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Purification of antiestrogen binding site by affinity chromatography

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Abstract : Antiestrogen binding protein has been purified from mouse mammary gland microsomal membranes by affinity chromatography on epoxy activated tamoxifen sepharose. The purification achieved was about 200-fold. The molecular weight of the protein as determined by SDS-PAGE was 90,000 daltons. The protein was also immunoprecipitable by anti-prolactin receptor antibody.

Keywords : Affinity chromatography, antiestrogen binding site. Abbrevia tion : ER—estrogen receptor, AEBS—antiestrogen binding site.

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1. Introduction

The role of estrogen noncompeteable microsome site for antiestrogen binding is still unknown. Their presence in both ER positive and ER negative breast cancer samples and normal mammary tissue of rat evoked considerable interest to delineate its normal physiological function. The microsomal lactogenic hormone receptors have been demonstrated in both normal andtransformed mammary tissue. The role of these hormones in etiology of cancer though not clear but their role in normal mammary gland development is well established. Recently we have shown antiestrogens block the prolactin induced growth in ER negative rat lymphoma cell line and lactogenic hormone binding is inhibited by antiestrogens in these cells (Biswas and Vonderhaar 1987, Biswas and Vonderhaar 1989). Antiestrogens showed similar effect on ER positive human breast cancer cell line MCF-7. To study this interaction between AEBS and lactogenic hormone binding site, in present paper we purified and partially characterized the AEBS.

2. Materials and methods

2.1. *Microsomal membrane preparation :*

Mammary glands were isolated aseptically from CH_3/HeN mice lactating for 10 to 12 days and stored at $-20°$ until use. The mammary glands were homogenized

with 8 volumes of 25 mM Tris-HCl, pH 7.4 with 0.3 M sucrose and centrifuged at 15,000 × g for 15 minutes at 4°C. Pellet obtained after centrifugation of the low speed supernatant at 105,000 × g for 60 minutes at 4°C was resuspended in 25 mM Tris-HCl, pH 7.4 to give final protein concentration of 10-20 mg/ml.

For solubilization the microsomal membranes thus obtained were diluted to protein concentration of 5 mg/ml and treated with 0.5% of powdered Chaps (Liscia and Vonderhaar 1982). After centrifugation for 60 minutes at 105,000 × g the resultant supernatant was the solubilized microsomal membrane.

Protein concentrations were determined by the method of Lowry *et al* (Lowry *et al* 1951).

2.2. ^{3}H-*tamoxifen binding assay* :

Binding of ^{3}H-tamoxifen (100,000 cpm/reaction) to the membranes (100-200 μg protein) was determined in triplicates by overnight (16-18 hrs) incubation at room temperature in presence of 25 mM Tris-HCl, pH 7.4 containing 10 mM $MgCl_2$ and 0.1% BSA in total volume of 500 μl with or without 1000-fold excess unlabeled tamoxifen. The reaction was terminated by adding 1 ml of ice cold buffer and the pellet obtained after centrifugation at 1500 × g for 20 minutes at 4°C was the membrane bound hormone receptor complex. The pellet was counted with liquid scintillation spectrometer. The specific binding was determined by substracting non-specific binding (with unlabeled hormone) from total binding (without unlabeled hormone).

2.3. *Preparation of the afinity column* :

The TAM-agarose was prepared by the conventional method (Van Oosbree *et al* 1984) 25 mg of TAM was dissolved in 5 ml of water and 5 ml of dioxane and pH adjusted to 11.0 with 0.1 N KOH and tracer ^{3}H-TAM was added to calculate the coupling efficiency. Freeze dried epoxy activated sepharose 4B was soaked in water for 15 minutes and washed in 300 ml of water and finally equilibrated to pH 11.

For coupling reaction the equilibrated sepharose and TAM solution was mixed so that the total volume is about 35 ml and kept shaking in a glass flask at 37°C for 16 hours. The affinity resin was washed in succession with water, 100 ml of 0.1M $NaHCO_3$ pH 8.0, 0.1M Na acetate pH 4.0, 0.5M formate buffer pH 4.0, 0.1M $NaHCO_3$ with 0.5M NaCl pH 8.0. The gel was then incubated for 4 hours with ethanolamine-HCl pH 8.0 and washed with 50 mM Tris pH 7.4 and stored at 4°–8°C.

2.4. *Purification of AEBS on affinity column* :

TAM-Sepharose

↓

Equilibrated with 50 mM Tris-HCl pH 7.4 containing 2% BSA and 0.5% Chaps.

↓

Mixed with solubilized mouse mammary gland microsomes (2mg/ml protein).

↓

Incubated overnight with 1 mM $PhMeSO_4$.

↓

Gel packed in a column.

↓

Washed with 4 M urea containing 0.1 M NaCl, and 0.5% Chaps.

↓

Eluted with 100 ml of 4M KCl with 0.5% Chaps.

2.5. SDS-PAGE :

The purified sample or iodinated purified samples were boiled for 5 minutes with 1% SDS/62 mM Tris-HCl pH 6.8/1 mM EDTA/5% mercapto-ethanol/10% glycerol and 0.001% bromophenol blue and electrophoresed for 4 hours on 12.5% polyacrylamide gel (Laemmli 1970). The gels were then fixed with 40% methanol/10% acetic acid for 1 hour and stained by rapid silver stain. For iodinated samples fixed gels were treated with scintillation fluid, dried and autoradiographed.

2.6. Immunoprecipitation :

The iodinated activity peak (1×10^6 cpm) was incubated with antiprolactin receptor antibody (1 : 200) for overnight. The antigen antibody complex was then precipitated by incubating the samples with anti-rabbit IgG beads for 2 hours. The reaction was stopped by PBS with 0.1% BSA and centrifuged at low speed. The pellet and supernatant were then analyzed on SDS-PAGE as described.

3. Results

The antiestrogen binding activity was eluted as two sharp peaks from TAM-agarose with urea and KCl. Coupling efficiency of the TAM to agarose was 30-40% which was not very good but allow us to purify AEBS. The antiestrogen binding activity peak represents 0.015% of the total membrane protein. The purification achieved is summerised in Table 1. Since we were interested in the

Table I. Purification of antiestrogen binding protein from lactating mouse mammary gland membranes.

Fractions	Specific binding activity	Fold purification
Microsomal membranes	1.5×10^3 cpm/100 μg protein	--
Solubilised microsomal membranes	5.6×10^3 cpm/100 μg protein	3.6
TAM-agarose fraction	10×10^5 cpm/100 μg protein	176

relationship between lactogenic hormone receptor and AEBS, the antiestrogen binding activity thus eluted was tested for lactogen binding activity. The microsomal antiestrogen binding protein has high affinity for lactogenic hormones.

On PAGE lactogen binding and antiestrogen binding peak as eluted by urea showed several protein bands when stained with silver stain with two major bands at 90K and 40K region. Lactogen binding and antiestrogen binding peak as eluted by KCl when stained with silver stain showed a major protein band at 90K region. As the protein concentration was low, we iodinated the KCl eluted both activity peaks and electrophoresed on polyacrylamide gel which after autoradiography showed two bands at 80K and 90K region.

As our interest was relationship between lactogen and antiestrogen receptors we immunoprecipitated both activity peaks with antiprolactin receptor antibody and found that both lactogenic hormone binding protein and antiestrogen binding protein are precipitated by the antibody.

4. Discussion

The goal of the present paper was to identify the microsomal antiestrogen binding protein as revealed by an ability to be retained by antiestrogen affinity column and to evaluate whether or not the candidate protein also exhibit affinity for lactogen.

The AEBS thus purified has lactogen binding activity and has molecular weight similar to lactogen binding protein (VanOosbree *et al* 1984). The AEBS appears to be lactogenic hormone binding protein as it is also immunoprecipitable by antiprolactin receptor antibody. This protein also appear to be distinct from Ly117018 (another antiestrogen) binding protein which has a molecular weight of 68K (Miller and Katzenellenbogen 1983). Since Ly117018 has low affinity for AEBS, it is likely that Ly117018 binding protein and AEBS are different proteins. The function of AEBS is completely unknown at this time. The presence of this protein in normal and transformed mammary tissues suggests that they may play in hormone related and/or other aspects of cell biology.

The localization and characterization of this protein may provide a new access to therapy of selected tumors, and new prespective in study of anti-estrogen biology. Studies of this possibility are in progress in more refined cell system.

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References

Biswas R and Vonderhaar B K 1987 *Cancer Res.* **47** 3509

—— 1989 *Cancer Res.* **49** 6295

Laemmli U R 1970 *Nature* **227** 680

Liscia D and Vonderhaar B K 1982 *Proc. Natl. Acad. Sci.* (USA) **79** 5930

Lowry O H, Rosebrough N J, Rarr A L and Randall R J 1951 *J. Biol. Chem.* **193** 265

Miller M A and Katzenellenbogen B S 1983 *Cancer Res.* **43** 3094

Van Oosbree T R, Olsen M R Tate A C, Jordan V C and Muller G C 1984 *Mol. Cell Endocrinol.* **35** 143