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CHEMICAL CHARACTERIZATION BY PROTEIN SEQUENCE ANALYSIS OF THE BOVINE ESTROGEN RECEPTOR

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SUMMARY Tryptic peptides generated from bovine estrogen receptor have been fractionated and purified using microbore column high performance liquid chromatography. Sequence analysis performed on six of these peptides, derived from diverse structural regions of the receptor protein, yielded 73 unique assignments corresponding to approximately 12% of the molecule. The amino acid sequences of these peptides displayed a high degree of similarity with corresponding sequences from estrogen receptors of mammalian origin, but were only moderately conserved in receptors from non-mammalian species. The sequenced residues of one tryptic peptide, positioned in the estrogen binding domain, were fully conserved in all estrogen receptors.

Our laboratory has previously described an affinity chromatography-based method for purifying the molybdate-stabilized, 9-10S untransformed estrogen receptor from calf uterus (1). This heteromeric receptor complex is composed of receptor subunits of relative molecular mass (M_r) 65,000 (65K) associated noncovalently with a 90K heat shock protein (hsp 90) which does not bind steroid (2). Similar component structures have been established for the steroid receptors of chick oviduct (3,4), the heteromeric 8S form of the progesterone receptor from rabbit uterus (5) and the untransformed glucocorticoid receptors of uncultured cells from mouse (6,7).

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In two previous publications we reported on the sequential use of preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electroelution and microbore high performance liquid chromatography (HPLC) techniques to isolate in pure form the bovine hsp 90 component from affinity chromatography-purified estrogen receptor extracts (8, 9). Direct amino-terminal sequence analysis of the protein and sequence determination of peptide fragments generated by trypsin digestion demonstrated regions of high similarity between bovine hsp 90 and a number of previously identified heat-shock proteins (8, 9). We have now employed a similar strategy for the chemical characterization, by sequence analysis, of the estrogen binding subunit. In this report we describe the amino acid sequences of six tryptic peptides derived from the bovine estrogen receptor. These sequences display varying degrees of similarity with the cDNA-deduced amino acid sequences of estrogen receptors from five other species (10-15). One peptide, which by sequence alignment is placed in the putative estrogen binding domain of the protein, is fully conserved in all six estrogen receptors.

MATERIALS AND METHODS

Estrogen Receptor Purification

Bovine estrogen receptor was purified, as previously described (1), by affinity chromatography of calf uterine cytosol. The method, which utilizes sodium molybdate in purification buffers, isolates estrogen receptor associated with a major bovine heat shock protein (hsp 90) (1,8,9) and provides receptor preparations of 5-20% purity. As an aid to receptor detection during preparative SDS-PAGE approximately 1% of the affinity chromatography-purified receptor was recovered and tagged covalently with [^{3}H]tamoxifen aziridine (specific activity 23 Ci/mmol, Amersham) (18). Receptor from batches of these purified extracts was refined to homogeneity by preparative SDS-PAGE (8). The separated proteins were visualized by Coomassie Brilliant Blue staining (19) and the receptor band corresponding to [^{3}H]tamoxifen aziridine labeled protein was excised. The protein was recovered from the polyacrylamide gel by electroelution in 50 mM NH_4HCO_3 containing 0.1% SDS (8) and was shown to be homogeneous by analytical SDS-PAGE (20) after silver stain detection (21).

Preparation of Tryptic Peptides of Estrogen Receptor for Sequence Analysis

The electroeluate (~450 µl) containing purified estrogen receptor (20 µg) was made 10 mM with respect to dithiothreitol and the sample was heated to 100°C for 5 min. After cooling to room temperature the solution was transferred to a polypropylene tube containing 4 mg of recrystallized

iodoacetic acid dissolved in 6 µl of 1 M NaOH (final solution pH 9.0). The sample was kept in the dark at 37°C for 45 min and the S-carboxymethylated (SCM)-estrogen receptor was recovered from this mixture by precipitation with 9 volumes of prechilled (-20°C) methanol. Trypsin (Worthington TRTPCK) (1% w/w) was immediately added and after standing at -20°C overnight the mixture was centrifuged at 16,000 g (Beckman JA-18.1 rotor) for 45 min at -5°C in Beckman microfuge tubes. SCM-estrogen receptor was resuspended in 250 µl of 0.1 M NH_4HCO_3, 2 mM $CaCl_2$ buffer containing 0.02% (v/v) Tween 20 (Pierce Chemical Co.). An additional aliquot of trypsin (1% w/w) was added and the mixture was incubated at 37°C for 24 h.

Purification of Tryptic Peptides for Sequence Analysis

The tryptic digest of estrogen recpetor was fractionated by microbore chromatography employing a two-dimensional purification procedure (16, 17). Selected tryptic peptides were subjected to N-terminal sequence analysis as previously described (8, 9).

RESULTS AND DISCUSSION

Covalent labeling of the estrogen receptor with [^{3}H] tamoxifen aziridine in the endstage of affinity chromatography purification proved to be most useful for identifying the receptor protein after electrophoretic separation on preparative SDS-PAGE gels. After recovery of the protein from the excised gel by electroelution analytical SDS-PAGE showed a single band for the purified sample with a M_r = 65,000 (Fig. 1). This corresponds closely to most published molecular weight values for the estrogen receptor present in a number of target tissues (10-15, 18, 22).

The tryptic digest of SCM-estrogen receptor was fractionated by microbore RP-HPLC in low-pH (0.1 % v/v) aqueous trifluoroacetic acid (pH 2.1) mobile phase into a complex pattern of peptide-containing peaks (Fig. 2A). Fractions 1-5 (see Fig. 2A) were selected for further purification using a second chromatographic step. Chromatographic conditions were as described in Fig. 2A except that the mobile phase was 1% (w/v) aqueous sodium chloride, pH 6.5 (see Fig. 2B, 2C). These refinements yielded six peptides (T_1-T_6) for sequence analysis (Fig. 3A). Figures 2B and 2C show the peptide profiles resulting from rechromatography of peak fractions 4 and 5. The multiple forms of peptides T_4 and T_5 (see different retention times in Fig. 2) are due, presumably, to oxidation of methionine and tryptophan residues.

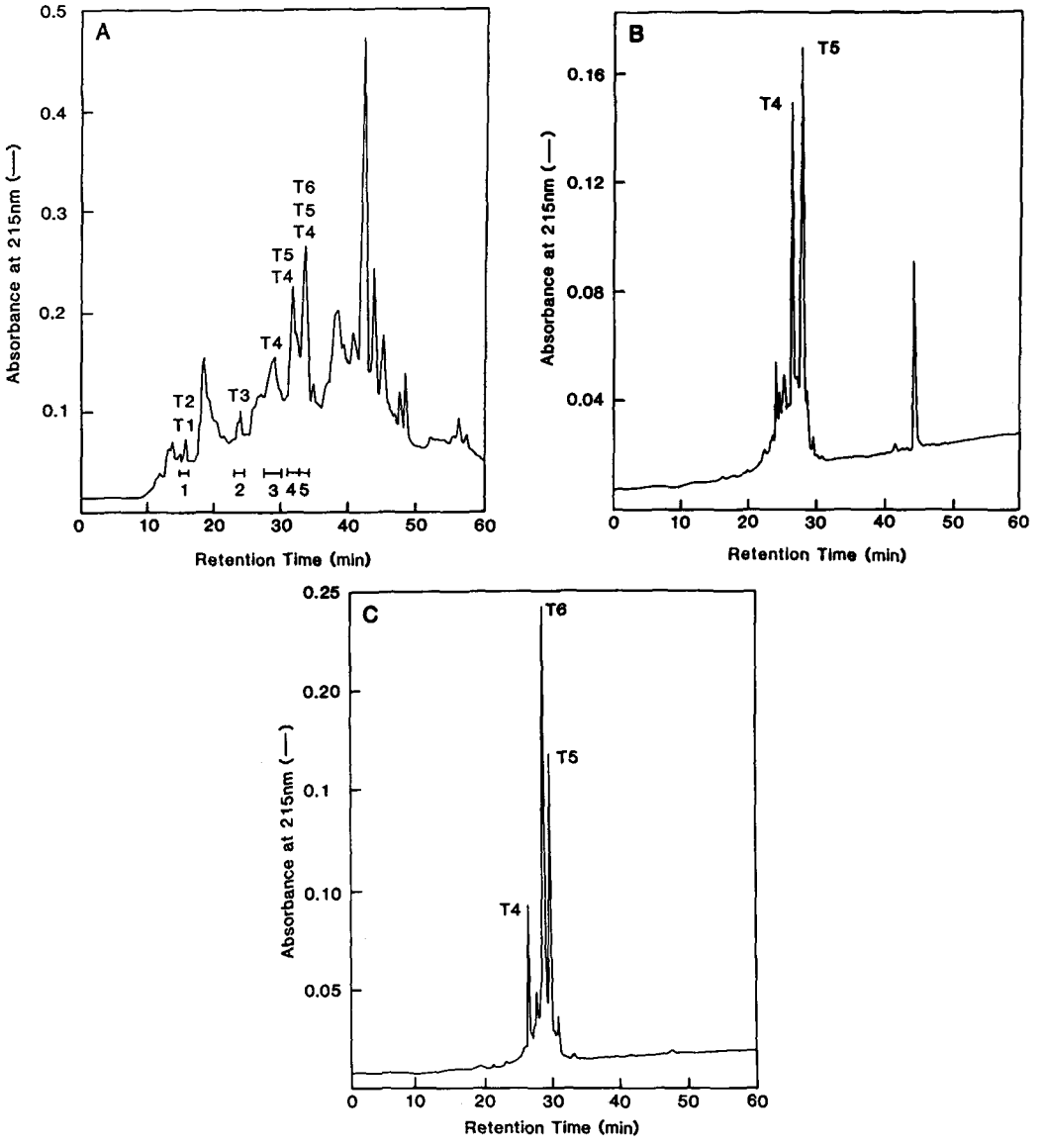

Figure 2A Separation of tryptic peptides of SCM-estrogen receptor by microbore RP-HPLC. Chromatographic conditions: column, Brownlee RP-300 (30 x 2.1 mm i.d.), linear 75-min gradient from 0 to 100% B, where solvent A was 0.1% (v/v) aqueous trifluoroacetic acid and solvent B was 75% acetonitrile/25% water containing 0.085% (v/v) trifluoroacetic acid, flow rate, 100 µl/min, column temperature, 45°C. Peaks (100-150 µl) were collected manually in Eppendorf tubes and stored at -10°C prior to further manipulation.

2B Rechromatography of peak fraction 4 from Fig. 2A by microbore RP-HPLC. Chromatographic conditions: column, Brownlee RP-300 (30 x 2.1 mm i.d.), linear 50-min gradient from 0 to 50% B, where solvent A was 1% (w/v) aqueous sodium chloride pH 6.5 and solvent B was 100% acetonitrile, flow rate, 100 µl/min, column temperature, 45°C.

2C Rechromatography of peak fraction 5 from Fig. 2A by microbore RP-HPLC. Chromatographic conditions were identical to those in Fig. 2B.

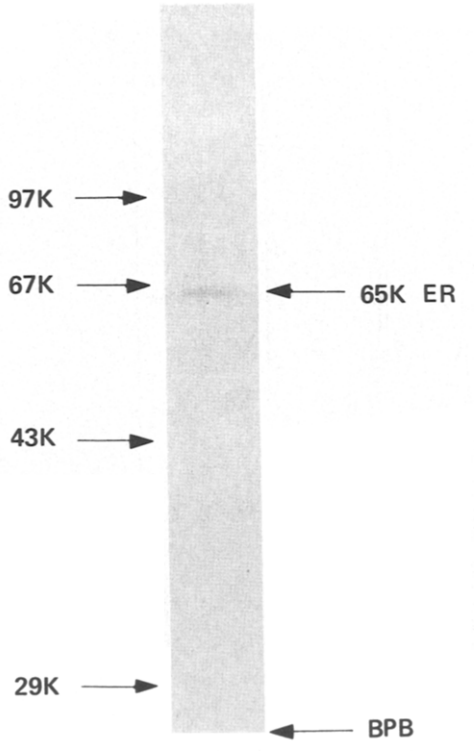

Figure 1 Analytical SDS-PAGE of bovine estrogen receptor recovered from preparative SDS-PAGE by electroelution. Proteins used were silver stained (21) and protein standards used were: 97 K, phosphorylase b, 67 K, bovine serum albumin, 43 K, ovalbumin, 29 K, carbonic anhydrase. BPB-bromophenol blue.

As expected the amino acid sequences of the tryptic fragments derived from the bovine estrogen receptor (T_1-T_6) share structural similarities with the sequences published for estrogen receptors from five different species (10-15) (Fig. 3A). Greatest amino acid sequence similarity was with human estrogen receptor (10,11) which displayed 81% identity with the sequenced residues (Fig. 3A). A slightly less extensive similarity (74%) was observed between the peptides and the estrogen receptors from mouse (15) and rat (13), while the corresponding regions of chicken (12) and frog (14) estrogen receptors were only 60% and 47% identical, respectively (Fig. 3A).

All estrogen receptors have been shown to have a structural organization similar to that initially described for human and chicken

A

Peptide T2													
bER		Y	L	E	N	E	S	S	G	Y	A	V	R
hER	131						P				T		
mER	135						P		A				
rER	136						P		A				
cER	125						Q	G	S	F	G	M	
fER	126				S		Q	G	T	F			

Peptide T1															
bER		E	A	G	P	P	A	Y	Y	R	P	N	S	D	N
hER	143							F							
mER	147	D	T					F			S				
fER	148	D	T					F			S				
cER	137			A				F				S			
fER	138			A			T	F			S	S			

Peptide T5													
bER		A	A	N	L	W	P	S	P	I	M	I	K
hER	288									L			
mER	293									L	V		
rER	293									L	V		
cER	282		P	T			T			L	V	V	
fER	284	S	I	W	V	N			V	K	S	M	

Peptide T4										
	E	L	V	H	M	I	N	W	A	K
353										
351										
358										
347										
345										

Peptide T6																
	L	H	A	P	A	N	F	G	S	A	P	P	E	D	V	N
549					T	S	R		G		S	V		E	T	D
545						S	R	M	G	V				E	P	S
554						S	R	M	G	V				E	P	S
543						A	R	S	A			M		E	E	
541	I		T		K	D	K	T	T	T	Q	E			S	R

Peptide T3										
	I	-	T	G	E	A	E	N	F	P
584		-						G		
588		-	P	P				G		
589		-	P	P				G		
577		N	S	K		E		S	M	Q

B

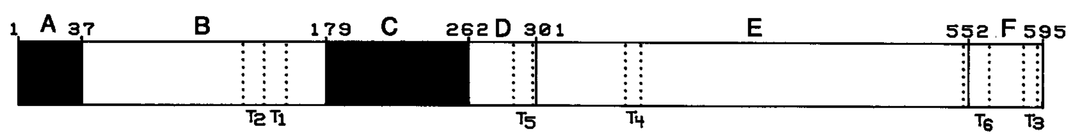

Figure 3A Sequence alignment of bovine estrogen receptor (bER) tryptic peptides with corresponding regions of the estrogen receptors from human (hER) (10,11), mouse (mER) (15), rat (rER) (13), chicken (cER) (12) and frog (fER) (14). Amino acids are identified by their single letter code and numbers represent the position of the amino acid residue in each sequence. Residues identical to bovine receptor amino acids are indicated by blank spaces. For peptide T_3 a gap (-) was introduced for maximal alignment of identical amino acids.

3B Structural organization of the human estrogen receptor (10,12). Bovine estrogen receptor tryptic fragments have been positioned in the structural regions according to sequence alignment with human receptor.

estrogen receptors by Krust et al. (12). Based on sequence homology the receptor can be divided into six structural regions (Fig. 3B). Between species domains A, C and E are highly conserved, while greater variability is displayed by B, D and F (10-15). Alignment of the peptide sequences with the cDNA-deduced amino acid sequence of the human estrogen receptor revealed that the peptides are derived from diverse regions of the bovine receptor protein (Fig. 3B). Peptides T_2 and T_1 are contiguous and are placed towards the carboxyterminus of domain B. Peptide T_5 comes from the D region, close to the dividing line with E, while peptide T_4 originates from the E domain. Peptides T_6 and T_3 are both derived from region F of the protein with T_6 partially overlapping domain E. Peptide T_3 represents the carboxyterminal tryptic peptide of the bovine estrogen receptor.

The homology patterns we have observed for the bovine receptor peptides are consistent with those reported generally for estrogen receptors (10-15). This is particularly evident for peptide T_4 from domain E - the steroid binding domain of the protein (23), and for peptides T_6 and T_3 from the carboxyterminal domain F. The residues of peptide T_4 are fully conserved in all six estrogen receptors (Fig. 3A). A similar trend is observed for the first four residues of peptide T_6 which also reside within the E domain (Fig. 3A,B). The above observations contrast with the generally low level of conservation noted for the residues from region F (part of peptide T_6 and peptide T_3) (Fig. 3A).

Domain E encompasses a stretch of approximately 250 highly conserved amino acids which form the steroid binding hydrophobic pocket in estrogen receptors (10-15). Computer-based studies (24) have defined similar segments within the steroid-binding domains of the estrogen and glucocorticoid (25) receptors and of the α-subunit of $(Na^+ + K^+)$ATPase (26). These studies favour residues 323-395 of the human estrogen receptor as the location of the estrogen binding site (24). Such a

location would place the residues of peptide T_4 close to the centre of the steroid binding region (Fig. 3A).

Region D, a segment containing approximately 40 amino acid residues (10-15), may function as a flexible hinge between the steroid binding (E) and DNA-binding (C) domains of the receptor (12) (Fig. 3B). While the amino acid residues in this region of the human estrogen receptor are 80% identical with the corresponding proteins from mouse and rat (10,11, 13, 15) less extensive homologies exist with the receptors from the non-mammalian species chicken (38%) and frog (30%) (12, 14). Apart from one conservative replacement (leucine for isoleucine) in the human receptor, and the same alteration plus a second substitution (valine for methionine) in the mouse and rat, the sequence of peptide T_5 is all but identical to all three proteins (Fig. 3A). This result from the bovine estrogen receptor therefore, extends the observed differences in region D, between the receptors of mammalian and non-mammalian origin.

The amino acid sequence in the contiguous peptides T_2, T_1 shows similarities with all published estrogen receptor sequences (Fig. 3A). Again, however, the residues were more highly conserved in the human, mouse and rat (Fig. 3A). Although a function for region B in estrogen receptors has not yet been defined (12) White et al., (15) have suggested that two small conserved regions (named B_1 and B_2), existing near the amino- and carboxytermini of the variable B domain, might be involved in the modulation of transcriptional activity. The first five residues of peptide T_2 are included in the B_2 region and are totally conserved in estrogen receptors except for a single substitution (serine for asparagine) in the frog (Fig. 3A).

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