 kDa -
Actin
B
180 kDa -
EGFR [pY992]
180 KDa -
EGFR
42 kDa -
Actin
C
180 kDa -
EGFR [pY1045]
180 kDa -
EGFR
42 kDa -
Actin
D
180 kDa -
EGFR [pY1068]
180 kDa -
EGFR
42 kDa -
Actin
E
180 kDa -
EGFR [pY1086]
180 kDa -
EGFR
42 kDa -
Actin
F
180 kDa -
EGFR [pY1148]
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EGFR
42 kDa -
Actin
G
180 kDa -
EGFR [pY1173]
180 kDa -
EGFR
42 kDa -
Actin

activation of the EGFR tyrosine kinase and the reduced interaction with Grb2 and c-Cbl adaptors may contribute to disrupt trafficking of EGFR leading to decreased internalization through the clathrin-dependent pathway. To investigate this possibility, we performed confocal microscopy immunofluorescence analysis by using antibodies against early endosome antigen-1 (EEA-1) to study EGFR localization in early endosomes (typical of the clathrin-dependent pathway; Sigismund *et al.* 2005). As shown in Fig. 5 (right panels and higher magnification subpanel), a strong co-localization is evident following stimulation with EGF (10 min) in PC3-Neo cells but not in the AR-positive cell lines PC3-AR, 22Rv1 and LNCaP. These data indicate that in PC3-Neo cells, EGFR internalization occurs mainly through the clathrin-dependent pathway which is altered by the expression of a functional AR. The EEA-1 expression is similar in the four cell lines, as demonstrated by western blot analysis (Fig. 5, insert).

Evidence in the literature suggests that EGFR-caveolin interaction leads to reduced activation of EGFR signalling (Couet *et al.* 1997). Therefore, we sought to investigate EGFR–caveolin-1 interaction in PC3-Neo and PC3-AR cells by confocal immunofluorescence. As shown in Fig. 6 (left panels), EGFR–caveolin-1 interaction (yellow staining) is low in basal conditions (C) in both cell lines, in agreement with data showing that only a small fraction of EGFR is associated to caveolin-1 (Couet *et al.* 1997, Khan *et al.* 2006). Following stimulation with EGF, EGFR is redistributed into punctate vesicular compartments within PC3-Neo cells and yellow staining is completely lost (Fig. 6, left panel). Conversely, in PC3-AR cells EGFR remains slightly associated with caveolin-1 even after treatment with EGF. Right panels of Fig. 6 show a confocal section of the sum of the sections shown on the right to demonstrate surface localization of caveolin-1. Caveolin-1 expression is similar in the two cell lines (Fig. 6, insert). Overall, our data indicate that sustained EGFR–caveolin-1 co-association may be a result of suppressed EGFR activation in the presence of AR.

To assess whether reduced internalization of EGFR in AR-positive cells at 10 min is due to a different kinetic in the internalization process, we evaluated EGFR internalization at 60 and 120 min in PC3-Neo, PC3-AR, LNCaP and 22RV1 cells. As shown in Fig. 7, EGFR is almost completely internalized in PC3-Neo cells at both 60 and 120 min (as demonstrated by the shift to the left of the peak in response to EGF at both time points). In PC3-AR cells, EGFR is internalized at both time points, although the process appears to be slower with respect to PC3-Neo cells, as the shift of the peak to the left in response to EGF is less marked in particular at 60 min. Similar results are obtained in LNCaP cells where EGFR internalization appears to be slower with respect to PC3-Neo cells. Conversely, EGFR internalization is complete at 60 min in 22RV1 cells. Interestingly, in these cells, EGFR is present mostly on the surface at 120 min despite the continuous presence of the growth factor, suggesting a different kinetic of internalization/recycling.

Discussion

Understanding the molecular mechanisms through which growth factor receptors are internalized represents a major focus of cancer research, as mutations or altered expression of endocytotic proteins have often been detected in tumours (Di Fiore & Gill 1999). In particular, it is vital to understand how signal transduction and endocytosis pathways regulate each other and how the breakdown of this integrated regulation contributes to cancer development. Understanding such links may be of help in providing an intervention for cancer therapy. Indeed, internalization of receptor controls the duration, intensity and specificity of signalling (Sorkin 2001). The present paper reports strong evidence that internalization pathways of EGFR are altered in the AR-positive PC cell models used in this study (PC3-AR, LNCaP and 22Rv1). In particular, we demonstrate here that reduced autotransphosphorylation of the receptor in several tyrosine residues as well as interaction with adaptor proteins which mediate its sorting to early endosome vesicles and thus clathrin-dependent pathway of endocytosis are compromised in AR-positive PC cell lines. Since endosome-associated signalling relevant for activation of many specific cellular functions occurs when internalization of EGFR is driven through the clathrin-dependent route (Aguilar & Wendland 2005), and endosomal signalling of EGFR is required for EGF-mediated cell survival (Wang *et al.* 2002), our findings may give a possible explanation of the lower tumorigenic and invasive phenotype of AR-positive PC cell lines with respect to AR-negative (Bonaccorsi *et al.* 2000, Cinar *et al.* 2001,

Figure 3 EGF-induced phosphorylation in different tyrosine residues in PC3-Neo and PC3-AR cells. Cells were pre-treated or not (C) with R1881 (R, 0·1 nM, 3 days) and then treated with EGF (E, 10 ng/ml, 10 min) in the presence or absence of R1881 (E+R). After the different treatments, cells were scraped from the plate and lysated. After immunoprecipitation (IP) of EGFR with the monoclonal anti-EGFR antibody Ab1, SDS-PAGE and transfer to nitrocellulose, the different membranes were blotted with antibodies directed against the indicated tyrosine phosphorylated sites (panels A–G). Membranes were then stripped and re-blotted with an anti-EGFR antibody (middle blots) and an anti-actin antibody (lower blots). Representative of at least two similar experiments for each anti-tyrosine antibody. IB, immunoblot.

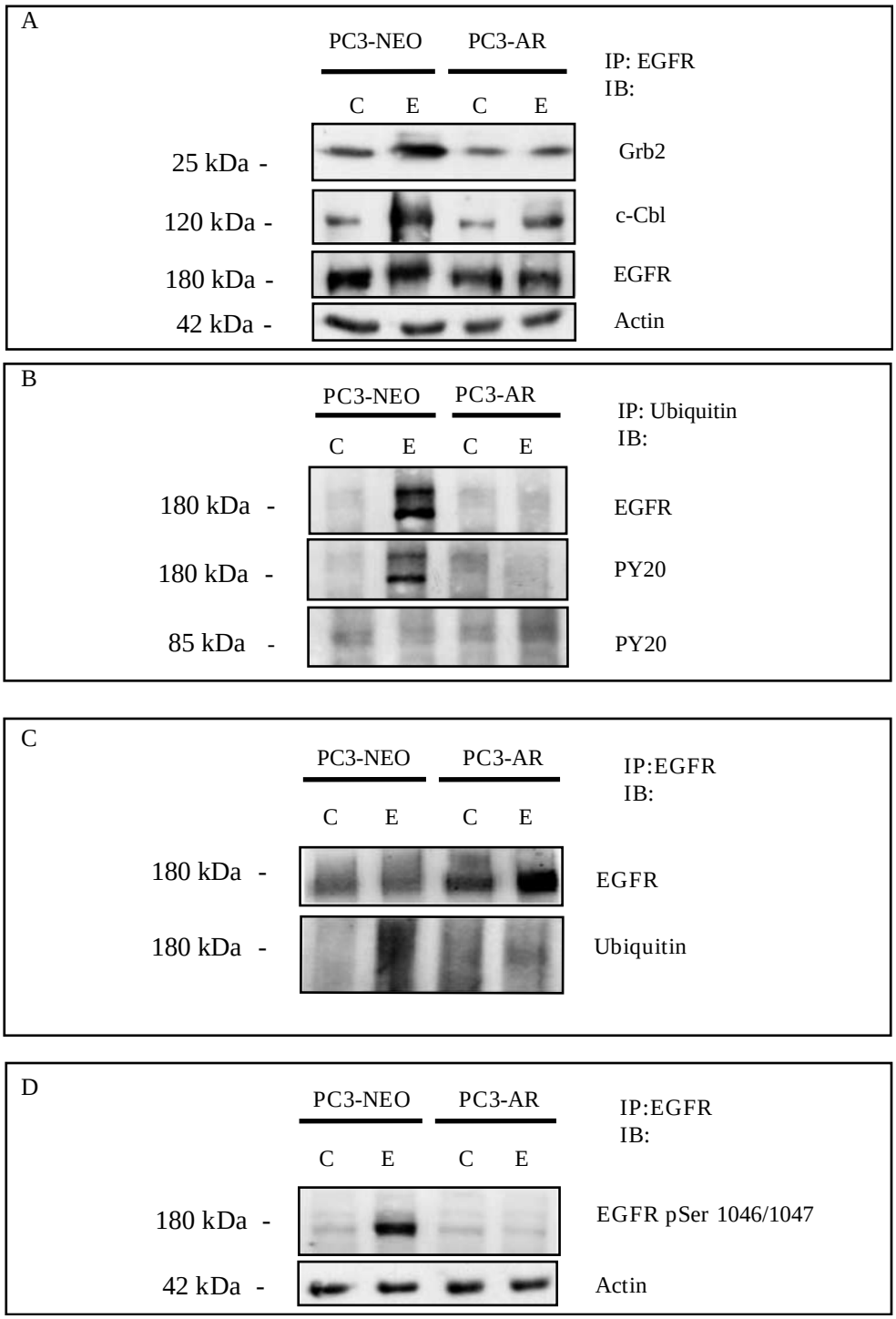

Figure 4 EGFR interaction with Grb2 and c-Cbl, EGFR ubiquitination and ser1046/1047 phosphorylation in PC3-Neo and PC3-AR cells. Cells were treated or not (C) with EGF (E, 10 ng/ml 10 min) and EGFR was immunoprecipitated (IP) with anti-EGFR antibody (Ab1) (panels A, C and D) (for details see Materials and methods) or anti-ubiquitin antibody (panel B). Western blot analysis was performed with the indicated antibodies. Representative experiments out of two similar are shown IB, immunoblot.

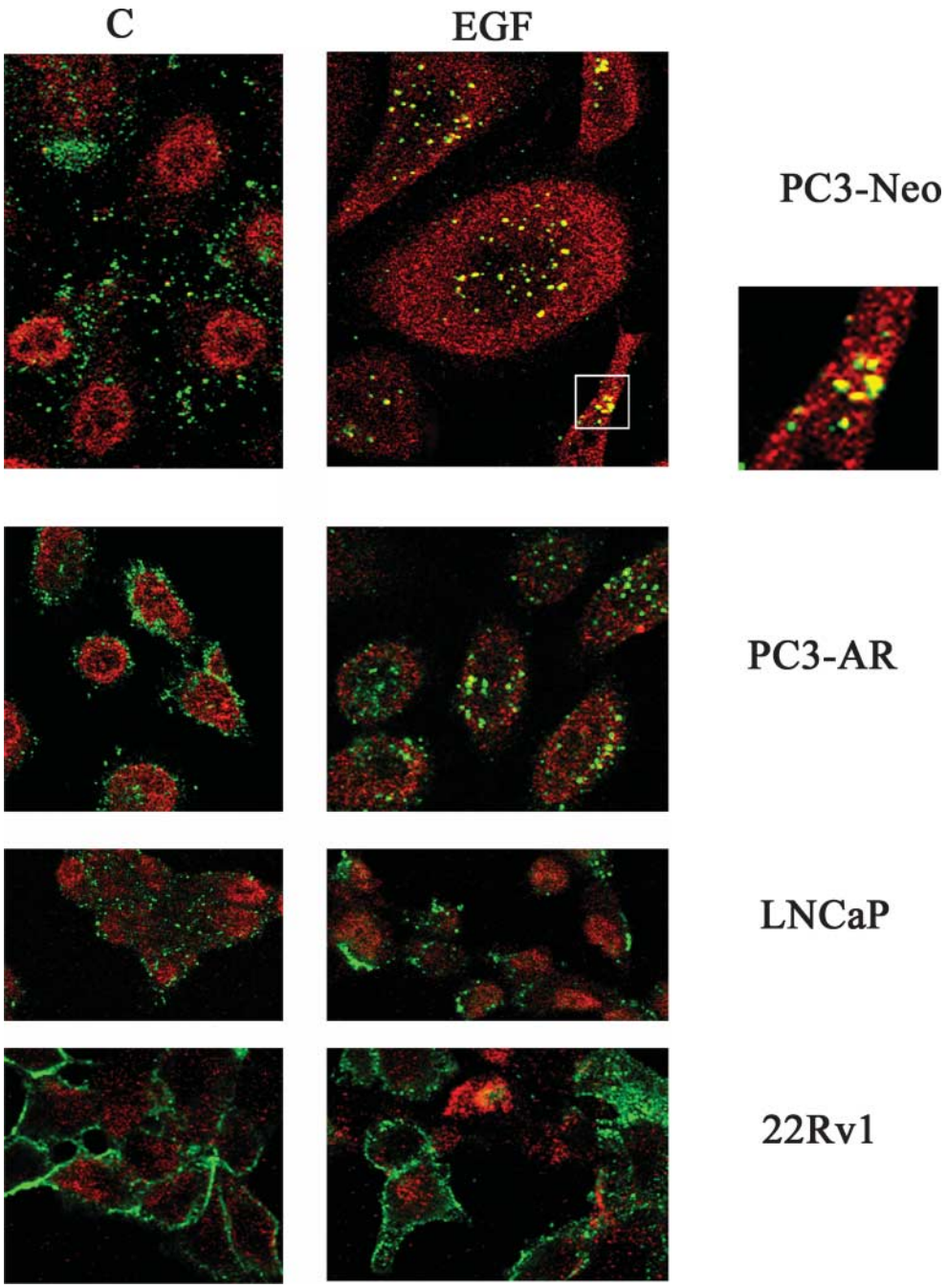

Figure 5 Confocal immunofluorescence of EGFR-EEA-1 interaction. Immunofluorescence of EEA-1 (red) and EGFR (green) is shown in basal conditions (C) and after treatment with EGF (10 ng/ml, 10 min) in PC3-Neo, PC3-AR, LNCaP and 22Rv1 cells. Cells were fixed, permeabilized and subjected to immunofluorescence using an anti-EGFR monoclonal antibody (Ab1) followed by FITC-conjugated secondary antibody and an anti-EEA-1 polyclonal antibody followed by Texas Red-conjugated secondary antibody. Co-localization analysis was performed by confocal microscopy. For simplicity only the merge images are shown. The yellow staining is indicative of co-localization of the two antigens. Representative of three different experiments. The panel of EGF treatment in PC3-Neo cells has been magnified threefold to show better the yellow staining of internal vesicular structures. The inset shows a higher magnification of the boxed area. Total cell lysates, obtained as described for Fig. 1, were separated onto SDS-PAGE and, after blotting, probed with the anti-EAA1 polyclonal antibody.

Nightingale *et al.* 2003, Guierini *et al.* 2005, Hatzoglou *et al.* 2005). Inline with our data, it has recently been shown that transfection of AI PC cell lines with the endocytosis protein REPS2 results in inhibition of EGFR internalization and decrease of related signalling (Oosterhoff *et al.* 2005). REPS2 is among the endocytosis regulating proteins whose expression is down-regulated in AI PC (Oosterhoff *et al.* 2005). Interestingly, REPS2 is highly expressed in LNCaP cells and in other AD cell lines (Oosterhoff *et al.* 2005) where EGFR

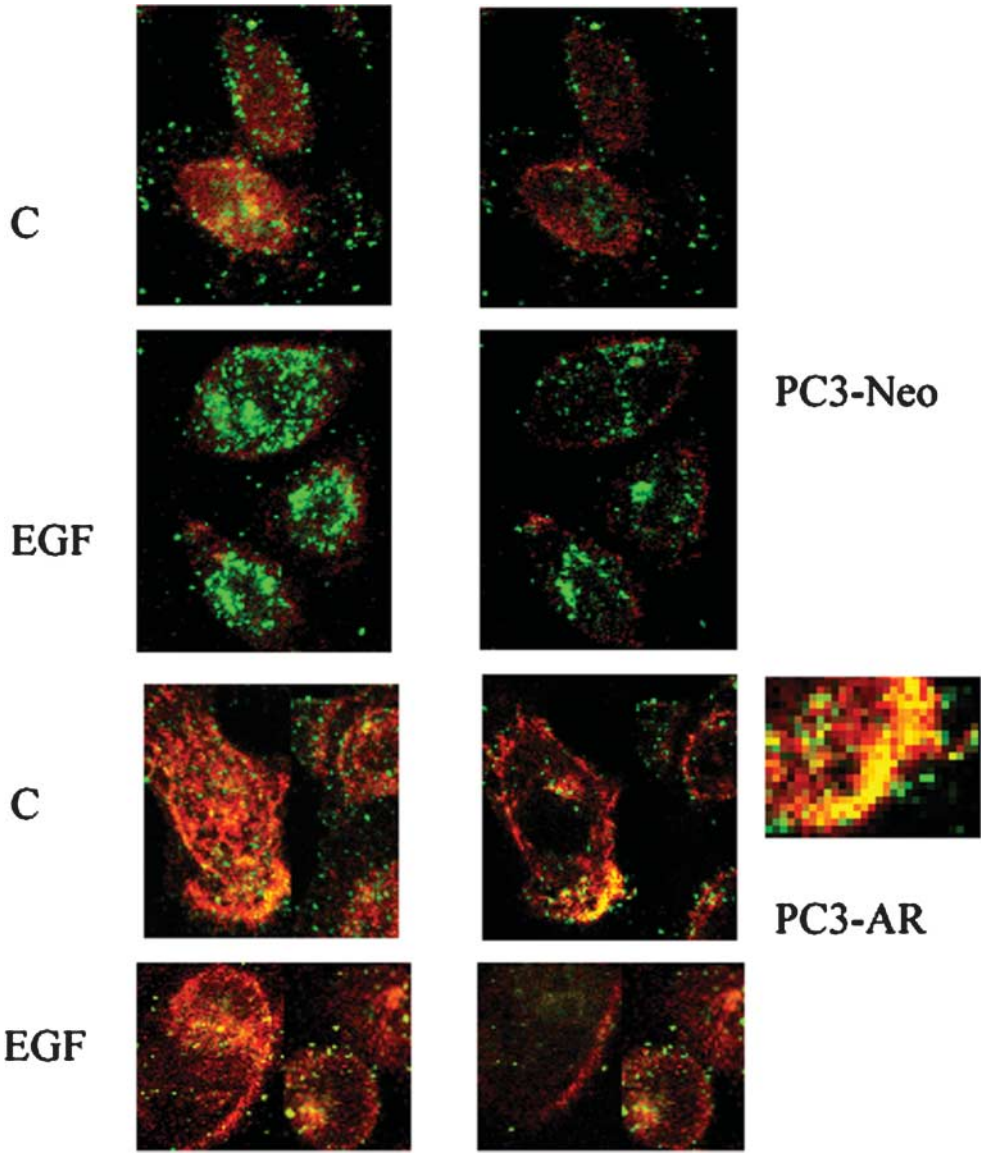

Figure 6 Confocal immunofluorescence of EGFR-caveolin-1 interaction. Immunofluorescence of caveolin 1 (red) and EGFR (green) is shown in basal conditions (C), and after treatment with EGF (10 ng/ml, 10 min) in PC3-Neo and PC3-AR cells. Cells were fixed, permeabilized and subjected to immunofluorescence using an anti-EGFR monoclonal antibody (Ab1) followed by FITC-conjugated secondary antibody and an anti-caveolin-1 polyclonal antibody followed by texas red-conjugated secondary antibody. Co-localization analysis was performed by confocal microscopy. For simplicity only the merge images are shown. The yellow staining is indicative of co-localization of the two antigens. Representative of three different experiments. Right panels show an internal confocal section of the sum of the sections shown on the left. The inset shows a higher magnification of the yellow area in control PC3-AR cells. Cell lysates, obtained as described for Fig. 1, were run onto SDS-PAGE and, after blotting, probed with anti-caveolin-1 polyclonal antibody.

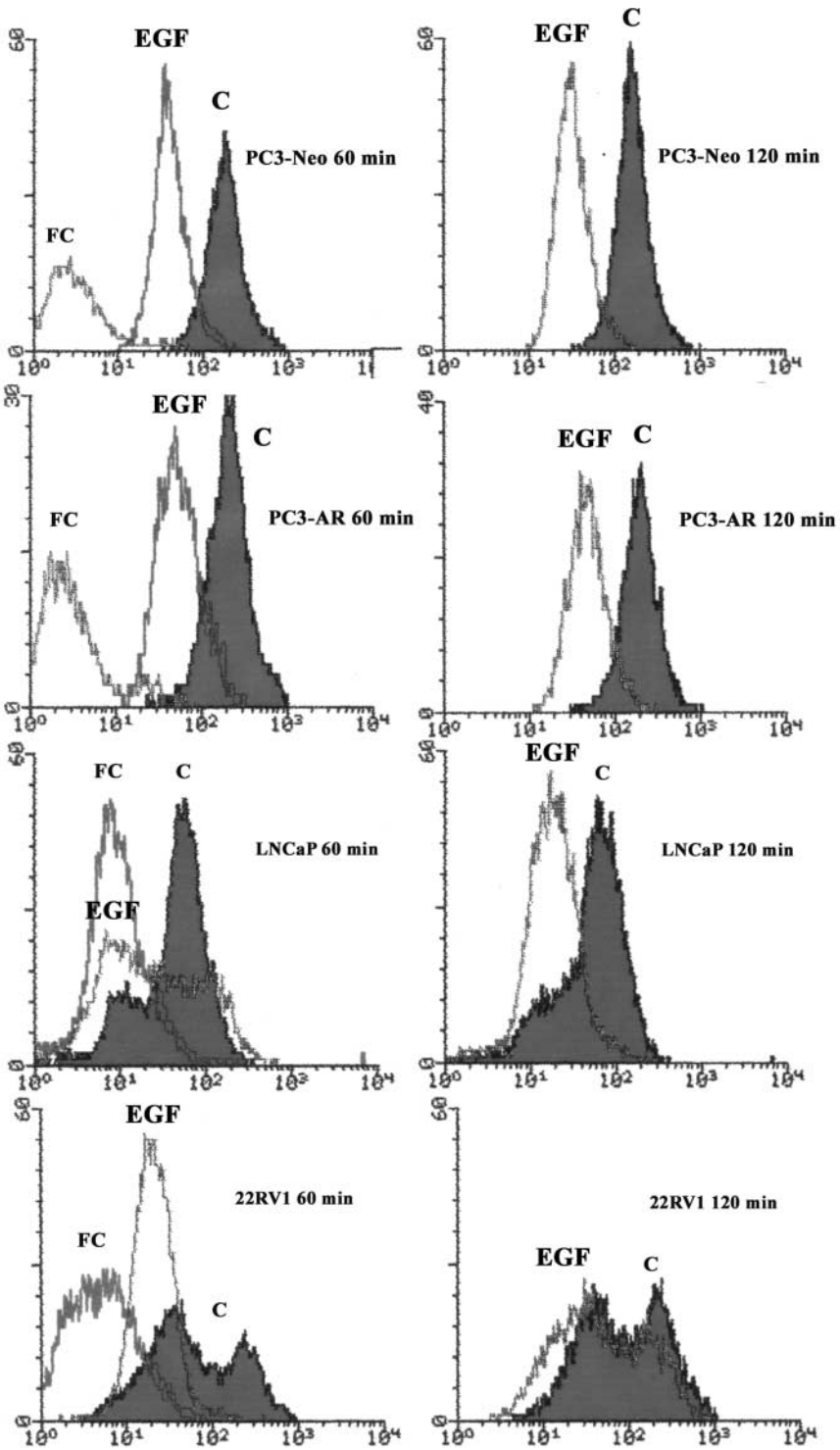

Figure 7 Internalization of EGFR at 60 and 120 min in PC3-Neo, PC3-AR, LNCaP and 22 RV1 cells. PC3-Neo, PC3-AR, LNCaP and 22RV1cells were treated with EGF (10 ng/ml) for the indicated times. EGFR internalization was evaluated by FACScan analysis (see Materials and methods for details). Left panels, control; right panels, EGF-treated cells. Representative of two similar experiments.

internalization is found to be substantially reduced (Oosterhoff *et al.* 2005; present study). Of interest, it has recently been shown in a neuroblastoma cell line that internalization of the tyrosine kinase receptor RET is required for complete activation of downstream signalling proteins mediating proliferation (Richardson *et al.* 2006). Noteworthy, decreased EGFR internalization was observed in all the three AR-positive cell lines, despite the fact that AR is mutated in different sites respectively in LNCaP (Gaddipati *et al.* 1994) and in 22Rv1 cells (Tan *et al.* 1997). This result suggests that the mutations of the two cell lines do not impair the ability of AR to affect EGFR internalization. In addition, EGFR internalization in AR-positive cell lines does not appear to be altered, since, when evaluated at 60 min after EGF treatment, internalization occurs in all the three cell lines. Rather, it appears that the kinetic of EGFR internalization is affected by the steroid receptor. Another important point concerns the need for activation of AR in affecting EGFR internalization. Here, we show that incubation with 5-hydroxyflutamide (HF) does not restore EGFR internalization in PC3-AR cells, suggesting that the genomic pathway of activation of AR is not required for such an effect. On the other hand we have shown previously that AR interacts with EGFR in PC3-AR and LNCaP cells (Bonaccorsi *et al.* 2004*a*). The lack of effect of HF further suggests that the inhibitory effect of AR on EGFR signalling and internalization (Bonaccorsi *et al.* 2004*a* and present study) is due to an extragenomic effect of the receptor. However, a genomic effect on EGFR internalization and signalling cannot be excluded, since in our previous paper (Bonaccorsi *et al.* 2004*a*) we have shown that treatment with a synthetic androgen further reduces the two processes.

As mentioned above, internalization of EGFR may occur through at least two different pathways, a clathrin-dependent and a clathrin-independent (or raft-mediated) one (Aguilar & Wendland 2005). According to several studies, key mediators of clathrin-mediated endocytosis are c-Cbl, that binds to the receptor and sustains its ubiquitination (de Melker *et al.* 2004, Stang *et al.* 2004), and Grb2, that mediates entry into coated pits (Huang & Sorkin 2005), although both effectors have been also demonstrated to play an active role in clathrin-independent EGFR endocytosis (Sigismund *et al.* 2005). Here, we demonstrate that EGFR interaction with both Grb2 and c-Cbl is strongly reduced in PC3-AR cells and thus we hypothesize that such reduced interaction is responsible for reduced internalization. Rather, the receptor appears to remain, at least in part, localized in a caveolar compartment (Fig. 6), where it can be maintained in an inactive state (Zhang *et al.* 2000).

As mentioned above, in our previous study (Bonaccorsi *et al.* 2004*a*), we have shown that the treatment with androgens further reduces signalling and internalization of the EGFR. Here, analyzing the single phosphorylated tyrosine residues in response to EGF in PC3-AR cells, we found a small increase only in residues 845 and 1068, whereas no increase was apparent in the other investigated residues. Interestingly, treatment with the synthetic androgen R1881 was able to decrease phosphorylation in 845 and 1068 tyrosine residues, indicating that the effect of the androgen on invasive properties of the cells (Bonaccorsi *et al.* 2000) and on EGFR internalization (Bonaccorsi *et al.* 2004*a*) might also be due to reduced signalling mediated by these two sites. On the other hand, tyr1068 directly binds Grb2 and is involved in the early steps of endocytosis (Chook *et al.* 1996, Johannessen *et al.* 2006) as discussed above. Recently, Mills *et al* (2005) have demonstrated that Huntingtin interacting protein 1 (HIP1), a nucleocytoplasmic protein involved in the early step of endocytosis (Hyun & Ross 2004), is overexpressed during progression towards AI PC. This protein has been shown to prolong activation and signalling of EGFR after the internalization step of endocytosis (Hyun & Ross 2004), further supporting an intriguing role of endocytotic pathways in the progression of PC (Oosterhoff *et al.* 2005).

Our data indicate that, in PC cells, the presence of the AR may have a regulatory function on EGF-mediated pathways by inhibiting EGFR activation. In PC, the role of androgens and AR appear to be twofold. In initial phases of the disease, AR and its ligand represent the first hint for uncontrolled cell growth, as also recently outlined by the identification of a chromosomal rearrangement, resulting in the fusion of an AR regulated gene (TMPRSS2) and ETS transcription factor family members ERG and ETV1, that is expressed at high frequency in PC (Tomlins *et al.* 2005). The fusion results in androgen-induced overexpression of these proto-oncogenes which are likely the cause of tumorigenesis. However, enhanced AR activity does not appear, *per se,* to be sufficient to cause aggressive growth and progression, as *in vitro* experiments indicate that androgens also induce differentiation of PC cells (Whitacre *et al.* 2002, Berger *et al.* 2004). In addition, there is clinical evidence indicating that correctly functioning AR during disease progression may maintain a more differentiated and less tumorigenic phenotype. Indeed, patients with AI PC displaying amplification of the AR gene survive longer than patients without amplification (Debes & Tindall 2004) and a higher level of AR protein expression correlates with a higher differentiation of tumours (Heinlein & Chang 2004). Moreover, it has recently been demonstrated that during PC progression from low grade to high grade and metastatic PC, a selective down-regulation of the AR-targeted genes that inhibit proliferation, induce differentiation or mediate apoptosis occurs (Hendriksen *et al.* 2006). Altogether this evidence supports the notion that a correct AR pathway

may maintain a more differentiated phenotype of PC, as observed *in vitro* in androgen-positive PC cell lines (Bonaccorsi *et al.* 2000, 2004*a*, Cinar *et al.* 2001, Davis *et al.* 2003, Nightingale *et al.* 2003, Guerini *et al.* 2005, Hatzoglou *et al.* 2005) both by regulating genes affecting growth and invasion (Bonaccorsi *et al.* 2000, Hendriksen *et al.* 2006) and by interfering with signal transduction of EGF (Bonaccorsi *et al.* 2004*a*, Oosterhoff *et al.* 2005; present study).

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