Antiandrogens prevent stable DNA-binding of the androgen receptor

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Accepted 16 June 2005
Journal of Cell Science 118, 4187-4198 Published by The Company of Biologists 2005
doi:10.1242/jcs.02546

Summary

The androgen receptor (AR) is essential for development of the male gender and in the growth of the majority of prostate cancers. Agonists as well as most antagonists induce translocation of the receptor to the nucleus, whereas only agonists can activate AR function. Antagonists are therefore used in the therapy of metastasized prostate cancer. To obtain insight into the mechanism by which antagonists block AR function in living cells, we studied nuclear mobility and localization of green fluorescent protein (GFP)-tagged AR in the presence of either the agonist R1881 or the antagonists bicalutamide and hydroxyflutamide. As controls we investigated a non-DNA-binding AR mutant (A573D) and two mutants (W741C and T877A) with broadened ligand specificity. We demonstrate that in the presence of R1881, AR localizes in numerous intranuclear foci and, using complementary fluorescence recovery after photobleaching (FRAP) approaches and computer modelling, that a fraction of AR (~10-15%) is transiently immobilized in a DNA-binding-dependent manner (individual ARs being immobile for ~45 seconds). By contrast, antagonist-bound GFP-AR showed no detectable immobile fraction and the mobility was similar to that of the R1881-liganded non-DNA-binding mutant (A573D), indicating that antagonists do not induce the relatively stable DNA-binding-dependent immobilization observed with agonist-bound AR. Moreover, in the presence of bicalutamide and hydroxyflutamide GFP-AR was homogeneously distributed in the nucleus. Binding of bicalutamide and hydroxyflutamide to GFP-AR(W741C) and GFP-AR(T877A), respectively, resulted in similar mobility and heterogeneous nuclear distribution as observed for R1881-liganded GFP-AR. The live cell studies indicate that the investigated antagonists interfere with events early in the transactivation function of the AR.

Key words: Androgen receptor, Antiandrogens, Fluorescence recovery after photobleaching, Prostate cancer, DNA binding

Introduction

The androgen receptor (AR) is a member of the family of steroid receptors. Functional ARs are required for development of the male gender (Cunha et al., 1987). In addition, ARs play a role in growth of prostate cancer (Feldman and Feldman, 2001; Trapman, 2001). Therefore, metastasized prostate cancers are frequently treated with antiandrogens, such as flutamide or bicalutamide (Casodex) (Small and Vogelzang, 1997). However, despite initial success, all patients eventually show tumour relapse. There may be several causes of therapy resistance, including changes in cell signalling pathways, AR overexpression and mutation of the AR (Feldman and Feldman, 2001; Trapman, 2001). The latter may lead to activation of the AR by ligands other than the androgens testosterone and 5α-dihydrotestosterone (DHT), including estrogens, glucocorticoids and adrenal androgens (Veldscholte et al., 1992; Brinkmann and Trapman, 2000; Zhao et al., 2000; Mizokami et al., 2004). Therapy also selects for AR mutants that are activated by the applied antiandrogens (Veldscholte et al., 1990; Hara et al., 2003). One of the AR mutations most frequently found in antiandrogen-treated patients is a mutation in codon 877, resulting in the replacement of threonine by alanine AR(T877A) (Veldscholte et al., 1990; Taplin et al., 2003). The mutation results in agonistic activity of OH-flutamide (Veldscholte et al., 1990), the active metabolite of flutamide (Katchen and Buxbaum, 1975). Recently, in a bicalutamide-treated patient, a novel mutation was found, resulting in substitution of tryptophan at position 741 by cysteine AR(W741C) (Taplin et al., 2003). It was demonstrated that this mutation enabled bicalutamide to act as an agonist (Hara et al., 2003).

The intracellular distribution of ARs in the absence and presence of ligand has been extensively studied in cell lines using both immunocytochemistry (Jenster et al., 1991; Simental et al., 1991) and green fluorescent protein (GFP)-tagging (Georget et al., 1997; Poukka et al., 2000; Tyagi et al., 2000; Avancès et al., 2001; Farla et al., 2004). In the absence of ligand, ARs are predominantly localized in the cytoplasm associated with a chaperone complex containing heat shock proteins (Smith and Toft, 1993; Pratt and Toft, 1997; Stenoien et al., 1999), keeping the AR in a high-affinity ligand-binding conformation (Vanaja et al., 2002). Ligand binding induces release from this complex and rapid translocation of AR to the nucleus within 15-60 minutes of addition of androgen (Georget et al., 1997; Poukka et al., 2000; Tyagi et al., 2000; Avancès et al., 2001; Farla et al., 2004). In the nucleus ARs bind as

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dimers to androgen-response elements (AREs) in promoters of target genes (Roche et al., 1992; Claessens et al., 2001). Addition of the antiandrogens OH-flutamide and bicalutamide also resulted in translocation of ARs from the cytoplasm to the nucleus (Jenster et al., 1993; Poukka et al., 2000; Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001), although the translocation in the presence of bicalutamide was slower and incomplete (Poukka et al., 2000; Tyagi et al., 2000; Avancès et al., 2001).

Ligand-activated steroid receptors, including estrogen receptor α (ERα) (Htun et al., 1999; Stenoien et al., 2000), glucocorticoid receptor (GR) (van Steensel et al., 1995; Htun et al., 1996) and mineralocorticoid receptor (MR) (Fejes-Tóth et al., 1998) were distributed in the nucleus in a focal pattern. Likewise agonist-liganded ARs were shown to accumulate in foci in the nucleus (Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001; Ochiai et al., 2003; Farla et al., 2004). Interestingly, ARs only accumulated into foci with agonistic and partial agonistic ligands, whereas antagonist-bound ARs showed a more homogeneous nuclear distribution (Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001). Recently, we showed that ARs carrying a mutation in the DNA-binding domain show a homogeneous intranuclear distribution, indicating that the focal pattern depends on the DNA-binding ability of the AR (Farla et al., 2004).

GFP technology and quantitative live cell imaging have provided new insights in the mechanism of gene activation by steroid receptors. We showed using fluorescence recovery after photobleaching (FRAP) that agonist-bound ARs are immobilized in a DNA-binding-dependent manner, the average immobilization of a single AR being 1-2 minutes (Farla et al., 2004). Others have shown that GFP-tagged GRs exchange rapidly between the nucleoplasmic compartment and a mouse mammary tumour virus (MMTV) promoter array (McNally et al., 2000). The p160 coactivator glucocorticoid receptor interacting protein 1 (GRIP1) displayed similar dynamic interactions on this promoter repeat (Becker et al., 2002). GFP-tagged GR (Schaaf and Cidlowski, 2003; Elbi et al., 2004), ERα and the p160 coactivator steroid receptor coactivator 1 (SRC-1) (Stenoien et al., 2001b) showed reduced intranuclear mobility in the presence of agonistic ligands suggesting that they dynamically interact with immobile elements in the nucleus, similar to the interaction of GRs with the MMTV promoter array. The coactivators SRC-1 and CREB binding protein (CBP) were shown to rapidly exchange on a *lac* repressor ERα chimera immobilized on an array of *lac* operators (Stenoien et al., 2001a), again demonstrating that interactions between steroid receptors and coactivators are very dynamic. Likewise in chromatin immunoprecipitation (ChIP) experiments, ERα and AR as well as associated coactivators have been shown to associate with transcription initiation complexes in a cyclic manner, albeit with much longer cycling times (in the order of minutes) (Kang et al., 2002; Métivier et al., 2003; Reid et al., 2003) compared to the residence times observed with FRAP (in the order of seconds). In addition, ChIP results suggest that the AR in the presence of bicalutamide binds to promoter regions of androgen regulated genes, although it is unable to form an active transcription complex (Kang et al., 2002; Masiello et al., 2002; Shang et al., 2002; Kang et al., 2004), in contrast to DHT-activated ARs.

To investigate the mechanism of action of antagonists, we studied the intranuclear dynamics and localization of GFP-tagged ARs in the presence of the non-steroidal antagonists OH-flutamide and bicalutamide, and compared them with the effects of the agonistic ligand R1881. We also studied mutant ARs containing mutations in codons 741 or 877 of the AR found in prostate cancer patients (Taplin et al., 1999; Taplin et al., 2003). As mentioned above, certain AR antagonists can activate transcription by these mutant ARs (Veldscholte et al., 1990; Hara et al., 2003). Mobility, transcriptional activation and the intranuclear focal distribution pattern of wild-type and mutant androgen receptors in the presence of activating ligands were highly correlated. We found the behaviour of wild-type AR in the presence of bicalutamide or OH-flutamide to be similar to that of the non-DNA-binding mutant AR(A573D), suggesting that antiandrogens act by interfering with the stable DNA binding of the AR.

Materials and Methods

Constructs

Generation of pGFP-AR and pGFP(A573D) constructs, coding for N-terminally tagged GFP-AR fusion proteins of which the expression is driven by a CMV promoter, has been described previously (Farla et al., 2004). pGFP-AR(W741C) and pGFP-AR(T877A) were generated by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) on pGFP-AR using sense primers, 5′-CTGTCATTCAGTACTCCTG-TATGGGGCTCATGGTGTTTG-3′ and 5′-CTGCATCAGTTCG-CTTTTGACCTGCTA-3, and antisense primers 5′-CAAACACCAT-GAGCCCCATACAGGAGTACTGAATGACAG-3′ and 5′-TAG-CAGGTCAAAAGCGAACTGATGCAG-3′. Presence of mutations was verified by sequencing.

Stock solutions of hormones

R1881 (Methyltrienolone) was purchased from NEN (Boston, MA); OH-flutamide was obtained from Schering (Bloomfield, NJ). Bicalutamide (Casodex) was a gift from AstraZeneca (Macclesfield, UK). R1881 and OH-flutamide were diluted to 1 μM and 1 mM stock solutions respectively in ethanol. Stocks were stored at –20°C. Bicalutamide stocks of 1 mM in ethanol were freshly prepared directly before use.

Cell culturing and transactivation assays

Hep3B cells were cultured in αMEM (Cambrex, East Rutherford, NJ) supplemented with 2 mM L-glutamine, 100 U/ml Penicillin, 100 μg/ml Streptomycin and 5% FBS (PAN Biotech GmbH, Aidenbach, Germany). For confocal microscopy, cells were seeded on glass coverslips in six-well plates. For transactivation assays Hep3B cells were seeded at a density of 100,000 cells/well in 24-well plates. Cells were transfected with 250 ng/well of AR expression construct and 500 ng/well of a luciferase reporter expression vector gene using FuGENE 6 (Roche, Indianapolis, IN). Four hours prior to transfection, the medium was changed to medium containing 5% dextran charcoal-treated hormone-depleted FBS in the absence or presence of 1 nM R1881, 1 μM bicalutamide or 1 μM OH-flutamide. Twenty-four hours after transfection, cells were lysed in lysis buffer (15% glycerol, 25 mM Tris-phosphate, pH 7.8, 1 mM DTT, 1% Triton X-100 and 8 mM $MgCl_2$). Luciferase activity was measured by addition of an equal volume of lysis buffer containing luciferine to cell lysates using Fluoroscan Ascent FL (Labsystems Oy, Helsinki, Finland). Luciferase activities were normalized to the activity in the presence of 1 nM R1881.

Generation of stable cell lines

Stable cell lines expressing GFP-AR (mutants) were generated to ensure GFP-AR protein was expressed at physiological levels. Hep3B cells were transfected with 1 μg/well plasmid DNA using FuGENE6 1 day after plating in six-well plates. After 24 hours, cells were trypsinized and plated in medium supplemented with 800 μg/ml Geneticin (G418 sulfate, Sigma, St Louis, MO) in 10 cm tissue culture dishes. Clones were selected and checked for appropriate GFP-AR distribution and expression by confocal microscopy and western blotting. Stable cell lines were maintained as normal Hep3B cells in medium supplemented with 800 μg/ml Geneticin.

Western blotting

Stable cell lines expressing GFP-tagged AR constructs were cultured in 25 cm^2 flasks and allowed to grow until fully confluent. Cells were washed with DPBS and lysed in 250 μl Laemmli buffer (50 mM Tris, 10 mM DTT, 10% glycerol, 2% SDS and 0.001% Bromophenol Blue). Lysates were boiled and stored at –20°C. Lysates were subjected to electrophoresis on a 10% SDS polyacrylamide gel using β-actin expression as loading control. Following electrophoresis, proteins were transferred to nitrocellulose membranes. Blots were incubated with monoclonal antibodies F39.4.1, directed against the AR N-terminal domain (Zegers et al., 1991) or anti-β-actin (Sigma). Blots were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark). Proteins were visualized using Super Signal West Pico Luminol solution (Pierce, Rockford, IL), followed by exposure to X-ray film.

Confocal microscopy

Cell imaging and FRAP studies were performed using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss, Jena, Germany) using the 488 nm laser line of a 200 mW Ar laser with tube current set at 6.1 A. All images and FRAP results were obtained using a 40×/1.3 NA oil immersion lens using filters which pass emission light between 505 and 530 nm. One day prior to confocal microscopy, media were changed to αMEM containing 5% dextran charcoal-treated FBS. Prior to confocal microscopy, cell media were changed to αMEM containing 5% dextran charcoal-treated FBS with or without 1 nM R1881, 1 μM bicalutamide or 1 μM OH-flutamide. Cells were incubated with the ligands for at least 1 hour before they were imaged or used for FRAP analysis.

FRAP nuclear mobility studies

Nuclear mobility in the presence of the various ligands was studied using two different FRAP methods (Houtsmuller et al., 1999; Houtsmuller and Vermeulen, 2001; Farla et al., 2004). In the first method (strip-FRAP) fluorescence in a narrow strip (~0.75 μm) spanning the width of the nucleus was monitored every 21 milliseconds using 0.5% laser power of the 488 nm laser line, an intensity at which no significant monitor bleaching was observed. After 4 seconds the strip was bleached for 42 milliseconds at maximum laser power. Fluorescence intensity in the strip was expressed relatively to the fluorescence intensity before bleaching. All graphs were normalized to relative fluorescence of GFP-AR(A573D) in the presence of 1nM R1881 after complete redistribution (Farla et al., 2004).

The second FRAP method uses a combination of FRAP and fluorescence loss in photobleaching (FLIP), of which the principle has been described previously (Hoogstraten et al., 2002; Farla et al., 2004). Briefly, a strip of ~1.1 μm was bleached at one pole of the nucleus for 0.6 seconds at maximum laser power. Subsequent post-bleach images were taken at 3-second intervals. Fluorescence intensities in the bleached strip and in a strip 10 μm from the bleached area were normalized to prebleach intensities. Differences in fluorescence ratio between the bleached strip (FRAP) and distal region of the nucleus (FLIP) were calculated at each time point after bleaching. Maximal difference was set to 1 and values of the individual cells in the experiments were averaged. For both FRAP methods nuclei were selected with similar expression levels and similar dimensions.

For analysis of FRAP assays, experimental data were fitted to curves generated by computer software we developed to simulate FRAP of fluorescent molecules inside a finite ellipsoid volume representing the nucleus. Simulations were performed using fixed, experimentally obtained parameters, describing lens properties (beam shape and 3D intensity distribution, during monitoring and during bleach pulse), GFP properties (quantum yield, susceptibility to bleaching) and nuclear properties (size and shape). Details on the simulations have been reported (Farla et al., 2004). Three protein mobility parameters, diffusion coefficient, bound fraction and duration of binding of individual molecules were varied. The three-dimensional diffusion constant (D_{eff}) was defined as [(stepsize^2)/6 × cycletime]. The values of the parameters reported here are smaller than reported previously. This is because we used an improved microscope system, which enabled us to measure very soon after the bleach pulse. Furthermore the improved sensitivity of the detector allowed monitoring of the cells at low laser intensities, where no monitor bleaching occurs, whereas previously correction for monitor bleaching may have resulted in an overestimation of the binding times. The D_{eff} values reported here differ from those reported previously (Farla et al., 2004), because a one-dimensional model was used to fit the data. All simulations were performed five times and averaged; the average s.d. at each point of the simulation curves was <0.01. Least-square fitting of averaged simulated curves was used to determine which curves fitted best to the experimental data. Mobility parameters for all curves with $\Sigma(x_t - y_t)^2/n$ less than 0.002 were averaged, where x_t and y_t represent the value of the experimental data and the simulation at a given time point of the curve, respectively, and n is the number of time points. Mann-Whitney U-tests were performed to assess statistical significance of differences in mobility parameters of GFP-AR (mutants) compared with GFP-AR(A573D).

Results

In previous work we studied the transcriptional activity, intranuclear distribution and mobility of the GFP-tagged wild-type AR using live cell microscopy and FRAP (Farla et al., 2004; Houtsmuller, 2005). To determine the role of DNA binding, we compared the wild-type AR with an AR containing a mutation in the DNA-binding domain (A573D) that disrupts promoter binding, but is unaffected with respect to ligand binding (Brüggenwirth et al., 1998) and transport from cytoplasm to the nucleus (Farla et al., 2004). To investigate the mechanism of action of AR antagonists, we investigated the effect of bicalutamide and OH-flutamide on the intranuclear mobility and localization of GFP-AR and GFP-AR(A573D) (Fig. 1A). In addition, we studied two AR mutants, implicated in resistance to prostate cancer treatment with these non-steroidal AR antagonists (Veldscholte et al., 1990; Taplin et al., 1999; Hara et al., 2003; Taplin et al., 2003). The first mutant has a base substitution in codon 741 of the AR coding sequence, resulting in substitution of Trp741 by Cys (Hara et al., 2003). The second mutant has a base substitution of Thr877 by Ala (Veldscholte et al., 1990). The cDNA expression constructs coding for GFP-tagged versions of these AR mutants were transfected in the AR-negative cell line Hep3B and allowed to stably integrate. Western blot analyses revealed that all stable cell lines expressed full-length GFP-AR (Fig.

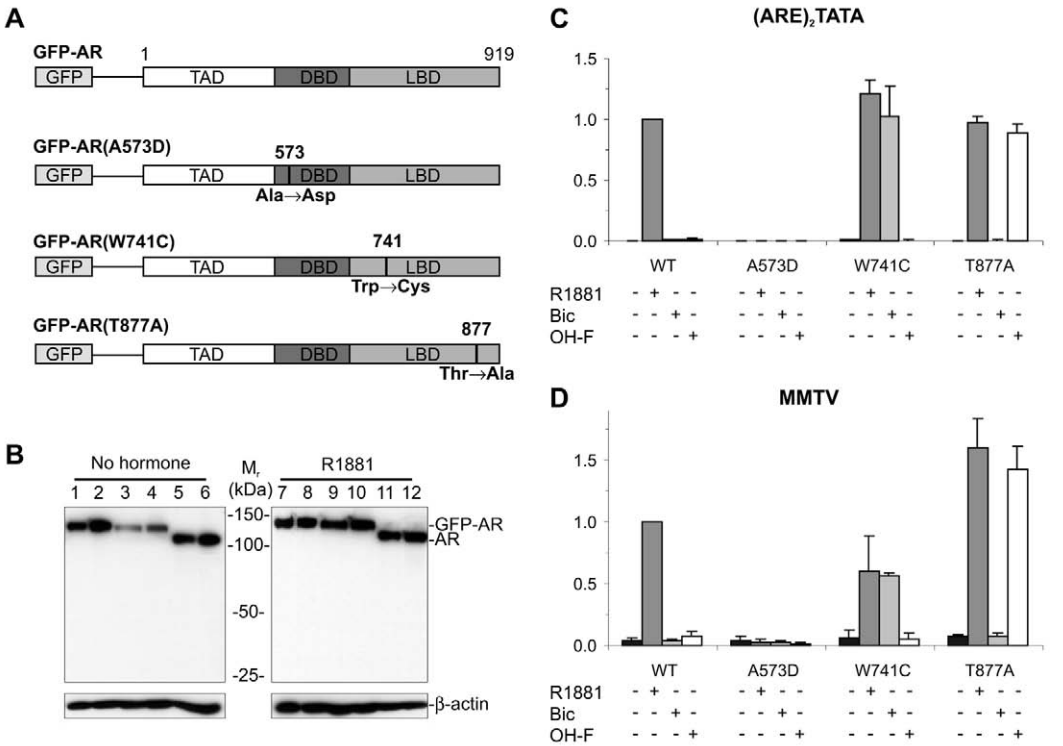

Fig. 1. Schematic representation, expression and transactivating capacity on androgen-regulated promoters of GFP-AR proteins investigated. (A) As well as GFP-tagged wild-type AR, ARs containing a mutation in the DBD disrupting DNA-binding (Brüggenwirth et al., 1998), or in helix 3 (W741C) or helix 12 (T877A) of the AR LBD, which result in altered ligand specificity (Veldscholte et al., 1990; Hara et al., 2003) were studied. TAD, Transactivating domain; DBD, DNA-binding domain; LBD, Ligand binding domain. (B) Hep3B cells containing stably integrated GFP-AR expression constructs and AR expressing prostate cancer cell lines were cultured for 1 day in the absence (lanes 1-6) or presence of 1 nM R1881 (lanes 7-12). Cell lysates were prepared and subjected to western blotting. Western blots of cell lysates from Hep3B cells (AR negative) containing GFP-AR (lanes 1 and 7), GFP-AR(A573D) (lanes 2 and 8), GFP-AR(W741C) (lanes 3 and 9) or GFP-AR(T877A) (lanes 4 and 10) and LNCaP (lanes 5 and 11) and PC346 (lanes 6 and 12) using an anti-AR or β-actin antibody. β-actin expression was used as a loading control. (C,D) Co-transfection assays of GFP-AR and the mutants depicted in A with androgen-regulated promoter constructs $(ARE)_2$-TATA-luciferase (C) and mouse mammary tumour virus (MMTV) luciferase (D) in the presence of 10^{-9} M R1881, 10^{-6} M bicalutamide (Bic), 10^{-6} M OH-flutamide (OH-F) or no ligand as indicated. Luciferase activity of the GFP-AR proteins is plotted relative to activity of GFP-AR in presence of 10^{-9} M R1881. Mean±s.e.m. of at least three independent experiments are shown.

1B). Expression in the GFP-AR stable cell line is comparable to the levels of AR in the prostate cancer cell lines LNCaP and PC346 (Fig. 1B) (Farla et al., 2004), indicating that GFP-AR is expressed at physiological levels. As expression levels of the mutants GFP-AR(W741C) and GFP-AR(T877A) were similar or slightly less than that of wild-type GFP-AR, the effects we observed were unlikely to be caused by overexpression.

To test the transactivating capacity of the GFP-tagged AR mutants, their corresponding cDNA expression plasmids were co-transfected with androgen-regulated promoters in AR-negative Hep3B cells. Activation of either the minimal promoter $(ARE)_2$TATA (Fig. 1C) or the mouse mammary tumour virus (MMTV)-promoter (Fig. 1D) in the presence of R1881, or the antiandrogens OH-flutamide or bicalutamide was measured. As expected, the non-DNA-binding mutant GFP-AR(A573D) was inactive. Wild-type AR could activate transcription of the $(ARE)_2$TATA promoter in the presence of R1881, whereas OH-flutamide and bicalutamide did not activate transcription, as expected. The prostate cancer-related GFP-tagged W741C mutant (Taplin et al., 2003) was activated by bicalutamide to the same extent as by R1881, whereas GFP-AR(T877A) in addition to R1881 was also activated by OH-flutamide (Fig. 1C). The same ligand specificity was observed on the MMTV promoter, although there were some quantitative differences in transactivating capacity between the mutants (Fig. 1D). These results show that the GFP tag does not interfere with the transactivating properties of the studied AR mutants, as the response is similar to results reported previously with untagged ARs (Veldscholte et al., 1990; Hara et al., 2003).

Antiandrogens OH-flutamide and bicalutamide do not reduce the mobility of wild-type androgen receptors

Previously we have shown using FRAP that binding of R1881 to GFP-AR resulted in a strongly reduced mobility of nuclear GFP-AR (Farla et al., 2004), when compared to a mutant GFP-AR(A573D), which is unable to bind DNA (Brüggenwirth et al., 1998). In this investigation we repeated these experiments as controls. Using two complementary FRAP assays (Fig. 2A-D) we show that fitting the data of the two assays to a model of free diffusion yields different diffusion coefficients,

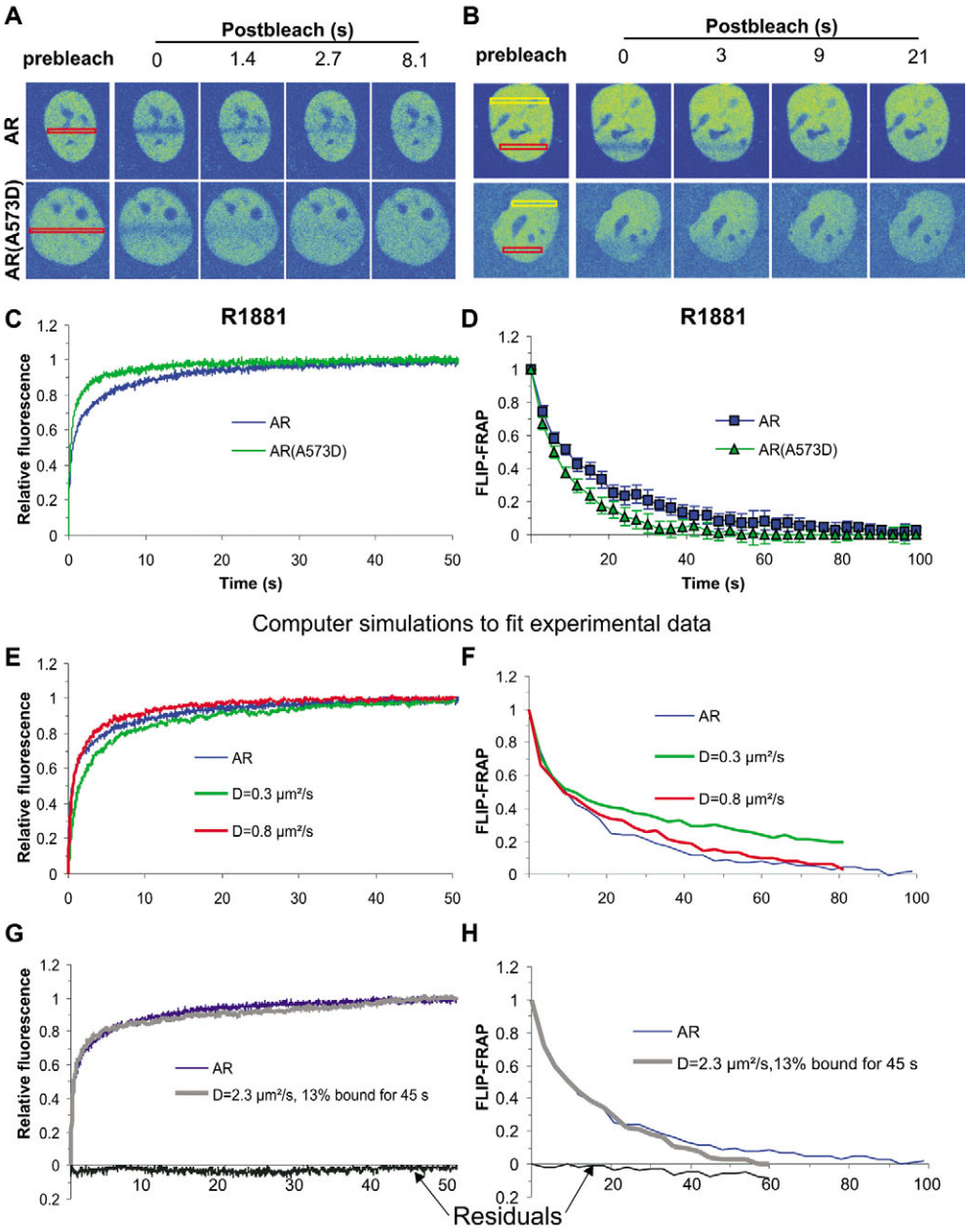

Fig. 2. Combined strip-FRAP and FLIP-FRAP reveal that a fraction of agonist-liganded GFP-ARs is transiently immobilized. (A) The strip-FRAP method. A strip in the centre of a nucleus is bleached (red rectangle) with high laser power. Subsequently, fluorescence in the strip is measured at regular time intervals. Images are shown in false colour to visualize fluorescence differences more clearly. (B) Combined FLIP and FRAP method (FLIP-FRAP). A strip at one pole of the nucleus was bleached for a relatively long period. The difference between fluorescence signals in the bleached region (FRAP, red rectangle) and a distal region at 10 μm from the bleached region of the nucleus (FLIP, yellow rectangle) was determined at regular time intervals. (C,D) Strip-FRAP and FLIP-FRAP experiments of GFP-AR or the non-DNA-binding mutant GFP-AR(A573D) in the presence of 10^{-9} M R1881. (C) Graph showing fluorescence intensities relative to complete redistribution of the non-DNA-binding mutant GFP-AR(A573D) in the presence of R1881 plotted as a function of time. Mean values of at least ten cells of a representative experiment are plotted. All experiments were performed at least three times. (D) Graph showing the difference between fluorescence intensity in the FLIP and FRAP regions (rectangles in B) relative to the difference directly after bleaching, plotted against time. Mean values±two times the s.e.m. of two independent experiments on at least ten cells are plotted. (E,F) Computer simulations (see Materials and Methods) of strip-FRAP and FLIP-FRAP of freely diffusing molecules do not explain the experimental FRAP data obtained with both methods. D is the effective diffusion coefficient. Experimental strip-FRAP data on wild-type GFP-AR lies in between curves representing indicated scenarios of free diffusion (E), whereas experimental FLIP-FRAP data on wild-type GFP-AR lies outside these boundaries (F). (G,H) Computer simulations representing a model where, next to freely diffusing molecules, a fraction is transiently immobilized, fitted to both strip-FRAP and FLIP-FRAP experimental curves on wild-type GFP-AR. Computer simulations correspond to the average of best fits of FRAP and FLIP-FRAP experiments respectively (Table 1), so are not necessarily the best fits of the individual experiments. Absolute value of residuals of the computer simulation fit and the experimental data on each time point are plotted below the *x*-axis.

indicating that the results cannot be explained by a simple model of free diffusion. Therefore, the observed slower mobility is probably not the result of an overall slow-down of diffusion (Fig. 2E,F). By contrast, a scenario where a ~15% fraction of ARs in the nucleus was immobilized for ~45 seconds (Fig. 2G,H and Table 1) fitted well to both strip-FRAP and FLIP-FRAP curves. The R1881-liganded DBD mutant GFP-AR(A573D) was freely mobile, similar to unliganded wild-type ARs (Farla et al., 2004), suggesting that the immobilization of wild-type AR was related to binding to its cognate sequences in the DNA. To investigate the mechanism by which AR antagonists OH-flutamide and bicalutamide interfere with proper transcription activation we set out to compare the behaviour of R1881-associated wild-type AR with that of antagonist-liganded wild-type ARs. First, we performed strip-FRAP and FLIP-FRAP assays on cells expressing wild-type ARs in the presence of OH-flutamide (Fig. 3A,B). Fitting of the experimental data to computer-simulated curves revealed no significant slow-down of AR mobility, compared to the non-DNA-binding GFP-AR(A573D) (Fig. 3A,B; Table 1). Diffusion constants in both strip-FRAP and FLIP-FRAP experiments were similar to that of GFP-AR(A573D). Similar to these results, the combined FRAP analysis of wild-type AR in the presence of the antagonist bicalutamide showed almost identical recovery kinetics as GFP-AR(A573D) indicating that no substantial immobilization occurred (Fig. 3C,D; Table 1). These results suggest that antagonist-liganded ARs have a similar mobility to the non-DNA-binding GFP-AR(A573D) and show no immobilization, whereas the agonistic ligand R1881 induces a transient immobilization of a fraction of ARs in a DNA-binding-dependent manner. These data suggest that antagonist-bound ARs cannot stably bind DNA.

Androgen receptor agonists induce intranuclear foci

In the absence of ligand, GFP-AR as well as GFP-AR(A573D) are predominantly localized in the cytoplasm, although not completely absent from the nucleus (Farla et al., 2004). As shown previously, R1881 induced translocation to the nucleus and in addition to AR immobilization, induced intranuclear AR accumulation in a focal pattern (Fig. 4A) (Farla et al., 2004). By contrast, the DBD mutant GFP-AR(A573D) was homogeneously distributed in the nucleus (Fig. 4D) (Farla et al., 2004). We studied the intranuclear distribution of GFP-AR and GFP-AR(A573D) in the presence of OH-flutamide or bicalutamide by high-resolution confocal microscopy. Addition of the antagonists translocated the receptor to the nucleus (although at a slower rate) and resulted in a homogeneous intranuclear distribution of wild-type AR (Fig. 4B,C) (see also Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001; Farla et al., 2004) as well as the DBD mutant GFP-AR(A573D) (Fig. 4E,F), indicating that the binding of antagonists prevented foci formation.

Next, we studied the intracellular distribution of GFP-AR(W741C) and GFP-AR(T877A). In the absence of ligand, those mutants similar to wild-type receptors were predominantly cytoplasmic. Exposure to R1881 as well as the antagonists OH-flutamide and bicalutamide resulted in translocation of the mutant receptors to the nucleus (Fig. 4G-L). In the presence of R1881, GFP-AR(W741C) and GFP-AR(T877A) displayed a very similar focal distribution (Fig. 4G,J). GFP-AR(W741C), which activates transcription on androgen-regulated promoters in the presence of bicalutamide (Hara et al., 2003) (Fig. 1C,D), in addition showed bicalutamide-induced intranuclear accumulations (Fig. 4I), whereas OH-flutamide treatment resulted in a homogeneous intranuclear distribution (Fig. 4H), supporting the hypothesis that lack of transactivating capacity results in homogeneous distribution. Treatment of the mutant GFP-AR(T877A) with OH-flutamide induced intranuclear accumulation (Fig. 4K), but bicalutamide did not result in accumulation of this AR mutant (Fig. 4L), consistent with transactivation of androgen-regulated target genes by OH-flutamide and not by bicalutamide (Fig. 1) (Veldscholte et al., 1990).

Table 1. Fit of experimental data to curves generated by computer simulation

Construct	Parameter	Ligand		
		R1881	OH-F	Bic
GFP-AR	Diffusion constant[†]	2.3±0.3**	3.4±0.2*	3.7±0.2
	Immobile fraction[‡]	0.13±0.01**	–	–
	Binding time[§]	45±5	–	–
GFP-AR(A573D)	Diffusion constant	3.0±0.2	3.7±0.2	3.5±0.2
	Immobile fraction	–	–	–
	Binding time	–	–	–
GFP-AR(W741C)	Diffusion constant	2.2±0.3**	3.6±0.3	2.8±0.3**
	Immobile fraction	0.13±0.01**	–	0.10±0.02**
	Binding time	52±7	–	33±5
GFP-AR(T877A)	Diffusion constant	2.4±0.3**	2.6±0.2**	3.5±0.2
	Immobile fraction	0.14±0.01**	0.11±0.01**	–
	Binding time	45±6	39±5	–

Data are the mean±two times s.e.m. of best fitting parameters from Strip-FRAP and FLIP-FRAP. Values in shaded areas indicate conditions with transcriptionally active ARs (see Fig. 1).

[†]Diffusion constant in μm^2/second of mobile fraction.

[‡]Fraction of receptors immobilized owing to interaction with subnuclear structures. –, no detectable immobile fraction (fraction <0.05).

[§]Mean immobilization of individual ARs in seconds.

P-values of Mann-Whitney U test comparing the value of the parameter with that of GFP-AR(A573D) with the same ligand, *P<0.05 and **P<0.005.

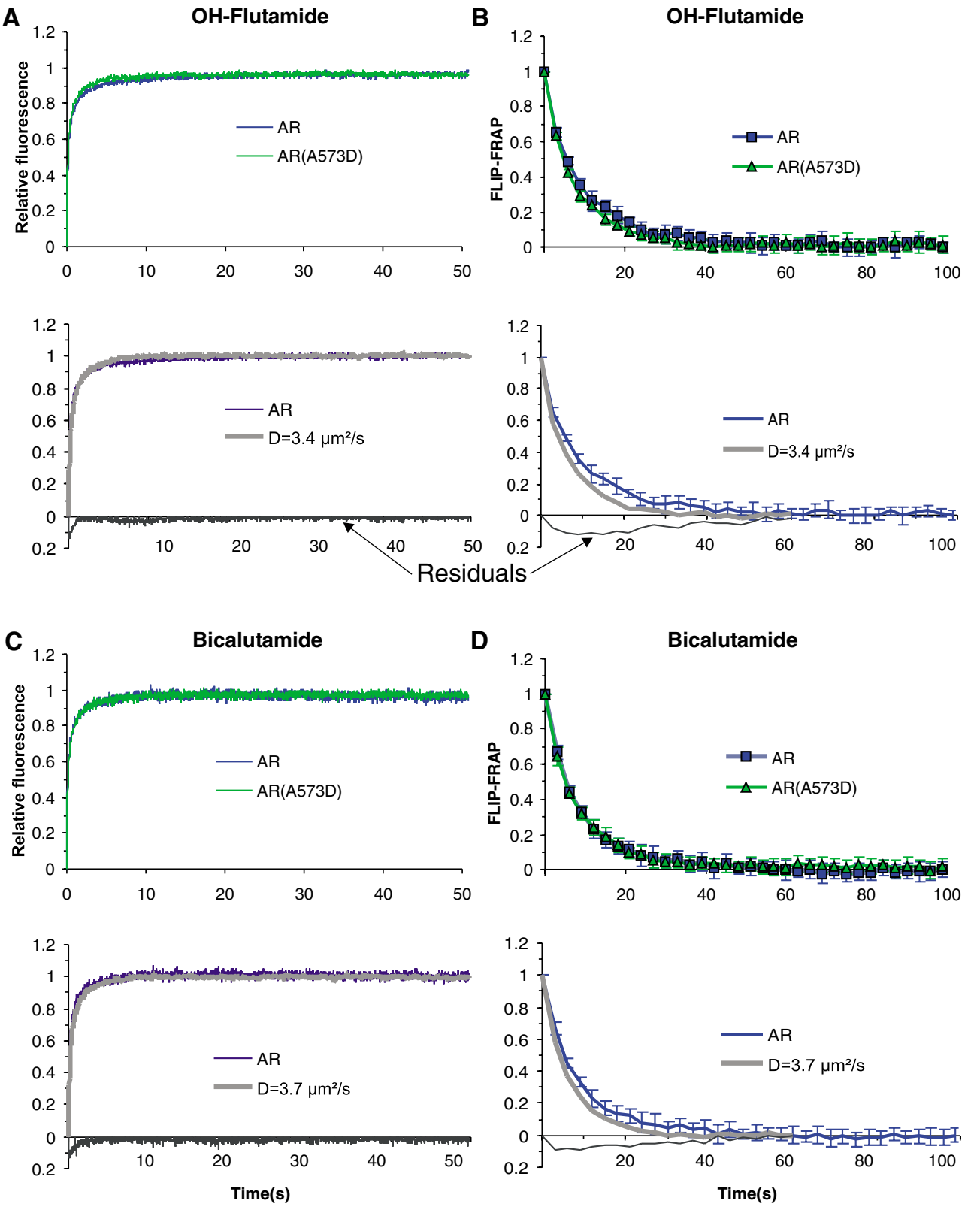

Fig. 3. In the presence of antagonists OH-flutamide and bicalutamide GFP-AR shows no or little DNA-dependent immobilization. Strip-FRAP (A,C) or combined FLIP and FRAP (B,D) of GFP-AR or the non-DNA-binding mutant GFP-AR(A573D) in the presence of 10^{-6} M OH-flutamide (A,B) or 10^{-6} M bicalutamide (C,D). Experimental settings were identical to those described in Fig. 2. Lower graphs show computer simulations corresponding to the average of best fits of strip-FRAP and FLIP-FRAP models of wild-type GFP-AR (see Table 1). The absolute values of the residuals of the fit and the experimental data are plotted below the *x*-axis. Larger residuals in the first second of strip-FRAPs are probably due to larger variation in the beginning of the experiment, when fluorescence changes rapidly.

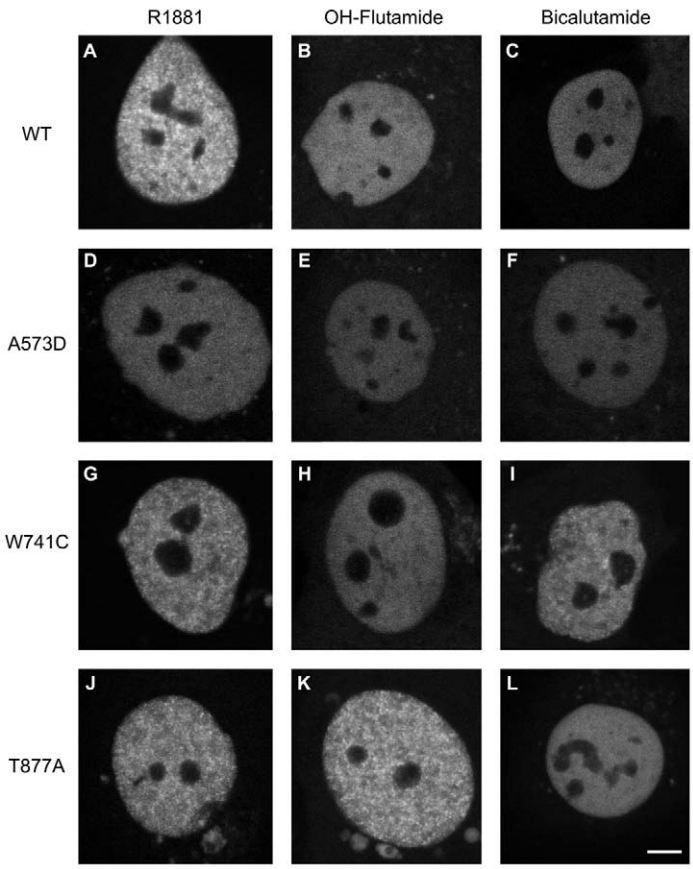

Fig. 4. Activation of AR by agonistic ligands results in intranuclear localization in foci. Confocal laser-scanning microscope images showing representative nuclei of Hep3B cell lines stably expressing GFP-AR (A-C), the non-DNA-binding mutant GFP-AR(A573D) (D-F) or GFP-AR proteins with mutations in the LBD (which result in altered ligand specificity), GFP-AR(W741C) (G-I) and GFP-AR(T877A) (J-L). Subnuclear localization was observed in the presence of 10^{-9} M R1881 (A,D,G,J), 10^{-6} M OH-flutamide (B,E,H,K) or 10^{-6} M bicalutamide (C,F,I,L). With all ligands, androgen receptors are localized in the nucleus, but are excluded from nucleoli (dark areas in the nucleus). In situations where AR is able to activate transcription intranuclear foci are observed (A,G,I,J,K, see Fig. 1). Bar, 5 μm.

Prostate cancer-related androgen receptor mutants show reduced mobility in the presence of their agonistic ligands

The intranuclear mobility of the mutant receptors GFP-AR(W741C) and GFP-AR(T877A) in the presence of R1881 was similar to wild-type AR (compare Fig. 5A,B with Fig. 2C,D). Fitting to computer-simulated curves revealed that ~10-15% of ARs were immobilized for ~45 seconds (Table 1). In addition, diffusion of the mobile fraction was slowed down significantly compared to GFP-AR(A573D). The antiandrogen OH-flutamide retarded redistribution of GFP-AR(T877A) (Fig. 5C,D), in agreement with OH-flutamide acting as an agonist of this mutant (Fig. 1C,D). Computer simulations showed a fraction (13%) of OH-flutamide-liganded GFP-AR(T877A) was immobilized in a similar manner to R1881-bound wild-type AR (Table 1). In addition, diffusion of the mobile fraction

Fig. 5. Prostate cancer-related AR-LBD mutants display reduced mobility in the presence of their agonistic ligands. Nuclear mobility of antiandrogen-resistant prostate cancer mutants AR(T877A) and AR(W741C) was investigated using two complementary FRAP assays (see also Fig. 2): strip-FRAP (A,C,E) and combined FLIP and FRAP (B,D,F). Intranuclear mobility of these mutants in the presence of 10^{-9} M R1881 (A,B), 10^{-6} M OH-flutamide (C,D) or 10^{-6} M bicalutamide (E,F) was studied. Mobility of non-DNA-binding GFP-AR(A573D) is plotted as a reference. Experimental settings were identical to those described in Fig. 2. Lower graphs in C-F show computer simulations corresponding to the average of best fits of strip-FRAP and FLIP-FRAP models (data in Table 1) of the experimental curves of GFP-AR(T877A) (C,D) and GFP-AR(W741C) in the presence of 1 μM OH-flutamide (C,D) or bicalutamide (E,F). Absolute values of the residuals of computer-simulated curves and experimental data are plotted below the *x*-axis.

was significantly slower compared to the other GFP-ARs in the presence of OH-flutamide (Table 1). Similarly, bicalutamide slowed down nuclear redistribution of GFP-AR(W741C) (Fig. 5E,F). By contrast, GFP-AR(T877A) showed the same FRAP kinetics as GFP-AR(A573D), suggesting that no substantial stable DNA binding occurs in the presence of bicalutamide. In conclusion, the slower recovery of fluorescence in the presence of agonistic ligands is probably caused by DNA-binding-dependent immobilization of ~10-15% of ARs for ~45 seconds, usually accompanied by a slow-down in diffusion of the mobile fraction (Table 1, Fig. 5).

Discussion

To obtain insight in the mechanism of the blocking of AR transcription activation by antagonists, we investigated the behaviour of the AR in living cells in the presence of bicalutamide and OH-flutamide. Using FRAP on GFP-tagged ARs to determine intranuclear mobility, we show that a fraction (~10-15%) of agonist R1881-liganded ARs are immobilized for ~45 seconds (Fig. 2C-H, Table 1). Immobilization is dependent on DNA binding, as the mutant GFP-AR(A573D) containing a mutation that completely disrupts DNA binding (Brüggenwirth et al., 1998) did not show an immobile fraction (Fig. 2C,D) (Farla et al., 2004). Similar high mobility was observed for other AR-DBD mutants (V581F and R585K), which do not bind DNA (data not shown). Binding of the AR antagonists bicalutamide and OH-flutamide resulted in a similar relatively high mobility as observed for the non-DNA-binding mutant AR (Fig. 3), strongly suggesting that the antagonists interfere with early steps in the mechanism of AR transcription activation: stabilizing binding to promoters and enhancers of androgen regulated genes. This is supported by previous observations in vitro showing that bicalutamide-liganded AR, but not R1881-liganded AR, could be removed from the nuclear fraction by detergent treatment (Berrevoets et al., 1993). However, studies using ChIP suggested that bicalutamide-bound ARs (Kang et al., 2002; Masiello et al., 2002) were present in DNA-protein complexes containing the corepressors N-CoR and SMRT on the promoter/enhancer region of androgen-regulated prostate-specific antigen (PSA) (Cleutjens et al., 1997; Shang et al., 2002; Kang et al., 2004). Assuming that the formation of DNA-bound repressor complexes is not a unique feature of the PSA gene, it is

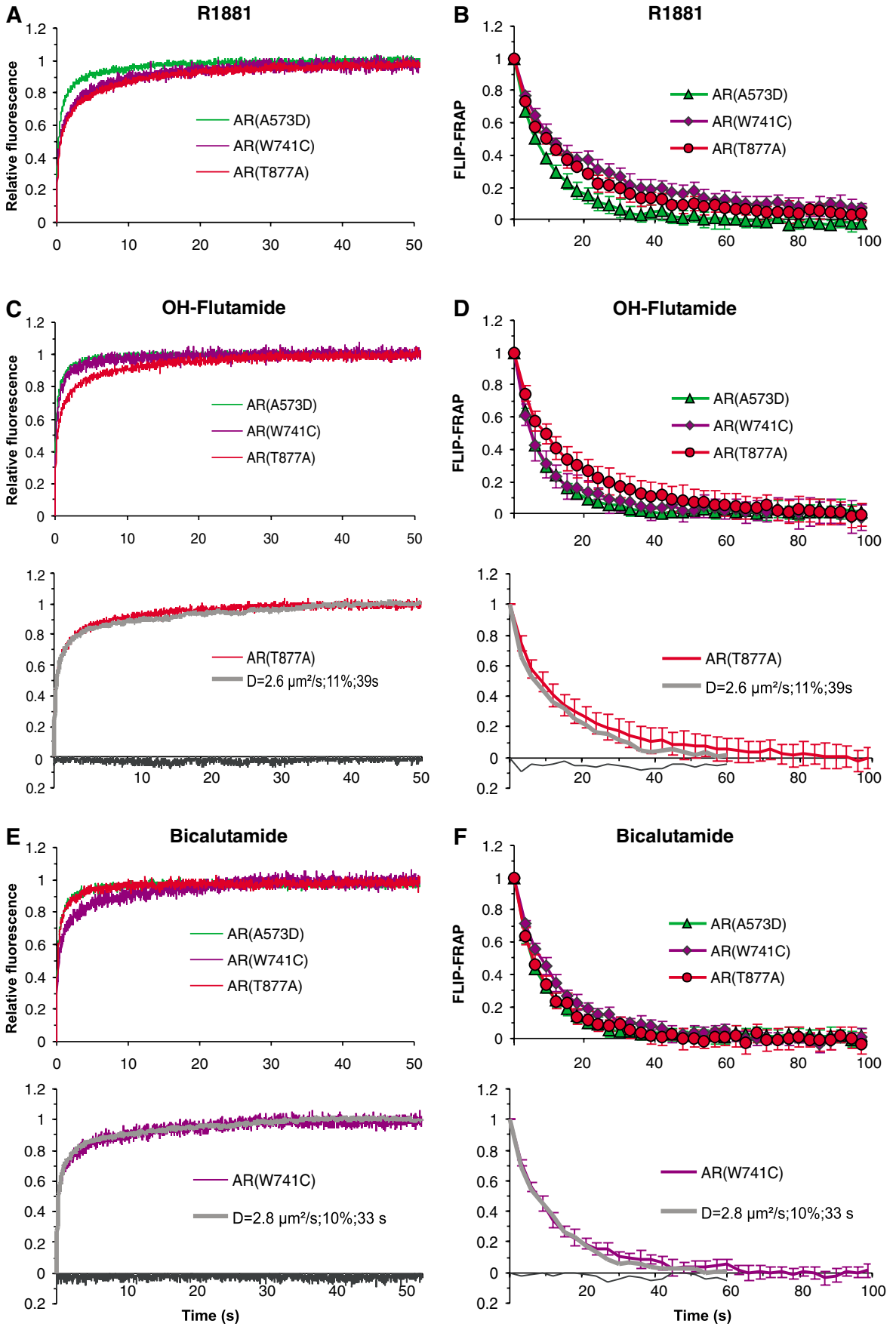

Fig. 5. See previous page for legend.

expected that antagonist-bound ARs also bind to other AR-specific promoters or enhancers. Although this seems to contradict the data presented here (Fig. 3C,D and Table 1), it may be that repressor complexes on promoters are very short-lived (<1 second) and escape detection by FRAP.

In addition to the observed transient immobilization, R1881 also induced a slow-down of the effective diffusion of the mobile AR fraction, which was not observed after antagonist binding (Table 1). There are two explanations for this observation. First, mobile agonist-bound ARs in the nucleoplasm may, in contrast to antagonist-bound ARs, associate with coactivators forming large complexes exhibiting slower diffusion owing to their size. This view is supported by published data indicating that binding of antagonists results in a conformation of the AR-LBD that does not allow AR amino/carboxyl-terminal (N/C) interaction (Doesburg et al., 1997; Chang and McDonnell, 2002) and interactions with coactivators (Chang and McDonnell, 2002; Shang et al., 2002). A second explanation may be that the R1881-induced ability to bind DNA not only leads to relatively stable binding to AR-regulated promoters, but also to very transient, highly frequent binding to non-specific regions in the DNA. Such a scenario in which DNA interacting proteins 'scan' DNA in order to find their cognate binding sites has been suggested previously (Karpova et al., 2004; Phair et al., 2004; Sprague et al., 2004). For instance, it was reported that the glucocorticoid receptor binds very transiently (<200 milliseconds) to DNA with a very high frequency, such that on average 80% of the GR is associated with DNA (Sprague et al., 2004). Although such a model does not completely explain our results of combined strip-FRAP and FLIP-FRAP experiments (as our data fit better to a model in which a small fraction is more stably immobilized), it is possible that the observed large mobile AR fraction (85-90%) exhibits this type of rapid interaction, resulting in the measured slow-down of this mobile fraction.

We further studied the effect of OH-flutamide and bicalutamide on the nuclear behaviour of two mutant ARs (T877A and W741C) (Figs 4, 5), which were found in prostate cancer patients treated with OH-flutamide and bicalutamide, respectively (Taplin et al., 2003). OH-flutamide can activate the transcription function of the T877A mutant and similarly, bicalutamide can activate the W741C mutant (Veldscholte et al., 1990; Hara et al., 2003) (Fig. 1C,D). It has been suggested that binding of OH-flutamide to AR containing the T877A mutation induces a conformation of the AR-LBD that allows AR N/C interaction and coactivator interaction (Doesburg et al., 1997; Chang and McDonnell, 2002; Shang et al., 2002), which results in agonistic activity of OH-flutamide. A similar mechanism may explain the agonist effect of bicalutamide on AR(W741C). Our data are in agreement with this hypothesis as both mutants, when exposed to bicalutamide (W741C) or OH-flutamide (T877A), showed the same kinetics as the R1881-liganded AR, i.e. all three mobility parameters measured here were in the same range (~10-15% transiently immobile for 30-40 seconds, as well as a slow-down of the mobile fraction (Fig. 5 and Table 1). As discussed above, this slow-down of diffusion can either be explained by very transient binding events or engagement of AR in larger complexes, or both.

The focal nuclear distribution pattern observed for agonist-bound wild-type AR is a common feature of steroid receptors. It has been described not only for AR (Tyagi et al., 2000; Tomura et al., 2001), but also for the ERα (Stenoien et al., 2000) and GR (Htun et al., 1999). Here we show that there is a direct relationship between transactivating capacity, reduced mobility (as a consequence of transient binding) and the occurrence of the focal pattern: in the presence of an agonistic ligand all three features are observed together, irrespective of whether the agonist is an antiandrogen activating a mutant or R1881 activating wild-type AR or AR(W741C) and AR(T877A) mutants. By contrast, in the presence of an antagonist, all three features were absent, i.e. the nuclear distribution was diffuse, mobility was not reduced, and no transactivating capacity was observed. Previously, it was suggested that the focal pattern reflects binding to the nuclear matrix (Stenoien et al., 2000; Schaaf and Cidlowski, 2003; Stavreva et al., 2004). However, this is contradicted by observations that bicalutamide-bound ARs were also found in the operationally defined nuclear matrix fraction (Tyagi et al., 2000), although they did not appear in foci (Fig. 4) (Tyagi et al., 2000; Tomura et al., 2001). For the aryl hydrocarbon receptor it was shown that foci correlated with transcription sites (Elbi et al., 2002) whereas for the GR, no clear correlation of foci with pre-mRNA synthesis sites could be demonstrated (van Steensel et al., 1995). Our results (Fig. 4) and that of our previous studies (Farla et al., 2004) strongly suggest a role for DNA binding in foci formation.

In conclusion, we have shown that, in contrast to R1881 binding, binding of the antagonists bicalutamide and OH-flutamide to AR did not induce detectable DNA-binding-related immobilization; did not give rise to a slow-down of the effective diffusion of the mobile fraction; and did not induce the formation of heterogeneous intranuclear distribution (foci). These three observations strongly suggest that the investigated antagonists interfere with events early in the transactivation function of AR leading to the absence of stable DNA-binding-dependent immobilization. This may be due to the absence of appropriate stabilizing interactions with cofactors. In cell lines expressing AR mutants GFP-AR(W741C) and GFP-AR(T877A) bicalutamide and OH-flutamide induce intranuclear immobilization and localization in numerous irregular shaped foci respectively, suggesting that these mutations restore the appropriate AR configuration and the capacity to stably bind to DNA.

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