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THE AMINO-TERMINAL SEQUENCE OF THE 85-90K NONHORMONE BINDING COMPONENT OF THE MOLYBDATE-STABILIZED ESTRADIOL RECEPTOR FROM CALF UTERUS

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SUMMARY The first six N-terminal amino acid residues of the 85-90K non-estrogen binding component of the calf uterine, molybdate-stabilized estradiol receptor have been determined by Edman degradation. After affinity chromatography of the stabilized receptor oligomer, the 85-90K unit was purified to homogeneity by preparative gel electrophoresis using electroelution for protein recovery. Inverse-gradient high performance liquid chromatography provided the 85-90K protein suitable for amino-terminal sequence analysis.

Estrogen responsive tissues such as uterus contain estrogen receptors (1,2). Some 25-30% of human breast cancers also depend on steroid sex hormones for their continued proliferation (3). These tumours are responsive to endocrine manipulation (3).

Structure analysis of the estradiol receptor is required for understanding the mechanisms by which this important regulatory protein exerts its effects

Abbreviations: M_r, Relative molecular mass; PMD buffer, 10mM potassium dihydrogen orthophosphate containing 20mM sodium; molybdate and 1mM dithiothreitol adjusted to pH 7.4 at 22°C; SDS, Sodium dodecyl sulphate; EDTA, Ethylenediaminetetraacetic acid; HPLC, High performance liquid chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PTH, Phenylthiohydantoin; Meth A, methylcholanthracene-induced sarcoma.

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on gene transcription. The cloning and sequencing of the human and chicken estrogen receptors has recently been reported (4-6).

We have previously reported on the purification of the calf uterine estrogen receptor as the oligomeric complex stabilized in vitro by sodium molybdate (7). Preliminary characterization of the complex gave a sedimentation coefficient (9.3S) and a Stokes radius (74 A) which together were used to give a calculated molecular weight of ~300,000 for the structure (7). In this communication we report the purification to homogeneity of an M_r 85-90,000 (85-90K) nonsteroid binding component of the oligomeric estrogen receptor complex and present our results of amino-terminal sequence analysis of this protein.

MATERIALS AND METHODS

Purification of the Oligomeric Estrogen Receptor Complex

The molybdate-stabilized, oligomeric form of the estrogen receptor was purified according to a modification of the procedure described in detail by Atrache et al., (7). Briefly, 200g of calf uterus was homogenized at 4°C in 4 volumes of PMD buffer. Cytosol was treated with heparin-sepharose and the adsorbed protein was recovered with PMD buffer containing 0.4M KCl. The eluate was incubated with 10ml of estrogen-linked affinity resin. After 2h at 4°C the resin was washed as described (7), suspended in 2 volumes of PMD buffer containing 5µM estradiol and incubated overnight at 4°C. The affinity chromatography eluate was dialyzed against 10mM ammonium acetate, pH 7.4 buffer, concentrated in a protein dialysis concentrator (Model 315/320. Pierce Chemical Company, U.S.A.) and lyophilized.

Purification of the 85-90K Nonhormone Binding Component

The lyophilized affinity chromatography-purified estrogen receptor extract was taken up in 700µl of Laemmli SDS sample buffer (10mM Tris.HCl, 1mM EDTA, 1% SDS, 5% β-mercaptoethanol and 20% glycerol, pH 8.0) (8) and heated at 65°C for 15 min. The sample was subjected to electrophoresis on a 10% acrylamide gel (0.75mm x 110mm x 150mm) using the standard Laemmli system with 0.05% thioglycollic acid in the cathode buffer. After separation the proteins were visualized by staining with Coomassie Brilliant Blue (9). The major band corresponding to the 85-90K protein was excised with a scalpel and equilibrated in 0.1M Tris-HCl pH 8.0 buffer containing 5mM dithiothreitol and 0.1% SDS. The protein was recovered from the acrylamide gel slice by electrophoretic elution using 50mM ammonium bicarbonate buffer containing 0.1% SDS (10), and was shown to be homogeneous by analytical SDS-polyacrylamide gel electrophoresis (8) and silver stain detection (11).

Preparation of Purified 85-90K Protein for Sequence Analysis

The recovered extract (600µl) containing approximately 9µg of the 85-90K protein was lyophilized, resuspended in 400µl of water and loaded directly (8 x 50µl aliquots), at a flow rate of 200µl/min, onto a Hypersil octadecylsilica column (5µm particle size, 100 x 2.0mm internal diameter, Hewlett Packard, Waldbronn, FRG). Primary solvent A was 90% n-propanol-10% H_2O and secondary solvent B was 50% n-propanol-50% H_2O containing 0.1% trifluoroacetic acid (v/v). The column was developed with a linear 1.5 min

gradient from 100% A to 100% B at a flow rate of 200µl/min. After 2 min the flow rate was reduced to 20µl/min and maintained for 60 min. Column temperature was 40°C. A Hewlett Packard liquid chromatograph (Model 1090) equipped with a diode-array detector (Model 1040 A) and a Rheodyne sample injector (Model 7125) was used. The column eluant was monitored for ultraviolet absorption at 280nm and fractions were collected manually. Protein eliciting the major peak was collected in a volume of 60µl and applied directly to a polybrene treated (12) glass fibre disc. Amino terminal sequence analysis was performed on an Applied Biosystems gas phase sequencer (Model 470A) and PTH amino acids were analyzed as described by Simpson et al., (13).

RESULTS AND DISCUSSION

Our group has previously described a reliable affinity chromatography-based method for purifying the molybdate-stabilized calf uterine estrogen receptor to apparent homogeneity (7). The purification conditions used ensured the isolation of an oligomeric receptor complex which closely resembles the native intact receptor (7). Our results, which indicated a unique polypeptide of Mr ~89,000 for the purified protein, were consistent with two structural alternatives for the stabilized oligomeric receptor: A tetramer composed of homologous estrogen binding units of M_r = 85-90,000 (85-90K) or a hetero-oligomer containing perhaps two receptor subunits associated with two nonsteroid binding proteins - all of similar size (7,14,15). Recent evidence suggests that the 85-90K protein, observed by our group in highly purified extracts of the molybdate-stabilized receptor, is structurally different from the estrogen binding monomer, but associates noncovalently with the protein as a nonhormone binding component of the oligomeric receptor (T. Ratajczak, M. Comber and R. Hähnel, in preparation). A similar nonhormone binding species (M_r = 90,000) has been shown to be associated with the molybdate-stabilized steroid receptors of chick oviduct (16,17), the heteromeric 8S form of the progesterone receptor from rabbit uterus (18) and the untransformed glucocorticoid receptors of cultured cells from mouse (19,20).

Preparative SDS-PAGE using electrophoretic recovery has been used to purify the 85-90K component from the oligomeric estrogen receptor complex obtained after affinity chromatography. The technique is well suited for the purification and efficient recovery (yields of approximately 90% have been

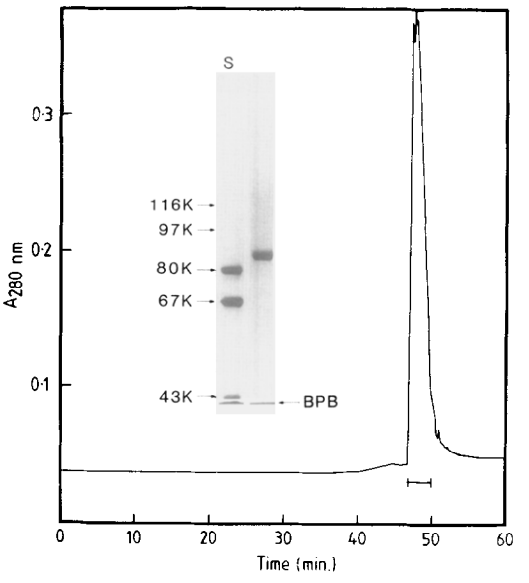

Figure 1. Inverse-gradient HPLC of purified 85-90K receptor-binding component in preparation for sequence analysis. The 85-90K protein extract (400µl), isolated by preparative SDS-PAGE and electroelution, was absorbed onto a 100 x 2.0mm internal diameter Hypersil octadecylsilica column. After column development (see Materials and Methods) the eluant was monitored for ultraviolet absorption at 280nm. The 85-90K protein, recovered manually, in 60µl of eluant was enriched in concentration and free of SDS and electrophoretic contaminants. Inset: analytical slab SDS-PAGE of 85-90K protein purified by preparative SDS-PAGE. Proteins were silver stained according to Merril (11). S, protein standards - 116K, β-galactosidase; 97K, phosphorylase b; 80K transferrin; 67K bovine serum albumin; 43K, ovalbumin.

achieved) of very small amounts of protein and the recovered sample can be used directly for amino-terminal sequence analysis (9). Analytical SDS-PAGE with silver-staining showed a single band for the electroeluted protein with a M_r = 87,000 (Fig.1) in close agreement with previous molecular weight determinations for the protein (7).

Although the electroelution technique is generally applicable for recovering samples suitable for Edman degradation (9) the eluate frequently contains intolerably high levels of SDS and polyacrylamide gel related artifacts. In this communication we describe a further application of inverse-gradient HPLC (21) which allows recovery of electroeluted proteins free of these contaminants. Utilizing this procedure the pure 85-90K protein (~90 pmole) was obtained in a form suitable for sequence analysis (Fig. 1).

Amino-terminal sequence analysis permitted the unambigous assignment of the first six residues (Fig. 2). The analysis was limited to 6 amino acid

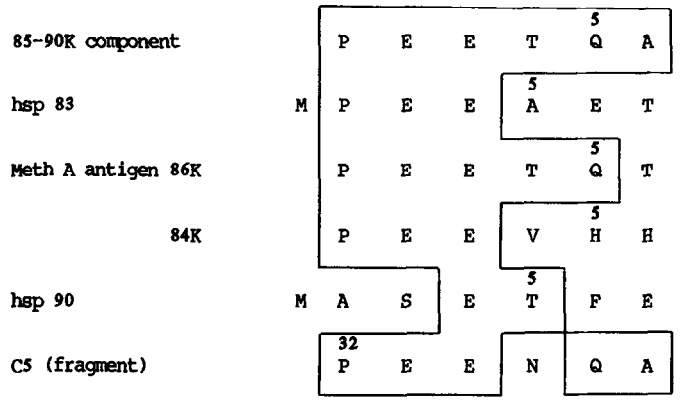

Figure 2. NH_2-terminal sequences of the 85-90K receptor-binding component, Drosophila heat-shock protein hsp83, MethA 86K and 84K antigens and yeast heat-shock protein hsp90 and residues 32-37 of human complement C5 (fragment).

residues since there was a rapid build-up of sequencer derived PTH amino acids. The initial yield during analysis was approximately 25 pmole.

A comparison of the sequence with 3,477 sequences described in the NBRF database[1] revealed a striking homology with residues 32-37 of human complement C5 (fragment) (22). More significantly a high degree of homology was noted between the assigned sequence and the amino-terminal structure published for the M_r 83,000 heat-shock polypeptide (hsp 83) of Drosophila melanogaster (23) (Fig. 2). The 85-90K component was also shown to share N-terminus sequence homology with the heat-shock related protein isoforms (Meth A antigens 86K and 84K) of a mouse tumour-specific transplantation antigen (24) (Fig. 2). A similar comparison with the amino-terminal sequence of the equivalent heat-shock inducible protein of yeast (hsp 90) (25) however showed only limited homology (Fig. 2). Although the short sequence described here for the isolated 85-90K protein precludes a detailed evaluation of the significance of these homologies our results are consistent with the recent identification of the 90K nonhormone binding component of molybdate-stabilized steroid hormone receptors as a heat-shock protein (hsp 90) (18, 26, 27).

[1] Protein sequence database, National Biomedical Research Foundation, Georgetown University Medical Center, 3900 Reservoir Road, N.W., Washington, D.C. 20007.

Hsp 90 is an abundant cytoplasmic phosphoprotein of most eukaryotic cells (26, 28, 29). Recent reports indicate that the association of the protein with steroid receptors is not artifactual and that the resultant oligomeric receptor complexes represent the native untransformed receptors in intact cells (15, 16, 30). Evidence also suggests that biological activation of steroid receptors requires the separation of the 90K component from receptor monomers (20). Biological functions suggested for the hsp 90 - steroid receptor interaction include the mediation of receptor intracellular transport and the regulation of receptor activity by modulating receptor phosphorylation and/or preventing association of receptor to DNA by sterically masking the DNA-binding domain (18, 26, 31).

The receptor-binding protein hsp 90 has previously been implicated in highly stable non-covalent complexes with a group of oncogenic viral tyrosine kinases (26, 31-36) and a similar association of the mammalian hsp 90 with actin has been more recently reported (37). Cloning and nucleotide sequence analysis of heat-shock inducible genes encoding proteins of M_r 80 - 110,000, from different species, may define more clearly the role of hsp 90 and the association of this component with steroid receptors as well as other protein families. Sequence data already obtained indicate considerable homology between the chick heat-shock proteins, hsp 90 and hsp 108 and those of yeast (hsp 90) and Drosophila (hsp 83) (38, 39).

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REFERENCES

1. Jensen, E.V., DeSombre, E.R. and Jungblut, P.W. (1967) Endogenous Factors Influencing Host-Tumour Balance, pp.15-30, University of Chicago Press, Chicago.
2. Evans, L.H. and Hahnel, R. (1971) J. Endocrinol. 50, 209-229.
3. Jensen, E.V. (1981) Cancer 47, 2319-2326.
4. Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.M., Argos, P. and Chambon, P. (1986) Nature 320, 134-139.
5. Greene, G.L., Gilna, P., Waterfield, M., Baker, A., Hort, Y. and Shine, J. (1986) Science 231, 1150-1154.
6. Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J.-M. and Chambon, P. (1986) EMBO J. 5, 891-897.
7. Atrache, V., Ratajczak, T., Senafi, S. and Hähnel, R. (1985) J. Biol. Chem. 260, 5936-5941.
8. Laemmli, U.K. (1970) Nature (Lond.) 227, 680-685.

9. Hunkapiller, M.W., Lujan, E., Ostrander, F. and Hood, E.L. (1983) Meth. Enzymol. 91, 227-236.
10. Stearne, P.A., van Driel, I.R., Grego, B., Simpson, R.J. and Goding, J.W. (1985) J. Immunol. 134, 443-448.
11. Merril, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981) Science 211, 1437-38.
12. Klapper, D.G., Wilde, C.E., III and Capra, J.D. (1978) Anal. Biochem. 85, 126-131.
13. Simpson, R.J., Smith, J.A., Moritz, R.L., O'Hare, M.J., Rudland, P.S., Morrison, J.R., Lloyd, C.J., Grego, B., Burgess, A.W. and Nice, E.C. (1985) Eur. J. Biochem. 153, 629-637.
14. Raaka, B.M. and Samuels, H.H. (1983) J. Biol. Chem. 258, 417-425.
15. Sherman, M.R. and Stevens, J. (1984) Ann. Rev. Physiol. 46, 83-105.
16. Joab, I., Radanyi, C., Renoir, M., Bichou, T., Catelli, M.-G., Binart, N., Mester, J. and Baulieu, E.-E. (1984) Nature 308, 850-853.
17. Dougherty, J.J., Puri, R.K. and Toft, D.O. (1984) J. Biol. Chem. 259, 8004-8009.
18. Catelli, M.G., Binart, N., Jung-Testas, I., Renoir, J.M., Baulieu, E.E., Feramisco, J.R. and Welch, W.J. (1985) EMBO J. 4, 3131-3135.
19. Housley, P.R., Sanchez, E.R., Westphal, H.M., Beato, M. and Pratt, W.B. (1985) J. Biol. Chem. 260, 13810-13817.
20. Mendel, D.B., Bodwell, J.E., Gametchu, B., Harrison, R.W. and Munck, A. (1986) J. Biol. Chem. 261, 3758-3763.
21. Simpson, R.J., Moritz, R.L. Nice, E.C. and Grego, B. (1987) Eur. J. Biochem. in press.
22. Lundwall, A.B., Wetsel, R.A., Kristensen, T., Whitehead, A.S., Woods, D.E., Odgen, R.C., Colten, H.R. and Tack, B.F. (1985) J. Biol. Chem. 260, 2108-2112.
23. Hacket, R.W. and Lis, J.T. (1983) Nucl. Acids Res. 11, 7011-7030.
24. Ullrich, S.J., Robinson, E.A., Law, L.W., Willingham, M. and Apella, E. (1986) Proc. Natl. Acad. Sci. USA 83, 3121-3125.
25. Farrelly, F.W. and Finkelstein, D.B. (1984) J. Biol. Chem. 259, 5745-5751.
26. Schuh, S., Yonemoto, W., Brugge, J., Bauer, V.J., Riehl, R.M., Sullivan, W.P. and Toft, D.O. (1985) J. Biol. Chem. 260, 14292-14296.
27. Sanchez, E.R., Toft, D.O., Schlesinger, M.J. and Pratt, W.B. (1985) J. Biol. Chem. 260, 12398-12401.
28. Riehl, R.M., Sullivan, W.P., Vroman, B.T., Bauer, V.J., Pearson, G.R. and Toft, D.O. (1985) Biochemistry 24, 6586-6591.
29. Kelley, P.M. and Schlesinger, M.J. (1982) Mol. Cell. Biol. 2, 267-274.
30. Baulieu, E.-E., Binart, N., Buchou, T., Catelli, M.-G., Garcia, T., Gasc, J.-M., Groyer, A., Joab, I., Moncharmont, B., Radanyi, C., Renoir, M., Tuohimaa, P. and Mester, J. Nobel Symposium No. 57: Steroid Hormone Receptors: Structure and Function (Eriksson, H. and Gustafsson, J.-A., eds.) pp.45-72, Elsevier Science Publishers, Amsterdam.
31. Ziemecki, A., Catelli, M.-G., Joab, I. and Moncharmont, B. (1986) Biochem. Biophys. Res. Commun. 138, 1298-1307.
32. Brugge, J.S., Erikson, E. and Erikson, R.L. (1981) Cell 25, 363-372.
33. Opperman, H., Levinson, W. and Bishop, J.M. (1981) Proc. Natl. Acad. Sci. USA 78, 1067-1071.
34. Lipsich, L.A., Cutt, J.R. and Brugge, J.S. (1982) Mol. Cell. Biol. 2, 875-880.
35. Courtneidge, S.A. and Bishop, J.M. (1982) Proc. Natl. Acad. Sci. USA 79, 7117-7121.
36. Brugge, J., Yonemoto, W. and Darrow, D. (1983) Mol. Cell. Biol. 3, 9-19.
37. Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H. and Yahara, I. (1986) Proc. Natl. Acad. Sci. USA 83, 8054-8058.
38. Catelli, M.-G., Binart, N., Feramisco, J.R. and Helfman, D.M. (1985) Nucl. Acids Res. 13, 6035-6047.
39. Sargan, D.R., Tsai, M.-J. and O'Malley, B.W. (1986) Biochemistry 25, 6252-6258.