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THREONINE ON AMINO ACID POSITION 868 IN THE HUMAN ANDROGEN RECEPTOR IS ESSENTIAL FOR ANDROGEN BINDING SPECIFICITY AND FUNCTIONAL ACTIVITY

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The human androgen receptor gene in the androgen sensitive prostate tumor cell line (LNCaP) contains a point mutation in codon 868 resulting in the substitution of threonine by alanine. This amino acid change is responsible for the increased affinity of the mutant receptor protein for progestagens and estrogens.
To further elucidate the role of threonine 868 on androgen binding capacity, specificity and functional activity, threonine 868 was substituted by six different amino acid residues. Substitution by aspartic acid, lysine or tyrosine totally eliminated androgen binding and the mutated androgen receptors did not have any transcriptional activating potential with either R1881, R5020 or estradiol. Introduction of a serine or an alanine broadened the steroid specificity, as did the introduction of a cysteine to a lesser degree. It is concluded that threonine on position 868 of the human androgen receptor limits the ligand specificity of the receptor to androgens.

Androgen regulated gene expression is mediated by the androgen receptor (AR), a protein belonging to the superfamily of receptors for steroid hormones, thyroid hormones and retinoids (1). Several single base changes in the androgen receptor gene resulting in amino acid substitutions have been identified in subjects with the androgen insensitivity syndrome, indicating the importance of the androgen receptor in normal male sexual differentiation (2). We have reported a point mutation in the androgen receptor gene at codon 868 of an androgen-sensitive tumor cell line (LNCaP) derived from a metastatic lesion of a human prostatic carcinoma (3). This mutation resulted in a threonine to alanine substitution in the steroid binding domain rendering the receptor protein responsive to androgens as well as to estrogens, progestagens and anti-androgens (3). Similar mutations are implicated in prostate tumors which do not respond to hormonal therapy. Only recently a point mutation resulting in a valine to methionine substitution in the steroid binding domain of the human androgen receptor gene has been reported in prostate tumor tissue (4).

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To study the role of threonine 868 in the human androgen receptor, mutant androgen receptor expression constructs were generated in which the codon for threonine 868 was substituted for triplets encoding either alanine, aspartic acid, cysteine, lysine, serine or tyrosine. These receptor constructs were transiently expressed in COS and HeLa cells and their steroid binding specificity and their ability to activate transcription of an androgen receptor responsive reporter gene was studied using androgens, progestagens and estrogens as ligands.

MATERIALS AND METHODS

Construction of androgen receptor expression vectors: The wild type human androgen receptor cDNA expression vector pAR0 (5) was modified on amino acid position 868 by specific mutagenesis using the polymerase chain reaction (PCR) as described by Higuchi et al (6). Briefly: for each construct a sense and an antisense oligonucleotide primer were constructed where the mismatches in the primer sequence upon translation result in the incorporation of the desired mutant amino acid (Table 1). In the primary PCR reactions, an oligonucleotide located upstream of the EcoRI site in exon 6 (5' - TGAGGCACCTCTCTCAAGA - 3'), and the antisense mutant primer were used on pAR0 template, and an oligonucleotide primer located downstream of the EcoRI site in exon 8 (5'- CAAAGTCTGAAGGTGCCATG - 3') combined with the sense mutant primer were used on a genomic DNA fragment containing exon 8 sequences (phage 18.1 in ref 7). 1% of both products were mixed and used in a second PCR with the oligonucleotiodes flanking both EcoRI sites as described above. The 642 bp product was digested with EcoRI and the resulting 500 bp fragment was exchanged with the 500 bp wild type EcoRI-EcoRI fragment from pAR0. The T868A mutant expression vector used was the pARL expression vector as described in ref. 3. All mutant expression vectors were sequenced to verify the mutation and the correct reading frame. A typical PCR protocol was: 1 minute 94 ^{o}C, 2 minutes 55 ^{o}C, 1 minute 72 ^{o}C for 25 cycles using 2,5 units AmpliTaq according to specifications of the manufacturer (Cetus).
Cell culture and transfection conditions: COS-1 and HeLa cells were cultured in Eagles minimal essential medium (Gibco) supplemented with either 5% full or 5% dextran charcoal treated (DCC) fetal calf serum, antibiotics and non-essential amino acids. Cells grown to 50-60% confluency were transfected using the calcium-phosphate precipitation method (8) essentially as described before (9).
Hormone binding assays: COS cells were harvested 48 hours after transfection by scraping in buffer (10) and the cytosol fraction was prepared by 3 freeze (20 seconds

TABLE 1: Oligonucleotides used to create specific amino acid substitutions on position 868 in the human androgen receptor

construct	amino acid introduced	sense oligonucleotide used to introduce specific amino acid substitution
T868C	cysteine	5' - CTG CAT CAG TTC TGT TTT GAC CTG CT - 3'
T868D	aspartic acid	5' - CTG CAT CAG TTC GAT TTT GAC CTG CT - 3'
T868K	lysine	5' - CTG CAT CAG TTC AAG TTT GAC CTG CT - 3'
T868S	serine	5' - CTG CAT CAG TTC TCC TTT GAC CTG CT - 3'
T868Y	tyrosine	5' - CTG CAT CAG TTC TAT TTT GAC CTG CT - 3'

Mismatches are underlined.
Corresponding antisense oligonucleotides are complementary.
In the T868A (LNCaP) mutant threonine 868 is replaced by alanine.

liquid nitrogen) thaw (10 minutes 10 °C) cycles followed by centrifugation at 12,000xg for 10 minutes. Cytosol (supernatant) was incubated for 16 hours at 4 °C, with 10 nM of the radiolabeled synthetic androgen methyltrienolone (^{3}H)R1881 (NEN, Boston) in the presence of either unlabeled R1881, R5020 or estradiol in 1 or 100-fold molar excess. Alternatively after whole cell incubations at 37 °C for 2 hours, cells were harvested in 0.1% trypsin, the supernatant was discarded and cell pellets were solubilized in 0.5N NaOH. Non specific binding was determined using a 200-fold molar excess unlabeled R1881. Separation of bound and unbound steroid was done by protamine-HCl precipitation (10). Radioactivity was determined by liquid scintillation counting.

Cat assays: HeLa cells co-transfected with androgen receptor expression plasmids and the pG29GtkCAT reporter plasmid (11) were 24 hours after transfection incubated for an additional 24 hours with either 1, 10 or 100 nM R1881, R5020 or estradiol, or 10 nM Dexamethasone before CAT activity in the cell lysates was measured as described (12). Data were corrected for the protein content of the cell lysates and the CAT activity induced by dexamethasone via the endogenous glucocorticoid receptors was used as an internal control.

Western blot analysis: The androgen receptor was immunoprecipitated from COS cells transfected as described above using the AR specific monoclonal antibody F39.4.1 and analyzed by SDS-PAGE and immunoblotting using the polyclonal antibody Sp061. Staining was done using alkaline phosphatase (9).

RESULTS

The wild type androgen receptor and six different androgen receptor proteins specifically mutated at amino acid position 868 were transiently expressed in COS-1 cells to study their steroid binding capacity and specificity. The wild type human androgen receptor protein on SDS-PAGE has an apparant molecular mass of 110 kDa and migrated as a tightly spaced doublet which is the result of post-translational phosphorylation, in the N-terminal domain of the receptor (13,14). Immunoblots demonstrated that all the constructs were expressed in COS cells showing the expected pattern after SDS-PAGE and in virtually equal amounts (Figure 1).

Substitution of threonine 868 by either alanine, serine, or cysteïne did not markedly influence the R1881 binding capacity as determined in a whole cell binding assay at 37 °C. The introduction of tyrosine, aspartic acid or lysine eliminated specific R1881 binding

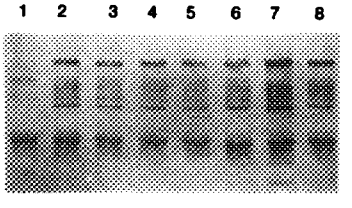

FIGURE 1.
SDS-PAGE analysis of immunoprecipitated androgen receptor from transiently transfected COS cells. Lane 1: mock transfected COS cells, lane 2: pARo, lane 3: T868A, lane 4: T868K, lane 5: T868C, lane 6: T868S, lane 7: T868Y, lane 8: T868D. The position of the 110 kDa androgen receptor protein specific doublet is indicated by an arrow.

TABLE 2: Specific R1881 binding capacity of androgen receptor protein mutated on amino acid position 868

construct	specific R1881 binding sites (fmoles/mg protein)
pAR0	165 ± 58 (3)
T868A	205 ± 79 (3)
T868C	131 ± 63 (3)
T868S	169 ± 66 (3)
T868K	nd (3)
T868Y	nd (3)
T868D	nd (2)

Hormone binding was determined in whole cell fractions of transfected COS cells at 37 °C using 10 nM (^{3}H) R1881. Non specific binding was determined using a 200-fold molar excess unlabeled ligand. Values are the mean ± SD. The number of determinations is in parenthesis.
nd = not detectable.

completely (Table 2). Similar results were obtained in a cytosol binding assay at 4 °C (data not shown).

To investigate the ligand binding specificity of the wild type and the mutant androgen receptors expressed in COS cells, competition experiments of (^{3}H)R1881 labeled receptor with the synthetic progestagen R5020 and with estradiol were performed in cytosol fractions. At 1-fold molar excess, both R5020 and estradiol as compared to R1881, did not markedly influence specific R1881 binding in the case of the wild type receptor protein, and only partly in case of the alanine, cysteine and serine mutants (Table 3). At 100-fold molar excess of competitor, the cysteine mutant resembled the wild type androgen receptor protein while the serine mutant displayed a pattern comparable with the alanine (LNCaP) androgen receptor mutant (Table 3).

The transactivating potential of the wild type androgen receptor and the receptors mutated at position 868 were tested in HeLa cells in a co-transfection assay with the pG29GtkCAT reporter gene which can function as an androgen responsive gene. The mutant receptors that did not demonstrate specific R1881 binding were also unable to

TABLE 3: Competition for R1881 binding by androgen receptor proteins mutated on amino acid position 868 by different ligands

	(^{3}H)R1881 bound, % of control					
	R1881		R5020		E_2	
	1-fold	200-fold	1-fold	100-fold	1-fold	100-fold
pAR0	49	0	99	12	103	35
T868A	32	4	67	0	74	13
T868C	51	3	89	8	94	47
T868S	47	0	77	0	90	9

Androgen binding was determined in cytosol fractions of transfected COS cells at 4 °C using 10 nM (^{3}H)R1881.
Competition of R5020 and E_2 for specific R1881 binding was determined using different molar excesses of cold competitor. One of three typical experiments is presented. Percentages are the average of duplicate determinations. (^{3}H)R1881 bound in the absence of competitor was set at 100% for each construct (=control).

TABLE 4: Functional activity, induced by R1881, of androgen receptor proteins mutated at amino acid residue 868

	% CAT activity induced by R1881			
	0 nM	1 nM	10 nM	100 nM
pAR0	-	100	80	91
T868A	-	61	43	41
T868C	-	45	60	25
T868S	-	45	45	35
T868K	-	-	-	-
T868Y	-	-	-	-
T868D	-	-	-	-

Androgen receptor mediated hormone induction of CAT activity determined in HeLa cells after co-transfection with androgen receptor expression constructs and the reporter gene pG29GtkCAT.
Presented data are one of three typical experiments.
Background activity (<1%) is indicated by - .

acquire activation potential under the influence of R1881 (Table 2 and Table 4). The alanine, serine and cysteïne mutants did acquire functional activity in the presence of R1881, but not to the same extent as the wild type receptor (Table 4), despite equal binding capacities (Table 2).

The wild type androgen receptor gains functional activity in the presence of 10-100 nM estradiol, but not at similar high R5020 concentrations (Table 5). This difference however is not a reflection of the relative binding affinities of estradiol and R5020 according to Table 3, indicating that a decreased binding affinity for the receptor (as measured in a competition assay) does not prohibit the generation of a functional receptor-ligand complex. The T868A (LNCaP) mutant responded equally well to R5020 and to estradiol, as has been shown before (3). The T868S mutant receptor was more responsive to both R5020 and estradiol as compared with the T868C mutant receptor protein. The T868C mutant responded better to R5020 compared with the wild type receptor protein (Table 5).

TABLE 5: Functional activity, induced by either R1881, R5020 or estradiol (E_2) of androgen receptor proteins mutated at amino acid residue 868

	% CAT activity induced by					
	nM R1881		nM R5020		nM E_2	
	0	1	10	100	10	100
pAR0	-	100	2	1	20	32
T868A	-	100	64	63	62	62
T868C	-	100	6	18	9	22
T868S	-	100	35	50	58	58
T868K	-	-	-	-	-	-
T868Y	-	-	-	-	-	-
T868D	-	-	-	-	-	-

Functional activity of wild type and mutant androgen receptors co-transfected in HeLa cells with the pG29GtkCAT reporter gene. The CAT activity resulting from incubation with 1nM R1881 was arbitrarily set at 100%.
Presented data are those of one of two representative experiments. Individual determinations were performed in triplicate.
Background activity (<1%) is indicated by - .

DISCUSSION

The ligand binding domain of steroid receptors in general is an independent functioning entity (15). The high degree of homology between the ligand binding domains of the steroid hormone receptors suggests that conserved amino acids are involved in shaping of the hydrophobic pocket and that ligand specificity is determined largely by the non-homologous amino acid residues. The integrety of a domain in the mouse estrogen receptor, corresponding to hAR residues 844-878, may be involved in dimerization. This region consists of hydrophobic residues arranged in a heptad repeat which is conserved in all nuclear receptors (16).

The markedly changed steroid specificity of the human androgen receptor in LNCaP cells, where threonine 868 was substituted for alanine, led to several hypotheses concerning the underlying function of threonine 868. The threonine residue at position 868 is unique for the human, rat and mouse androgen receptor. The human progesterone and glucocorticoid receptor carry a cysteïne at this position and the human estrogen receptor a serine residue (Figure 2). In the human estrogen receptor receptor cysteïne 530 can be covalently labelled by aziridine analogs of estrogens and amino acid residues in this area of the receptor may be involved in discrimination between estrogens and anti-estrogens (17). The equivalent of hAR residue 868 in the mouse and rat glucocorticoid receptor is a cysteïne which can be crosslinked by triamcinolone acetonide (18,19). Although the residues corresponding to hAR amino acid 868 are not totally conserved for a particular receptor within the different species it could be that a cysteïne or serine residue at position 868 would shift the steroid specificity towards a higher binding affinity for progestagens, estrogens or glucocorticoids respectively. In general this does not seem to be the case. Only the relative functional activity of the T868C mutant for R5020 is slightly increased. The effect of glucocorticoids was not analyzed since HeLa cells contain a substantial amount of glucocorticoïd receptors.

All steroid hormone receptors are phosphoproteins (20-24) and it has been shown that basal phosphorylation is indispensable for ligand binding activity of the estradiol

							868						
hAR	Arg	Glu	Leu	His	Gln	Phe	Thr	Phe	Asp	Leu	Leu	Ile	Lys	(862-874)
hPR	Lys	Gln	*	*	Leu	Tyr	Cys	Leu	Asn	Thr	Phe	*	Gln	(885-897)
hGR	Glu	Asn	*	Leu	Asn	Tyr	Cys	*	Gln	Thr	Phe	Leu	Asp	(730-742)
hMR	Ser	Asp	*	Leu	Glu	*	Cys	*	Tyr	Thr	Phe	Arg	Glu	(936-948)
hER	Gly	Met	Glu	*	Leu	Tyr	Ser	Met	Lys	Cys	Lys	Asn	Val	(521-533)

FIGURE 2.
Amino acid homology of part of the hormone binding domain of the steroid hormone receptors. The amino acid sequence of the human androgen receptor residues 862-874 (hAR, ref 27) was aligned with the sequences of the human progesterone receptor (hPR, ref 28), the human glucocorticoid receptor (hGR, ref 29), the human mineralocorticoid receptor (hMR, ref 30) and the human estrogen receptor (hER, ref 31). Identical amino acids are indicated by asterisks.

receptor (23). The phosphorylation on tyrosine in the steroid binding domain is essential for the activation of hormone binding of in vitro synthesized hER (25). For the LNCaP androgen receptor it has been established that over 90 percent of the phosphorylation sites are localized in the N-terminal part of the androgen-receptor protein (14) but this does not rule out the possibility that in the wild type androgen receptor basal phosphorylation of threonine 868 could be involved in ligand binding and/or specificity. Introduction of the aromatic side chain containing tyrosine, which can also be phosphorylated, or the acidic side chain containing aspartic acid, which mimics the negative charge of a phosphorylated residue, totally inhibited specific androgen binding and these mutated androgen receptors were unable to activate transcription of a CAT reporter gene under the influence of either R1881, R5020 or estradiol. This is also the case when a basic side chain containing lysine was substituted at this position. These data strongly suggest that basal phosphorylation on threonine 868 is not essential for androgen specificity and that the introduction of a charged or relative bulky amino acid at this position is detrimental for androgen binding and receptor functional activity.
The substitution of cysteïne 656 in the rat glucocorticoid receptor by either glycine or serine resulted in mutant receptors with a higher affinity for glucocorticoids compared to the wild type receptor accompanied by a decreased relative affinity for cross-reacting steroids (26). The results in this study indicate that a threonine residue at position 868 limits the ligand specificity to androgens and that the introduction of either an alanine, a serine or a cysteïne at this position leaves the androgen binding capacity intact, but broadens the binding specificity for other ligands, while the introduction of either a lysine, a tyrosine or an aspartic acid destroys the binding capacity for androgens progestagens and estrogens.

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