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Mechanism of antiandrogen action: conformational changes of the receptor

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Abstract

Androgen receptor mRNA was translated in vitro, and androgen- and antiandrogen-bound receptor complexes were studied using limited proteolytic digestion by trypsin. Partial proteolysis of androgen-bound receptor protein resulted in a 29-kDa proteolysis-resisting fragment, whereas antiandrogen binding stabilised a 35-kDa fragment. Both fragments contain the entire ligand binding domain, and the 35-kDa fragment extended into the hinge region of the receptor. Several antiandrogens show agonistic properties for a mutated androgen receptor (LNCaP cell variant); trypsin digestion of antiandrogen-bound mutated receptor also resulted in a 29-kDa fragment. Our results point to an important difference between antiandrogens and antagonists of other steroid hormone receptors. Antiandrogens result in protection of both the hinge region and C-terminus of the androgen receptor against proteolytic attack, whereas other studies showed that antiestrogens and antiprogestagens expose the C-terminal end of the ligand binding domain of their respective receptors to protease. Differences in conformation of the hinge region distinguish androgen-bound from antiandrogen-bound receptor complexes, which represents an important feature of antiandrogen action.

Key words: Androgen receptor; Antiandrogen; LNCaP cell; Prostate; Steroid hormone

1. Introduction

The study of the mechanism of antiandrogen action is of great interest, not only because of the therapeutic potential of antiandrogens, but also because these compounds are important tools to elucidate the molecular mechanism of action of androgens. Androgens initiate effects in target cells through a ligand-activated transcription factor, the androgen receptor (AR). Like all members of the steroid hormone receptor family, the AR binds to its responsive element after a ligand-dependent activation process and interacts with the transcription complex to regulate gene transcription (Carson-Jurica et al., 1990; Smith and Toft, 1993).

Antiandrogens compete with androgens for binding to the AR, but binding of antagonists does not result in full transformation of the receptor to a transcriptionally active form. Several mechanisms for the resulting inhibitory effect have been postulated, and recently a subdivision of antagonists in two distinct classes has been made (Reese and Katzenellenbogen, 1991; Klein-Hitpass et al., 1991; Gronemeyer et al., 1992). One class of antagonists does not, or does so with decreased efficiency, promote DNA binding of the receptor, whereas the other class of antagonists promotes DNA binding but induces an abnormal conformation of the ligand-binding domain. The latter class of antagonists may give rise to a partial agonistic effect, through a transcription activation function in the N-terminal domain of the receptor.

For the progesterone receptor it was recently shown that binding of progestagens and antiprogestagens results in different susceptibility of the receptor to proteolytic digestion. Antagonists induced protection of a smaller progesterone receptor fragment than did agonists (Allan et al., 1992a,b), and studies with antibodies indicated that a short region at the C-terminal end of the progesterone receptor is involved in this difference (Allan et al., 1992a; Weigel et al., 1992).

The presented data concern differences in susceptibility of androgen- and antiandrogen-bound receptor complexes to proteolytic digestion.

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2. Materials and methods

2.1. Materials

RNA transcription kit and pBluescript II KS- were obtained from Stratagene (La Jolla, USA). Nuclease-treated rabbit reticulocyte lysate was purchased from Promega (Madison, USA). L-[^{35}S]methionine (s.a. > 1000 mCi/mM) was obtained from Amersham (Buckinghamshire, UK). Trypsin (type III), soybean trypsin inhibitor (type I-S), goat-anti-mouse agarose and goat-anti-rabbit agarose were obtained from Sigma (St. Louis, USA). R1881 (methyltrienolone) was purchased from NEN (Boston, USA). Cyproterone acetate was a gift from Schering AG (Berlin, Germany), hydroxyflutamide from Schering USA (Bloomfield, USA) and ICI 176.334 ("Casodex") from ICI Pharmaceuticals (Macclesfield, UK). Dihydrotestosterone and testosterone were obtained from Steraloids (Wilton, USA).

2.2. Plasmid construction

The coding sequence for the wild-type human AR (AR0) was excised from the expression vector pSVAR0 (Brinkmann et al., 1989) and subcloned in the *Sal*I site of pBluescript to obtain pBSAR0. The recombinant pBSAR0 615–910 was obtained from pBSAR0 after digestion with *Sac*I and religation. The recombinants were linearized with *Xho*I for transcription. The coding sequence for the mutant LNCaP AR (ARL) was subcloned between the *Sal*I and *Bam*HI sites of pBluescript (pBSARL). For linearization, the recombinant was digested with *Xba*I.

2.3. In vitro transcription and translation

Both in vitro transcription and in vitro translation in the presence of L-[^{35}S]methionine were performed according to manufacturer's instruction. For in vitro transcription of pBSAR0 and pBSAR0 615–910, T_7 RNA polymerase was used to produce sense mRNA, whereas pBSARL was transcribed with T_3 RNA polymerase.

2.4. Limited proteolytic digestion of in vitro-produced receptors

Two μl of labeled translation mix was pre-incubated for 1 h at room temperature with 3 μl of ligand solution diluted in water. For limited proteolytic digestion, 5 μl trypsin (40 μg/ml, dissolved in water) was added to the pre-incubation mix, followed by an incubation for 15 min at room temperature. After incubation, 20 μl SDS sample buffer was added. Samples were boiled for 3 min, and 15 μl was loaded onto 0.1% (w/v) sodium dodecyl sulfate-12.5% (w/v) polyacrylamide gels. After electrophoresis (Laemmli, 1970), the gels were vacuum-dried at 80°C for 45 min and autoradiography was performed overnight.

2.5. Immunoprecipitation

Labeled translation mix (20 μl) was hormone-treated and digested with trypsin as indicated above. After digestion, soybean trypsin inhibitor was supplemented to a final concentration of 200 μg/ml. Goat-anti-rabbit or goat-anti-mouse agarose (100 μl, diluted 1:4 in phosphate-buffered saline (PBS)) was incubated for 2 h at 4°C with 1 μl of the indicated polyclonal rabbit or

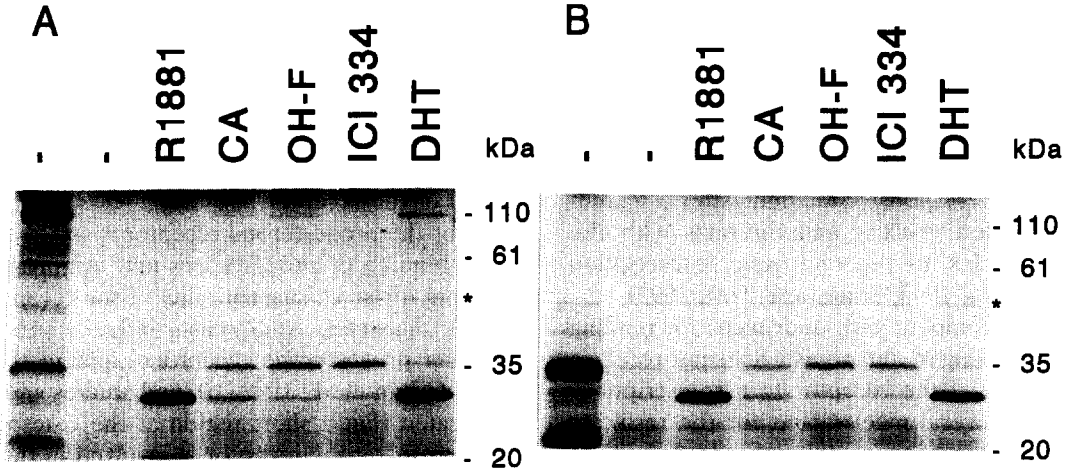

Fig. 1. Limited proteolytic digestion of in vitro-produced AR (A) and AR0 615–910 (B) bound with different androgens and antiandrogens. Lane 1: no trypsin added. Lane 2: control digestion without steroid (−). Lanes 3–7: 10 nM R1881, 1 μM cyproterone acetate (CA), 10 μM hydroxyflutamide (OH-F), 10 μM ICI 176.334 (ICI 334), and 10 nM dihydrotestosterone (DHT), respectively. Molecular mass markers are indicated at the right. * indicates a non-specific band.

monoclonal mouse antiserum. The polyclonal antiserum SP066 (epitope amino acids 892–910) and monoclonal antiserum F52 (epitope amino acids 593–612) were described previously (Zegers et al., 1991; Veldscholte et al., 1992). Following this incubation, the resin was washed three times with 1 ml PBS and added to the limited proteolytic digest of the receptor. After incubation for 2 h at 4 °C, the resin was washed three times with 1 ml PBS, and 25 μl of sample buffer was added. Electrophoresis was performed as described above.

3. Results and discussion

Limited proteolytic digestion of [^{35}S]methionine labeled, in vitro-produced AR was used to detect conformational changes upon androgen or antiandrogen binding to the receptor. The in vitro-produced AR showed steroid binding properties similar to those observed for AR isolated from mammalian cells (Kuiper et al., 1993). After incubation of the AR with androgen or antiandrogen, a limited amount of trypsin was added and the digestion products were analyzed by denaturing gel electrophoresis. Either in the absence of ligand (Fig. 1A) or in the presence of a steroid with no affinity for the AR (dexamethasone; result not shown), the AR was completely digested to fragments that were undetectable with electrophoresis. Proteolytic digestion of AR incubated either with the synthetic androgen R1881 or the natural ligands dihydrotestosterone and testosterone, resulted in a 29-kDa proteolysis-resisting fragment (Fig. 1A; result for testosterone not shown). Incubation of AR with the antiandrogens cyproterone acetate, hydroxyflutamide, or ICI 176.334 before tryptic digestion resulted in stabilisation of a 35-kDa fragment (Fig. 1A). The concentrations of the different ligands used varied according to their differences in relative binding affinities of the ligands for the AR (Veldscholte et al., 1992).

Formation of a 29-kDa fragment was the result of binding of an agonist, whereas stabilisation of a 35-kDa fragment indicated binding of an antagonist. When increasing concentrations of the antiandrogen ICI 176.334 were added together with a constant level of R1881, digestion with trypsin resulted in less an amount of the 29-kDa fragment and an increased amount of the 35-kDa fragment (Fig. 2). As predicted from the relative binding affinities of the ligands, 50% binding inhibition of 1 nM R1881 occurred at 1 μM ICI 176.334. When the concentration of R1881 was increased, the 29-kDa fragment reappeared (Fig. 2).

Several in vitro-produced AR fragments, including a 35-kDa fragment, were already present before the start of the proteolytic digestion, probably due to alternative translation initiation. Therefore, the effect of trypsin

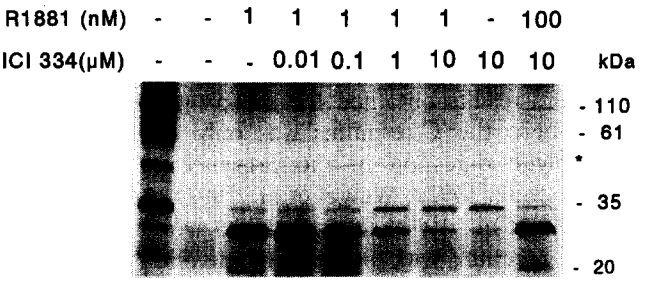

Fig. 2. Competition of 1 nM R1881 with increasing levels of ICI 176.334 (ICI 334). Ligands were bound to the in vitro-produced AR before digestion with trypsin. Lane 1: no trypsin added. Lane 2: control digestion without steroid. Lanes 3–9: indicated levels of R1881 and/or ICI 176.334. Molecular mass markers are indicated at the right. * indicates a non-specific band.

on the 35-kDa fragment was studied in detail in separate experiments. A mutant AR cDNA, deleted of the N-terminal and DNA-binding domains (AR0 615–910), was translated in vitro into a predominant 35-kDa protein (Fig. 1B). Also for this truncated receptor protein, binding of either R1881 or dihydrotestosterone resulted in formation of a 29-kDa fragment upon proteolytic digestion, whereas the antiandrogens predominantly stabilised the 35-kDa fragment. These results show that formation of the 29-kDa fragment is not dependent on interaction of the ligand binding domain with the N-terminal and DNA-binding domains.

From these experiments it can be concluded that trypsin treatment of androgen- or antiandrogen-bound AR results in different proteolysis-resisting fragments which suggest a different structural conformation. Comparable, but not similar, observations have been made for the progesterone and estrogen receptors (Vegeto et al., 1992; Allan et al., 1992a; Beekman et al., 1993). In contrast with the results found for the AR, antagonist binding to these receptors resulted in smaller proteolysis-resisting fragments than obtained after agonist binding.

Immunoprecipitation was performed to determine which part of the AR was removed upon conversion of the 35-kDa fragment into the 29-kDa fragment. After incubation of the full-length AR with ligand, followed by limited proteolytic digestion with trypsin, the fragments resisting proteolysis were immunoprecipitated with different antisera (Fig. 3). Neither the 35-kDa fragment nor the 29-kDa fragment could be immunoprecipitated with the antiserum F52 which recognizes an epitope in the DNA-binding domain (Zegers et al., 1991). Therefore, this epitope appears not to be present in either fragment. The antiserum SP066, raised against a peptide at the C-terminus of the AR, recognizes both fragments, which indicates that the difference in size of the 29-kDa and 35-kDa fragments is not located at the C-terminus of the AR.

These results indicate that, in the AR, a part of the hinge region is protected against degradation by trypsin in the presence of antiandrogens. This is in contrast with the results obtained for the progesterone receptor, where the C-terminus was only retained during proteolytic digestion in the presence of an agonist (Allan et al., 1992a; Weigel et al., 1992). Another study (Vegeto et al., 1992) showed that a truncated progesterone receptor, with a C-terminal deletion of 42 amino acid residues, could not bind a progestagen but still bound the antiprogestagen RU486. In addition, RU486 was able to act as an agonist in a transcription activation assay.

LNCaP prostate tumor cells contain an AR (ARL) with a single amino acid change in the steroid binding domain (codon 868; Thr–Ala (Veldscholte et al., 1990)). Both cyproterone acetate and hydroxyflutamide act as agonists for the ARL but ICI 176.334 still behaves as an antiandrogen with the ARL (Veldscholte et al., 1992). ARL is therefore a useful tool to study the mechanism of action of antagonists. The in vitro-produced ARL was treated with the same androgens and antiandrogens as described above for the wild-type AR (Fig. 4). For R1881, dihydrotestosterone and ICI 176.334, the results for ARL were comparable with those obtained with the wild-type AR. However, the antiandrogens cyproterone acetate and hydroxyflutamide, which gave rise to a 35-kDa fragment with the wild-type AR, both induced formation of a 29-kDa fragment with the ARL. As these antiandrogens act as agonists for the ARL, it can be assumed that the single amino acid change in the ARL made it possible for

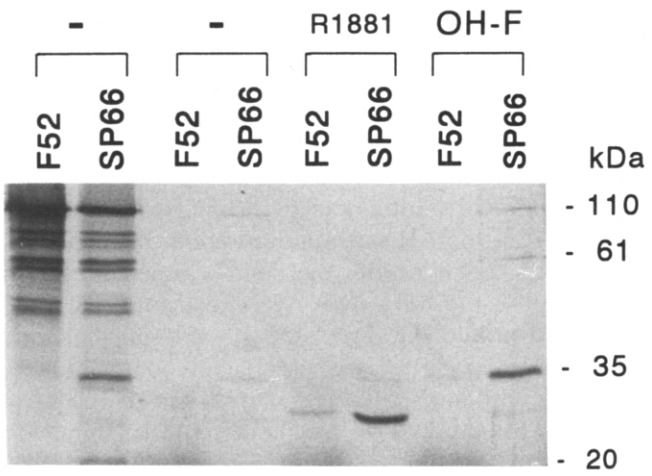

Fig. 3. Immunoprecipitation of AR fragments with different antisera. After treatment with vehicle (−; lanes 3–4), 100 nM R1881 (lanes 5–6) or 100 nM hydroxyflutamide (OH-F; lanes 7 and 8), the AR was digested with trypsin. Lanes 1 and 2 were controls without trypsin. After digestion, immunoprecipitation was performed with the monoclonal antiserum F52 (lanes 1, 3, 5 and 7) or the polyclonal antiserum SP066 (lanes 2, 4, 6 and 8). Molecular mass markers are indicated at the right.

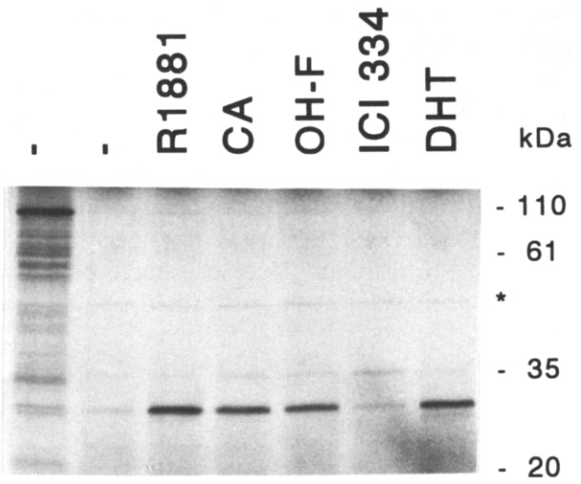

Fig. 4. Limited proteolytic digestion of in vitro-produced mutant ARL bound with different androgens and antiandrogens. Lane 1: no trypsin added. Lane 2: control digestion without steroid (−). Lanes 3–7: 10 nM R1881, 1 μM cyproterone acetate (CA), 10 μM hydroxyflutamide (OH-F), 10 μM ICI 176.334 (ICI 334), and 10 nM dihydrotestosterone (DHT), respectively. Molecular mass markers are indicated at the right. * indicates a non-specific band.

both antiandrogens to induce a comparable conformation as formed after the interaction of an androgen with the wild-type AR.

In conclusion, androgens and antiandrogens induce a different change in the conformation of the AR as detected by proteolytic digestion. This appears to involve the hinge region of the receptor, which is in contrast with studies on other steroid receptors. Allan et al. (1992a) proposed a general action of steroid receptor antagonists in preventing the formation of a transcriptionally competent conformation of the C-terminal end of the ligand binding domain. Conformational changes in the C-terminal end of the AR were not detected by trypsin treatment although several consensus sites for trypsin digestion are present. The results therefore suggest a difference in mechanism of antiandrogen action compared to other steroid hormone receptor antagonists.

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