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Assignment of the ligand binding site of the porcine estradiol receptor to the N-terminal 17 kDa part of domain E

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Ligand-filled porcine estradiol receptor was adsorbed to heparin-Sepharose, from which a 26 kDa fragment was released by papain. Its mass was reduced to a 17 kDa fragment by trypsin. The sedimentation velocities of both estradiol binding fragments increased after reaction with the MAB 13H2. The same antibody identified the denatured fragments on Western blots. The N-terminal 21 amino acid sequence was obtained from the 17 kDa peptide which corresponds to amino acids 303–323 of the human receptor with four amino acids exchanged. This indicates that the ligand binding site resides in the N-terminal 17 kDa portion of domain E.

Estradiol receptor; Steroid hormone receptor; Domain E; Estrogen

1. INTRODUCTION

The steroid binding domain E of the estradiol receptor was deduced from deletion experiments [1]. It extends from amino acids 302 to 552 of the human sequence [1] and is also involved in receptor dimerisation [2], interaction with heat-shock proteins [3], transcriptional activation [4] and may contain the proposed autoproteolytic center [5]. Detailed assignments of the various properties have been pursued by functional analyses of the products of large deletion mutants [1,4] as well as of point mutations [2,6,7]. The search for an estradiol binding site in the large domain can also proceed by analysing the fragments resulting from limited digestions of either unfilled or ligand-filled wild type receptor [8]. The hormone-labelled receptor is of greater promise than the unlabelled one, because the ligand-induced conformational change leaves fewer sites accessible to proteolytic attack [9]. Their number can be further reduced by attaching ligand-filled intact receptor to an insoluble matrix such as heparin-Sepharose, from which a 32 kDa peptide spanning most of domain E [10] is released by traces of endogenous plasmin [11]. This procedure leaves some 10–20% of the initially adsorbed receptor bound to the matrix. The recovery of the balance of estradiol-filled receptor as a 26 kDa fragment by papain, its digestion to 17 kDa by trypsin and the characterization of both peptides are described in this report.

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2. MATERIALS AND METHODS

[(6,7)-^{3}H]Estradiol, spec. act. 2.22 TBq/mmol, obtained from NEN (Dreieich), was diluted with unlabelled steroid to 75–222 GBq/mmol. Trypsin (TPCK treated) and papain were from Sigma. ProBlott membranes and all sequencer reagents were from Applied Biosystems.

2.1. *Monoclonal and polyclonal antibodies*

The antibodies are directed against a 32 kDa estradiol receptor fragment [10] containing the whole of domain E. The monoclonal antibody 13H2 [12,13] was purified from hybridoma supernatant by protein A-Sepharose chromatography. The IgG_1 fraction of the polyclonal anti-estradiol receptor antiserum #402 [10,14,15] was purified by protein G-Sepharose and Mono-Q chromatography.

2.2. *Preparation of receptor fragments*

The papain receptor fragment was obtained by an extension of the method described in [10,16]. All operations were carried out at 4°C in the presence of an excess of estradiol. Briefly, the procedure included the adsorption of ligand-filled receptor from porcine uterine extracts (approx. 20 l) to heparin-Sepharose (1 l) in batch operation and washes of the adsorbent, filled into a 10 × 13 cm column, with 0.2 M KCl buffered with 0.01 M glycylglycine/piperazine-HCl (Smith and Smith buffer, S&S) to pH 7.4, followed by 0.3 M KCl in the same buffer containing 0.05 M dithiothreitol. The column was then short-circuited through a reservoir and recycled for 24 h. After the elution of the first recycling fluid containing the 32 kDa fragment [10], the adsorbent was washed with 0.3 M KCl, 0.01 M S&S, pH 7.4, until OD_{280} reached baseline. The column was next fed with digestion buffer (1 l of 0.3 M KCl, 0.01 M S&S, pH 6.8, 0.002 M DTT containing 5 mg papain) and was again short-circuited for 24 h at a pumping rate of 150 ml/h. The recirculating fluid was then displaced from the column by buffer, and solid $(NH_4)_2SO_4$ was added to the eluate to 40% saturation. The precipitated material was collected by centrifugation, dissolved in 70 ml 0.3 M KCl, 0.01 M S&S, pH 8, dialysed against the same buffer, and the solution stored at 4°C. For tryptic digestion, the papain pool was concentrated 5-fold in a centriprep and 1% (w/w) of trypsin was added. After 24 h at 4°C, the digestion was terminated by the addition of 0.1 mM PMSF. The reaction mixture was stored at 4°C.

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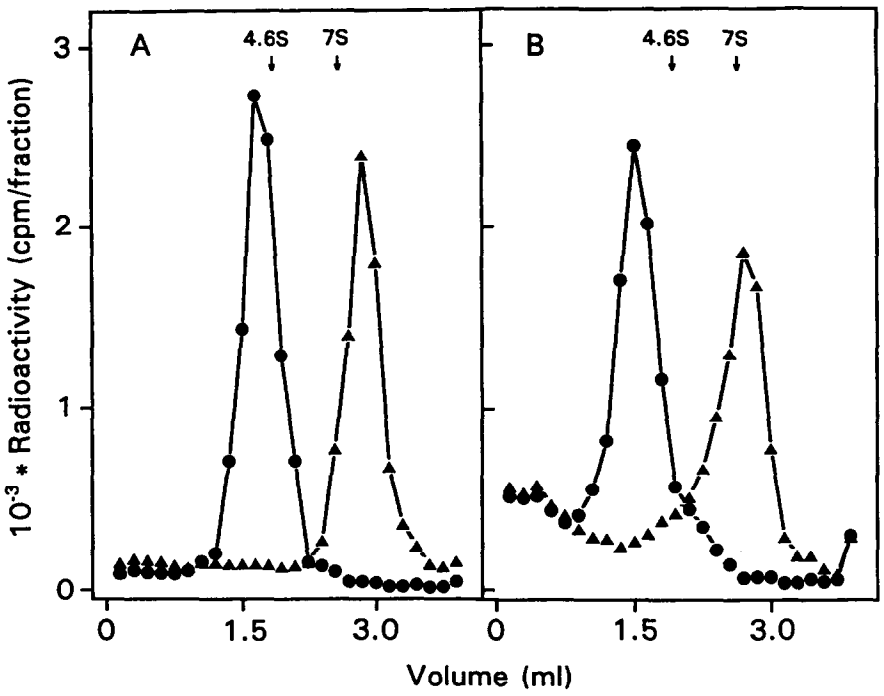

Fig. 1. Sedimentation analysis of the interaction of proteolytic fragments (A, papain; B, papain and trypsin) of porcine estradiol receptor with MAB 13H2. (A) Centrifugation of estradiol receptor fragment released from heparin-Sepharose by papain (papain pool). Density gradients: 200 μl of ● papain digest with ~30 ng of 26 kDa receptor fragment; ▲, as before but incubated for 4 h at 4°C with 5 μg of MAB 13H2 IgG_1 layered on top of the gradient. (B) Density gradient analysis of tryptic estradiol receptor fragment (tryptic digest of the papain pool, Fig. 1A). 200 μl of ● tryptic digest with ~30 ng of 17 kDa receptor fragment; ▲, as before but incubated for 4 h at 4°C with 5 μg of MAB 13H2 IgG_1.

2.3. *Density gradient centrifugations*

Samples of 0.2 ml, treated with dextran-coated charcoal (DCC) [17], were layered on 3.7 ml of linear 5–20% (w/v) sucrose gradients (containing 0.3 M KCl, 0.1 M sodium phosphate, pH 7.5) in SW 60 polyallomer tubes and spun at 257,000 × *g* for 15 h at 2°C. Fractionation was done by upward constant-volume displacements of 0.15 ml each into PE counting vials. Radioactivity was measured in β-counters with ^3H efficiency of 50–55%. Incubations with antibodies were performed as specified in the legends of the figures.

2.4. *SDS-PAGE and Western blotting*

SDS-PAGE was performed in 1.5 mm slab gels as described [18] using 4% acrylamide in the stacking and 16% in the separating gels. Samples were denatured according to [18] and run for 15 h at 60 V, 55 mA, at 8°C. The procedure for Western blotting and immunostaining with immunoglobulin-peroxidase conjugates have been described previously [10,12].

2.5. *Analyses of amino acid sequence*

Proteins were blotted on ProBlott membranes, stained with Coomassie R250 and the bands of interest excised with a scalpel. Pieces of ~ 2 × 4 mm were placed on top of a glas fiber filter and sequenced with an Applied Biosystems 477A/120A microsequenator using standard cycles and gradients.

3. RESULTS

3.1. *Partial purification and characterisation of a 26 kDa estradiol binding receptor fragment*

Our procedure for the isolation of estradiol binding receptor fragments starts with the adsorption of some 50 nmole of ligand-filled receptor from approx. 20 l of cytosol to heparin-Sepharose. After the washing steps (during which 8–10 nmol of receptor are shed), the subsequent recovery of the 32 kDa fragment amounts to 25–30 nmol [10], leaving 5–10 nmol of receptor at the adsorbent. We exposed this tightly bound receptor to a recirculated solution of different endopeptidases (trypsin, chymotrypsin and papain). Papain digestion was most effective and released all the remaining receptor while preserving the steroid attachment. The receptor fragment released by papain has a spec. act. of 230 nmol receptor/g protein with an enrichment from cytosol of ~ 770-fold. The steroid–protein complex sedimented as a single peak in 5–20% sucrose gradients (Fig. 1A) without dissociating during centrifugation. The monoclonal antibody (MAB) 13H2 binds to the estradiol-labelled material and gives rise to a faster sedimenting peak. The receptor fragment obtained by papain digestion migrates as a single 26 kDa peptide in SDS-PAGE which reacts both with MAB 13H2 and the polyclonal antibodies #402 in Western immunoblots (Fig. 2). The mouse MAB F1 raised against estradiol dehydrogenase [19] was used as a control for non-specific attachment (lane 3).

3.2. *Preparation of a 17 kDa estradiol binding receptor fragment*

The 32 kDa receptor fragment [10] and the 26 kDa peptide generated by papain were cleaved by 1% (w/w)

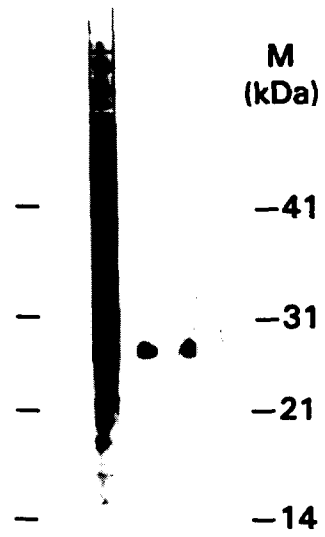

Fig. 2. Association of MAB 13H2 with denatured 26 kDa receptor fragment in papain digest. SDS-PAGE/Western blot of aliquots of papain digest containing ~15 ng of 26 kDa fragment. Lane 1 stained with colloidal gold [25]; lane 2 stained with 13H2 IgG_1-peroxidase conjugate; lane 3 exposed to IgG_1-peroxidase conjugate of MAB F1 directed against estradiol 17β dehydrogenase [19]; lane 4 stained with Fab-S-peroxidase of #402 [10]. Molecular masses of standards (Bio-Rad) are indicated.

of trypsin for 24 h at 4°C. The specific attachment of estradiol is preserved during the digestion. The former gives rise to 3 prominent immunoreactive fragments (identified with MAB 13H2) with MW's of 25, 23 and 17 kDa (not shown). An assignment of the steroid binding pocket to either of them is not possible. In contrast, Fig. 3 shows that the tryptic digestion of the 26 kDa peptide gives rise to a single, immunoreactive peptide of 17 kDa. Its sedimentation velocity is a little slower than that of the papain fragment (Fig. 1B). Again a faster sedimenting complex is seen after incubating the 17 kDa tryptic fragment with MAB 13H2. The 17 kDa fragment, identified by MAB 13H2 in the native and in the SDS-denatured state, must therefore contain the steroid binding pocket.

3.3. *N-Terminal sequence of the tryptic 17 kDa peptide and alignment to sequences of domain E known from other species*

The N-terminus of the 26 kDa peptide in the papain digest is blocked, which occurs probably during the precipitation by $(NH_4)_2SO_4$. Trypsin removes the blocked N-terminus and the 17 kDa peptide produced can be sequenced from the blot for a length of 21 amino acids. A comparison with sequences from the beginning of domain E of human, rat, mouse, chicken and *Xenopus* estradiol receptor (Fig. 4) indicates a high degree of similarity. The only remarkable exchange is that of a proline in the porcine for a leucine in the human sequence. The N-terminal lysine of the porcine peptide is preceded by another lysine in the human sequence. The upstream cut of the 26 kDa papain peptide by trypsin is either at the carboxyl of this lysine or of an arginine. The 17 kDa mass of the tryptic peptide measured by SDS-PAGE fits best with a downstream extension to Lys^{449} of the human sequence for a calculated mass of 16766 Da, which is a little more than half of the mass of region E.

4. DISCUSSION

The isolation of estradiol-filled receptor and receptor fragments from extracts of target tissues saturated with the hormone complicates the determination of their ligand binding affinities by, e.g. Scatchard plot analysis. This apparent disadvantage is compensated for by the ligand-induced conformational arrest, which offers the chance for retrieving the native binding site from receptor digests. Its preservation in the papain 26 kDa and the subsequently obtained tryptic 17 kDa fragment can be concluded from (i) the distinct peaks of macromol-

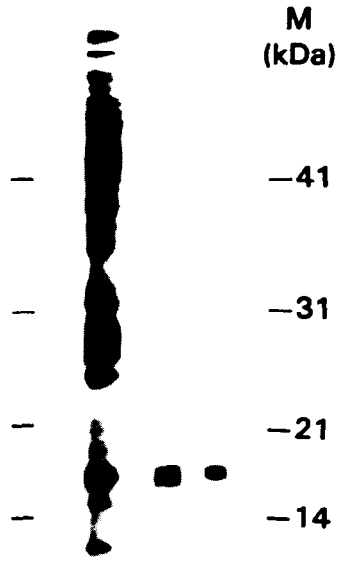

Fig. 3. Association of MAB 13H2 with denatured 17 kDa estradiol receptor fragment in tryptic digest. SDS-PAGE/Western blot of aliquots of tryptic digest of papain pool containing ~ 15 ng of 17 kDa fragment. Goldstaining/immunostaining as in Fig. 2.

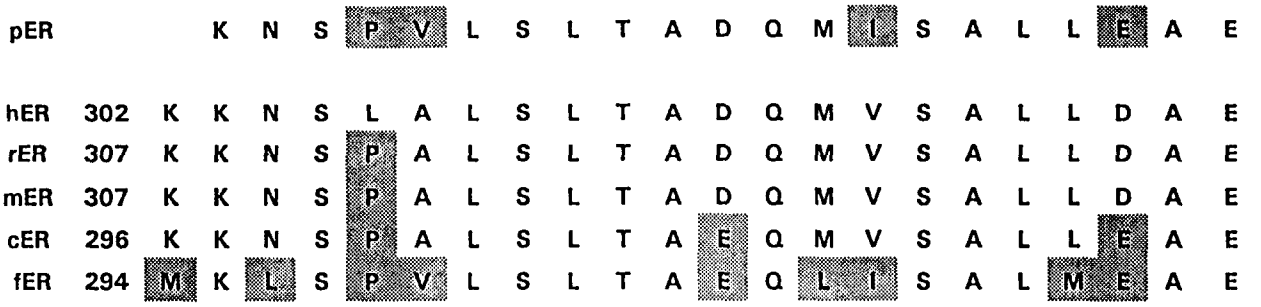

Fig. 4. Comparison of the N-terminal 21 amino acid sequence of the 17 kDa tryptic receptor fragment with the starts of domain E in receptors of other species. Starting positions of proposed domain E [1] of human (hER) [26] aligned to rat (rER) [30], mouse (mER) [29], chicken (cER) [28] and frog (fER) [27] sequences.

ecular-bound estradiol in the density gradients of charcoal-treated solutions, (ii) their shifts to higher velocities after association with the monoclonal antibody 13H2, and (iii) the identification on the SDS-PAGE/immunoblots with the same antibody. The N-terminal sequence of the tryptic peptide, corresponding to the beginning of domain E, localises the estradiol binding site in the N-terminal part of domain E.

Recently, Cys^{530} (which should not be included in the 17 kDa fragment) was identified as the covalent attachment site of the affinity-labeling ligands, Tamoxifen aziridine (Tam-Az) and Ketononestrol aziridine [20]. The importance of this particular Cys, however, has recently been attenuated. Neither one of the mutants C530A and C530S of the MCF_7 receptor showed a lesser affinity for estradiol binding and for (presumably Cys specific) covalent attachment of both affinity-labeling compounds [6]. This also raises the question of how previous studies on the proteolysis of the receptor, which used covalently attached Tam-Az for the identification of fragments [21, 22], can be interpreted. At least one cysteinyl side chain of the estradiol receptor is indispensable for estradiol binding [23]. This must be one of the 3 half cystines 381, 417 and 447, of the human sequence (out of 4 in domain E) which are preserved in the 17 kDa tryptic pig receptor fragment described here (unpublished observation).

The assignment of the binding site for estradiol to the upstream half of domain E apparently also disagrees with data obtained by Fawell et al. [2] from a panel of point mutations in the downstream half of domain E (amino acids 507–537) of the mouse estradiol receptor. This group reported major impairments of steroid binding ability, dimerisation and DNA binding. The Katzenellenbogen group [24] synthesized a 42-amino acid peptide encompassing amino acids 510–551 of the MCF_7 receptor, which, however, bound neither estradiol nor Tam-Az. This negative direct probing does not necessarily rule out the importance of amino acids 507–537 for high affinity estradiol binding. Some kind of long-range cooperativity might be responsible for the differing functional assignments, as concluded from the analysis of the estradiol-filled niche of wild-type porcine receptor fragments in comparison to the results of studying the affinities for estradiol and of other functions in receptor mutants.

The upstream half of domain E from Lys^{303} to Lys^{449} of the human sequence contains 7 arginyl and 3 lysyl peptide bonds. All of them are conserved in the porcine sequence (unpublished observation) but are not susceptible to attack by trypsin under the mild cleavage conditions chosen to retain the steroid bound. The question of whether one linear segment of the 17 kDa peptide or several contribute to the niche formed for estradiol, therefore, remains presently unanswered. However, our results leave no doubt that approximately half of the steroid binding domain E suffices for retaining bound ligand.

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