J. Steroid Biochem. Molec. Biol. Vol. 40, No. 1–3, pp. 349–352, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

Steroid Receptor and Enzyme Abnormalities

ANDROGEN RECEPTOR ABNORMALITIES

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Summary—The human androgen receptor is a member of the superfamily of steroid hormone receptors. Proper functioning of this protein is a prerequisite for normal male sexual differentiation and development. The cloning of the human androgen receptor cDNA and the elucidation of the genomic organization of the corresponding gene has enabled us to study androgen receptors in subjects with the clinical manifestation of androgen insensitivity and in a human prostate carcinoma cell line (LNCaP). Using PCR amplification, subcloning and sequencing of exons 2–8, we identified a G → T mutation in the androgen receptor gene of a subject with the complete form of androgen insensitivity, which inactivates the splice donor site at the exon 4/intron 4 boundary. This mutation causes the activation of a cryptic splice donor site in exon 4, which results in the deletion of 41 amino acids from the steroid binding domain. In two other independently arising cases we identified two different nucleotide alterations in codon 686 (GAC; aspartic acid) located in exon 4. One mutation (G → C) results in an aspartic acid → histidine substitution (with negligible androgen binding), whereas the other mutation (G → A) leads to an aspartic acid → asparagine substitution (normal androgen binding, but a rapidly dissociating androgen receptor complex). Sequence analysis of the androgen receptor in human LNCaP-cells (lymph node carcinoma of the prostate) revealed a point mutation (A → G) in codon 868 in exon 8 resulting in the substitution of threonine by alanine. This mutation is the cause of the altered steroid binding specificity of the LNCaP-cell androgen receptor. The functional consequences of the observed mutations with respect to protein expression, specific ligand binding and transcriptional activation, were established after transient expression of the mutant receptors in COS and HeLa cells. These findings illustrate that functional errors in the human androgen receptor have an enormous impact on phenotype and fertility.

INTRODUCTION

Androgens (e.g. testosterone and 5α-dihydrotestosterone) are indispensable for normal male sexual differentiation. Androgen action at the target cell level is accomplished via a low abundance nuclear protein: the androgen receptor. The androgen receptor belongs to the steroid/thyroid hormone/retinoic acid receptor family [1, 2].

It is generally accepted that in the X-chromosome linked androgen insensitivity syndrome defects in the androgen receptor gene have prevented the normal development of both internal and external male structures in 46, XY individuals [3, 4]. The recent cloning and characterization of cDNA encoding the human androgen receptor and the elucidation of the organization of the human androgen receptor gene have greatly increased our knowledge of the molecular structure of the human androgen receptor [5–9]. The information on the molecular structure of the human androgen receptor has facilitated the study of molecular defects associated with androgen insensitivity [10–15]. Moreover, naturally occurring mutations in the androgen receptor are a potentially interesting source for the investigation of receptor structure–function relationships. In addition, the variation in clinical syndromes provides the opportunity to correlate a mutation in the androgen receptor structure with the impairment of a specific physiological function of the androgen receptor. In the present investigation the molecular structure of the human androgen receptor was analysed after amplification by

Proceedings of the VIIIth International Congress on Hormonal Steroids, The Hague, The Netherlands, 16–21 September 1990.

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PCR and subsequent sequencing of the individual exons of the androgen receptor gene from three different subjects with the complete form of androgen insensitivity and from a human prostate cancer cell line (LNCaP).

PRIMARY STRUCTURE OF THE HUMAN ANDROGEN RECEPTOR

The primary structure of the human androgen receptor has been determined after molecular cloning and characterization of cDNA encoding the human androgen receptor [7, 8]. The cDNA sequence reveals an open reading frame of 2730 nucleotides encoding a protein of 910 amino acid residues with a calculated molecular mass of 98,500 Da.

STRUCTURAL ORGANIZATION OF THE HUMAN ANDROGEN RECEPTOR GENE

The human androgen receptor gene is located on the X-chromosome and has a length of >90 kb [9, 16, 17]. The information for the protein-coding region is separated over 8 exons (Fig. 1). The sequence encoding the N-terminal domain is present in 1 large exon (exon 1) [8]. The DNA-binding domain is encoded by exons 2 and 3, and the information for the steroid-binding domain is distributed over 5 exons (exons 4–8) [9].

ANALYSIS OF GENOMIC DNA OF SUBJECTS WITH ANDROGEN INSENSITIVITY

A mutation in the intron 4 splice donor site

Examination by Southern blotting of genomic DNA isolated from genital skin fibroblasts of a subject (index patient 20.1) with complete androgen insensitivity and no detectable androgen binding to receptors in target cells revealed the presence of the complete androgen receptor gene.

To investigate whether a point mutation or small gene deletion was responsible for the absence of hormone binding in genital skin fibroblasts of this patient's exons 4–8, coding for the steroid-binding domain of the androgen receptor were amplified from genomic DNA using the polymerase chain reaction. In addition, exons 2 and 3, coding for the DNA-binding domain were amplified. Exons 2–8 and all corresponding exon/intron junctions were sequenced. Sequences were found to be identical to the previously published wild-type sequence with one exception: a G → T mutation on position 1 in the splice donor site of intron 4.

The consequence of this point mutation on RNA-splicing was investigated after amplification of cDNA from subject 20.1 using specific primers for exons 4 and 5. This resulted in the identification of a mutant fragment, which was shorter than the corresponding fragment from the wild-type androgen receptor. This finding indicated an abnormal androgen receptor mRNA-splicing. Sequence analysis of the mutant fragment revealed the use of a cryptic splice donor site CAG/GTGTAG at position 2020/2021 in exon 4 of the human androgen receptor gene which is normally inactive. The use of this cryptic splice site results in the deletion of 123 nucleotides from the mRNA. Translation will consequently result in an in-frame deletion of 41 amino acid residues in the androgen receptor protein from this patient (see Fig. 2).

Transient expression of the mutant androgen receptor in COS cells resulted in a protein which was approx. 5 kDa smaller than the wild-type receptor. The mutant receptor was unable to bind androgens and did not activate transcription of an androgen-regulated MMTV-CAT

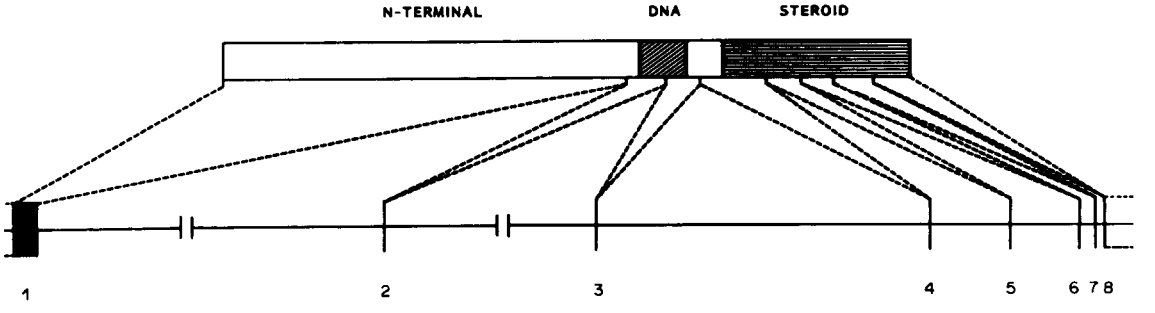

Fig. 1. Structural organization of the human androgen receptor gene. The 8 exons of the human androgen receptor gene are numbered 1–8 and their corresponding position in the human androgen receptor cDNA is indicated together with the location of the N-terminal, DNA-binding and steroid-binding domains, respectively.

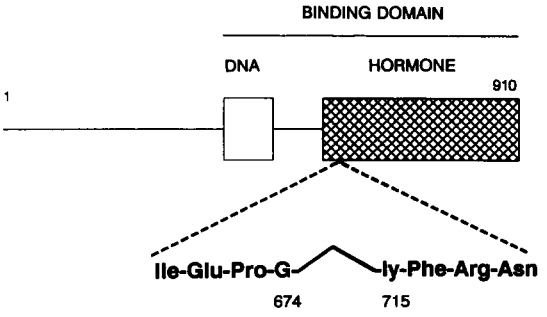

Fig. 2. Position of the deletion of amino acid residues 674–714 in the human androgen receptor protein of patient 20.1 as a result of an activation of a cryptic splice donor site in exon 4 due to a point mutation on position 1 of intron 4.

reporter gene construct (in the presence as well as absence of ligand). The deletion of 41 amino acid residues is partly located in a region that displays a high degree of sequence conservation in the family of steroid hormone receptors and is supposed to be involved in the transduction of the signal of hormone binding into derepression of receptor function [18].

Mutations of codon 686 in exon 4

In two unrelated patients with the complete form of androgen insensitivity, two different point mutations were found at the same nucleotide in codon 686 (aspartic acid) of exon 4 located in the steroid-binding domain (Fig. 3). Aspartic acid 686 is conserved in the steroid-binding domain of the progesterone, glucocorticoid and mineralocorticoid receptor. Both mutations eliminate a HinfI restriction site (Fig. 3). One mutation (G → C) results in an aspartic acid → histidine substitution. In this patient the androgen receptor displays low to negligible androgen binding. The other mutation (G → A) leads to an aspartic acid → asparagine substitution. The androgen receptor in the patient

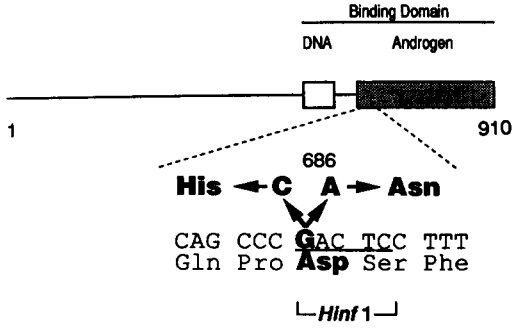

Fig. 3. Location of the G → C and G → A substitutions at codon 686 in exon 4 of the human androgen receptor of 2 subjects with the complete form of androgen insensitivity. The HinfI site of exon 4 is underlined.

with the latter mutation shows normal androgen binding but the half-life of the androgen receptor complex is 5 times shorter than of the wild-type androgen receptor complex.

Both mutant androgen receptors were transiently expressed in COS cells and displayed the same altered binding characteristics as those measured in genital skin fibroblasts from the affected subjects. In co-transfections in HeLa cells using a GRE-tk-CAT reporter gene construct both mutant receptors were tested for their transcription activation activity. A partial activation of transcription (approx. 30%) was observed only in the presence of a 100-fold higher concentration of ligand as compared with the wild-type androgen receptor. Both mutant receptors were devoid of any transcriptional activation activity in the presence of physiological hormone concentrations.

ANALYSIS OF GENOMIC DNA OF THE LNCaP CELL LINE

Previous studies have established that human LNCaP-cells can be stimulated with respect to growth by androgens, but surprisingly also by progesterone, R5020 (a synthetic progestagen) and oestradiol [19]. Characterization of the androgen receptor in these cells revealed an altered steroid-binding specificity with an increased preference for progestagens and oestradiol as compared with the steroid-binding specificity of the androgen receptor in normal cells [20]. These data strongly suggested a modification of the androgen receptor and particularly in the steroid-binding domain. To establish a possible small deletion or point mutation exons 2–8 of the LNCaP-cell androgen receptor were sequenced with the same approach as applied for the genomic DNA analysis of patients with androgen insensitivity. Sequence analysis revealed one point mutation in codon 868 (threonine) in exon 8 located at the C-terminal end of the steroid-binding domain (Fig. 4). The mutation (A → G) resulted in a threonine → alanine substitution. To determine whether the substitution affects the functional properties of the LNCaP androgen receptor the mutant cDNA was cloned in an expression vector and transiently expressed in COS-1 cells (for studying binding characteristics) and in HeLa cells (for studying transcription activation of the reporter gene construct: GRE-tk-CAT). In the transfection studies the mutant receptor displayed increased binding affinity for

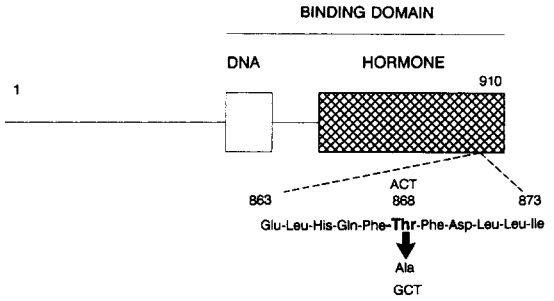

Fig. 4. Location of the point mutation (A → G) in codon 686 of exon 8 of the androgen receptor gene in human LNCaP-cells. The mutation results in the substitution of threonine by alanine in the C-terminal part of the hormone-binding domain.

progestagens and oestradiol. In addition, these ligands activate transcription at concentrations which are inactive with the wild-type androgen receptor. These results confirm that the observed point mutation in the LNCaP androgen receptors the cause of the broad steroid-binding specificity. Whether this point mutation can play a role in progressive prostate tumour growth remains to be established.

REFERENCES

1. Evans R. M.: The steroid and thyroid hormone receptor superfamily. *Science* **240** (1988) 889–895.
2. O'Malley B. W.: The steroid receptor superfamily: more excitement predicted for the future. *Molec. Endocr.* **4** (1990) 363–369.
3. Pinsky L. and Kaufman M.: Genetics of steroid receptors and their disorders. *Adv. Hum. Genet.* **16** (1987) 299–472.
4. Griffin J. E. and Wilson J. D.: The androgen resistance syndromes: 5α-reductase deficiency, testicular feminization, and related syndromes. In *The Metabolic Basis of Inherited Disease* (Edited by C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle). McGraw-Hill, New York, 6th Edn (1989) pp. 1919–1944.
5. Chang C., Kokontis J. and Liao S.: Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. *Proc. Natn. Acad. Sci. U.S.A.* **85** (1988) 7211–7215.
6. Lubahn D. B., Joseph D. R., Sar M., Tan J., Higgs H. N., Larson R. E., French F. S. and Wilson E. M.: The human androgen receptor: complementary deoxyribonucleic acid, cloning, sequence analysis and gene expression in prostate. *Molec. Endocr.* **2** (1988) 1265–1275.
7. Trapman J., Klaassen P., Kuiper G. G. J. M., van der Korput J. A. C. M., Faber P. W., van Rooij H. C. J., Geurts van Kessel A., Voorhorst M. M., Mulder E. and Brinkmann A. O.: Cloning, structure and expression of a cDNA encoding the human androgen receptor. *Biochem. Biophys. Res. Commun.* **153** (1988) 241–248.
8. Faber P. W., Kuiper G. G. J. M., van Rooij H. C. J., van der Korput J. A. G. M., Brinkmann A. O. and Trapman, J.: The N-terminal domain of the human androgen receptor is encoded by one large exon. *Molec. Cell. Endocr.* **61** (1989) 257–262.
9. Kuiper G. G. J. M., Faber P. W., van Rooij H. C. J., van der Korput J. A. C. M., Ris-Stalpers C., Klaassen P., Trapman J. and Brinkmann A. O.: Structural organization of the human androgen receptor gene *J. Molec. Endocr.* **2** (1989) R1–R4.
10. Brown T. R., Lubahn D. B., Wilson E. M., Joseph D. R., French F. S. and Migeon C. J.: Deletion of the steroid-binding domain of the human androgen receptor gene in one family with complete androgen insensitivity syndrome: evidence for further genetic heterogeneity in this syndrome. *Proc. Natn. Acad. Sci. U.S.A.* **85** (1988) 8151–8155.
11. Lubahn D. B., Brown T. R., Simental J. A., Higgs H. N., Migeon C. J., Wilson E. M. and French F. S.: Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 9534–9538.
12. Sai T., Seino S., Chang C., Trifiro M., Pinsky L., Mhatre A., Kaufman M., Lambert B., Trapman J., Brinkmann A. O., Rosenfield R. L. and Liao S.: An exonic point mutation of the androgen receptor gene in a family with complete androgen insensitivity. *Am. J. Hum. Genet.* **46** (1990) 1095–1100.
13. Marcelli M., Tilley W. D., Wilson C. M., Wilson J. D., Griffin J. E. and McPhaul M. J.: A single nucleotide substitution introduces a premature termination codon into the androgen receptor gene of a patient with receptor-negative androgen resistance. *J. Clin. Invest.* **85** (1990) 1522–1528.
14. Marcelli M., Tilley W. D., Wilson C. M., Griffin J. E., Wilson J. D. and McPhaul M. J.: Definition of the human androgen receptor gene structure permits the identification of mutations that cause androgen resistance: premature termination of the receptor protein at amino acid residue 588 causes complete androgen resistance. *Molec. Endocr.* **4** (1990) 1105–1116.
15. Ris-Stalpers C., Kuiper G. G. J. M., Faber P. W., Schweikert H. U., van Rooij H. C. J., Zegers N. D., Hodgins M. B., Degenhart H. J., Trapman J. and Brinkmann A. O.: Aberrant splicing of androgen receptor mRNA results in synthesis of a nonfunctional receptor protein in a patient with androgen insensitivity. *Proc. Natn. Acad. Sci. U.S.A.* **87** (1990) 7866–7870.
16. Brinkmann A. O., Faber P. W., van Rooij H. C. J., Kuiper G. G. J. M., Ris C., Klaassen P., van der Korput J. A. G. M., Voorhorst M. M., van Laar J. H., Mulder E. and Trapman J.: The human androgen receptor: domain structure, genomic organization and regulation of expression. *J. Steroid Biochem.* **34** (1989) 307–310.
17. Brown C. J., Goss S. J., Lubahn D. B., Joseph D. R., Wilson E. M., French D. S. and Willard H. F.: Androgen receptor locus on the human X-chromosome: regional localization to Xq11–12 and description of a DNA polymorphism. *Am. J. Hum. Genet.* **44** (1990) 264–269.
18. Pratt W. B., Jolly D. J., Pratt D. V., Hollenberg S. M., Giguere V., Cadepond F. M., Schweizer-Groyer G., Catelli M-G., Evans R. M. and Baulieu E-E.: A region in the steroid binding domain determines formation of the non-DNA-binding, 9S glucocorticoid receptor complex. *J. Biol. Chem.* **263** (1988) 267–273.
19. Schuurmans A. L. G., Bolt J., Voorhorst M., Blankenstein M. A. and Mulder E.: Regulation of growth and epidermal growth factor receptor levels of LNCaP prostate tumor cells by different steroids. *Int. J. Cancer* **42** (1988) 917–922.
20. Veldscholte J., Voorhorst-Ogink M. M., Bolt-de Vries J., van Rooij H. C. J., Trapman J. and Mulder E.: Unusual specificity of the androgen receptor in the human prostate tumor cell line LNCaP: high affinity for progestagenic and estrogenic steroids. *Biochim. Biophys. Acta* **1052** (1990) 187–194.