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Completion of the Amino Acid Sequence of the C-Terminal Half of the Porcine Estradiol Receptor by Edman Degradation: Reconfirmation of the Absence of O-Linked Sugars and Phosphates

Hubert H. Thole and Peter W. Jungblut[1]

Max-Planck-Institut für Experimentelle Endokrinologie, Postfach 610309, D-30603 Hannover, Germany

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The peptide A_{569}–Y_{582} of the porcine estradiol receptor containing the missing sequence T_{570}–M_{581} (1) was isolated and sequenced. The 4 seryl- and 2 threonyl-PTH amino acids were recovered in normal yields, excluding their posttranslational modification and reconfirming the absence of O-glycosylation and O-phosphorylation in H_{267}–I_{595}. © 1996 Academic Press, Inc.

We previously described the cDNA-derived sequence of the porcine estradiol receptor, which was proven for the C-terminal half (H_{267}–I_{595}) by Edman sequencing except for T_{570}–M_{581} (1). This dodecapeptide contains 4 seryl and 2 threonyl side chains. Since all other seryl- and threonyl (as well as tyrosyl) residues of the receptor half were found unmodified, a complete absence of O-linked sugars or phosphates could only be concluded from mass reading (SDS-PAGE) and calculation. We have now succeeded in recovering the missing peptide in quantities sufficient for analytical proof.

MATERIALS AND METHODS

The C-terminal "32" kDa estradiol receptor fragment was purified at 0–4°C. Parts of the procedures have been previously published (1,2). Uteri from prepubertal pigs were collected, minced and mixed with two volumes of 10 mM piperazine hydrochloride, 10 mM glycylglycine (S&S) buffer pH 7.4 containing 1 mM EDTA. After homogenization (3) and centrifugation (75 min) at 11,000 × g, the 'cytosol' was incubated with 1 ml/l of a 2×10^{-5} M (^{3}H) estradiol solution and stirred with heparin-Sepharose. The matrix was filled into a glass column and washed consecutively with S&S buffer pH 7.4 containing 0.3 M KCl and with ≈1 volume of S&S buffer pH 7.4 containing 0.3 M KCl and 0.05 M DTT (until OD reached the baseline). The column was then shortcircuited for 24 h at 250 ml/h. The recirculating buffer containing the "32" kDa estradiol receptor fragment was recovered and the eluate supplemented with 20 mM methylamine and 0.5 M urea. The solution was applied overnight at 35 ml/h to a 2.5 × 5 cm column of mab 13H2 (4) linked to tresyl-agarose. [The purified 13H2 IgG_1 was coupled at a concentration of 5 mg IgG_1/ml matrix.] The immunoadsorbent was washed with a) 0.1 M $NaHCO_3$, 20 mM methylamine buffer pH 9, containing 2 M urea b) 100 mM S&S buffer pH 7.4 and c) 0.5 M $MgCl_2$ in 20 mM S&S buffer. The "32" kDa estradiol receptor fragment was then eluted with ≈30 ml of 50 mM S&S buffer pH 7.4 containing 2.5 M $MgCl_2$ and immediately buffer exchanged on a Sephadex G25 column equilibrated in 0.5 M TRIS pH 8.2.

Some 5 nmoles (160 μg) of the pure protein were concentrated to 3 mg/ml and incubated with 8μg (0.15mg/ml) endopeptidase Lys-C (Achromobacter lyticus E.C. 3.4.21.50, Wako) at pH 9, 0–4°C for 18 hrs (2). Cleavage was stopped by the addition of DTT (1 mM) and Guanidinium/HCl (6 M). The digest was adjusted to pH, 2.5 with 25% TFA and applied to a Phenyl RP HPLC column (PH 300, 30 × 2.1 mm, 7μm, 300A, Brownlee) operated with narrow-bore 140B solvent delivery module, 785A absorbance detector (Applied Biosystems/-BAI). Argon-purged solvents were used: A = 0.1% TFA in H_2O; B = 60% acetonitrile, 20% 2-propanol and 0.085% TFA in H_2O; flow rate 100 μl/min; UV monitoring at 215 nm; linear gradients: 1–10% B for 1 min, 10–20% B for 15 min, 20–50% B for 50 min, 50–80% B for 15 min, 80–99% B for 10 min, 99% B for 10 min. Peptides were sampled manually, frozen and stored at −80 C. Selected peaks were concentrated in a Speed Vac; aliquots were neutralised, adjusted to 1% SDS, heated for 5 min at 95°C and subjected to SDS-PAGE; immunoblotting with antireceptor mab's 13H2 (4,5), HT65 (6), H222 (Abbott) and polyclonal ab #402 (7); other aliquots were subjected to Edman sequencing on a pulsed liquid phase sequenator Model 477A (Applied Biosystems) with 120A on-line HPLC system as described (1). The major portion of the 9 kDa fragment (N-terminus N_{532} VVP. . .) was denatured with 0.1% SDS and heating to 95°C for 5 min. The sample was then diluted 1:5 with 100 mM $(NH_4)_2CO_3$ buffer pH 7.8

[1] To whom correspondence should be addressed. Fax: 511/5359 203.

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and digested with chymotrypsin (sequencing grade, Boehringer Mannheim) (1:20) at 20°C for 18 hrs. The concentrated digest was adjusted to pH 2.5 with 25% TFA and passed in sequence over a Brownlee AX-300 anion exchanger precolumn and C18 RP column (20 × 1.6mm, 5μm, 300A, VYDAC). Its components were separated by gradient elution as described above.

RESULTS AND DISCUSSION

The estradiol-filled "32" kDa C-terminal receptor fragment was enriched by the heparin-sepharose technique (7) and purified to homogeneity by adsorbtion to a mab 13H2 immunoaffinity column as previously published (1,2) except for a speedy adsorbtion to the heparin-matrix and the inclusion of EDTA in all buffers. While EDTA inhibits most protein-seryl/threonyl phosphatases (PP2a,b,c; 8), heparin blocks protein-tyrosyl phosphatases as well as PP of type1 (8,9). The survey of purification is shown in Tab. 1. Calculated on a MW of 37.3 (1), the estradiol receptor fragment isolated is better than 99% pure. It appears as a homogenous band on (silverstained) SDS-gels as was previously shown for Coomassie-stained PVDF blots (2).

The pure receptor can be cleaved to four major fragments with calculated MW of 17, 9, 7 and 5 kDa on the Western Blot by endopeptidase Lys-C (2). We have now developed a method for separating and recovering these Lys-C fragments in good yields on a wide pore reverse phase phenyl HPLC matrix. The results are delineated in the flow sheet (Fig. 1). The missing sequence was found in the 9 kDa fragment (N-terminus N_{532}VVPL. . .), isolated from the Phenyl RP column at 55 min retention time. While CNBr processing and Glu-C digestion of this fraction proved unsatisfactory, digestion with chymotrypsin after SDS-denaturation produced 5 peptides separated by C18 RP HPLC [after removal of the anionic detergent with the aid of an anion exchange precolumn (10)]. The peak eluted at 31 min was identified as A_{569}TSGSTPSHSLQMY. Since all PTH aminoacids were recovered in normal yields, a glycosylation or phosphorylation of the 4 seryl- and 2 threonyl side chains can be safely excluded, which completes the analytical proof for H_{267}–I_{595} (1). The formerly suspected glycosylation of the estradiol receptor by short-chain hyaluronidase moities (11), therefore, does not exist in this part of the protein and likely not at all.

Testis hyaluronidase was first used by us for breaking the jellies formed by some uterine cytosols. This was accomplished, but in addition a dissociation of the "9S" receptor to "4S" (monomer) and "5S" (dimer) forms and a reduction in the electrophoretic mobility of "microsomal" receptor was observed. We suspected a contamination of the commercial product with proteases (e.g. acrosin) and therefore repeated the experiments with eliminase from streptomyces hyaluronolyticus, claimed protease-free by the supplier Seikagaku Ltd, Tokyo. Incubations of receptor-containing extract with 27 TRU/ml (turbidity reducing units), for 1 hr at 30°C gave virtually identical results. However, the slow passage (0.65ml/h) of an enzyme bolus (333 TRU/ml) at 0–4°C over a receptor-saturated heparin-sepharose column (2.5 × 48 cm) released "microsomal" type receptor well ahead of the enzyme (12). A reinvestigation of the enzyme preparation for protease content with ^{14}C-labelled Ehrlich ascites proteins at enzyme/substrate ratios of 30:1 revealed the presence of a seryl-type endopeptidase (13). Attempts to remove the trace of con-

TABLE 1
Purification of "32" kDa Estradiol Receptor Fragment from Porcine Uteri

Material /step	Volume (ml)	Receptor (nmol)	Recovery (%)	nmol/g of protein	Enrichment (-fold)
Cytosol	19600	49.2		0.3	
Heparin–Sepharose	630	26.5	53.8	65	217
mab 13H2-column	46	21.7	44.1	26790	89300

Concentrations of the receptor fragment were determined from [^{3}H]estradiol binding assuming a 1:1 ratio. The precise MW of the fragment is 37.3 kDa on calculation (1).

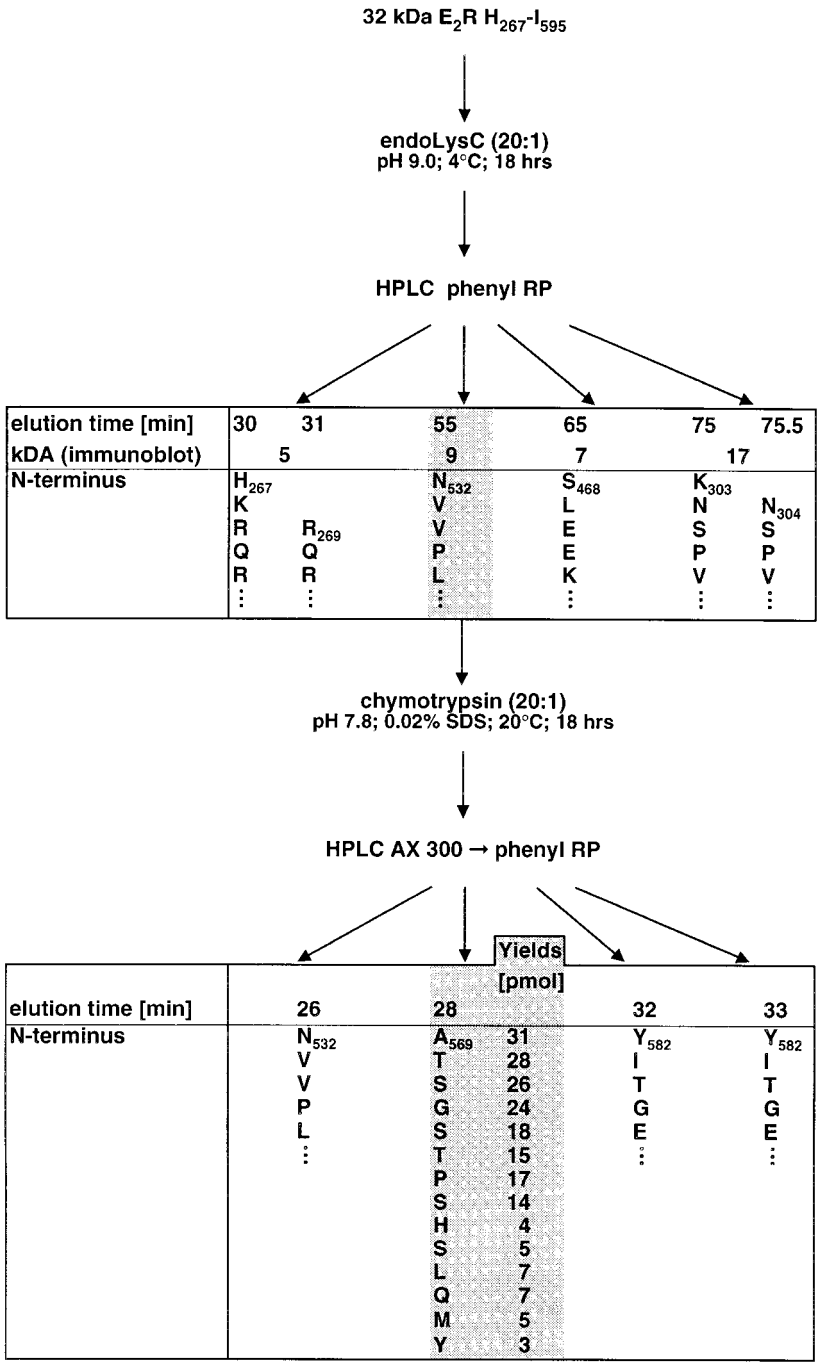

FIG. 1. *Isolation and sequencing yields of peptide A_{569}–Y_{582}.*

taminants were abandoned because of cost and frustration. It must be suspected that the extent of contamination varies with batches as was shown by Yen and Simons (14) who proved the absence of a posttranslational glycosylation of the rat glucocorticoid receptor.

The now established yields of seryl-, threonyl- and tyrosyl-PTH amino acids throughout H_{267}-

I_{595} also exclude the presence of phosphate esters in the analyzed material. It could of course be argued that in-vivo existing phosphates might be hydrolysed by admixed phosphatases over the course of isolation. However, such dephosphorylation in the presence of inhibitors like EDTA or heparin most likely would not go to completion. Any remaining phosphoryl-sidechains of peptides like the A_{569}TSGSTPSHSLQMY one would almost certainly change the overall hydrophobicity to the extent, that an additional peak should appear on the reversed phase column which was not observed. So far phosphorylation of the estradiol receptor has been detected mainly at certain seryl-residues in domains A and B (15,16) and phosphoserin or -threonin has never been observed within domain E or F which is in accordance with our results. There are other studies which indicate that phosphorylation in the vicinity of the ligand-binding niche at Tyr_{537} is essential for steroid binding (17). Since our isolation-protocol employs estradiol-tagged receptor and since the complex survives a number of proteolytic cleavages, one would then have to assume that although the phosphorylation of certain aa-residues is required for the high affinity attachment of estradiol, their persistence is not for maintaining the complex. The unlikelihood of this assumption is supported by our stripping/refilling experiments of phosphate-free fragments of domain E (18).

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