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TESTOSTERONE DESTROYS THE TRANSCORTIN-RECEPTOR COMPLEX

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Summary. Dissociation of the complex of transcortin receptor with immobilized transcortin in the presence of 10^{-5} M testosterone has been shown with the use of affinity chromatography on transcortin-Sepharose. The specificity of this effect is confirmed by its abrogation in the presence of cortisol. The testosterone effect has been used for the elution of transcortin receptor from affinity column. The receptor retained transcortin-binding capacity after the elution and removal of testosterone. Characteristics of the receptor obtained by testosterone elution were identical with those of the transcortin eluted preparation.

The role of the specific steroid-binding globulins of human plasma, transcortin and sex-hormone binding globulin (SHBG), in the steroid hormone action on target cells have been widely discussed in the recent years (see Refs. 1-3). These proteins have been shown to interact specifically with the plasma membrane of human steroid hormone target tissue, liver [4], decidual endometrium [5-8], prostate [9,10] and syncytiotrophoblast [11,12]. Protein components responsible for these interactions have been isolated and partially characterized [13-16]. However, the question whether the interaction of steroid-binding proteins with their membrane receptors is steroid dependent remains unclear. While the results obtained in different laboratories concerning the influence of steroid on SHBG-receptor binding are sufficiently contradictory [5,6,14,17], similar studies of transcortin-receptor interaction were not extensively performed.

Recently we have isolated transcortin-binding protein from plasma membrane of human syncytiotrophoblast [18]. The protein properties appeared to be very similar to those of another transcortin-binding protein previously isolated from human decidual endometrium [15]. Apparently, these proteins are the transcortin-recognizing subunits of the transcortin membrane receptor. The steroid dependence of the

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Abbreviations: SHBG, sex-hormone-binding globulin; TCA, trichloroacetic acid.

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interaction of transcortin with its receptor from decidual endometrium has been shown [15]. In the present study we have used the approach which has allowed us to elucidate whether the interaction of transcortin with its placental receptor is steroid dependent.

MATERIALS AND METHODS

Materials. Pronase P from *Streptomyces griseus*, cortisol, testosterone, sodium dodecylsulfate (SDS), sodium cholate, tris(hydroxymethyl)aminometane and Servalyts were purchased from Serva (Heidelberg, F.R.G.). CNBr-activated Sepharose 4B and calibration kit for molecular weight determination using electrophoresis were obtained from Pharmacia (Uppsala, Sweden). The techniques described previously were used for the isolation of transcortin [19] and syncytiotrophoblast plasma membranes [20].

Membrane preparation. The membrane components interacting with transcortin were isolated by affinity chromatography on transcortin-Sepharose as previously described [15,18]. Briefly, membranes (about mg membrane protein) were suspended in standard buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl) and incubated overnight with constant stirring at 4°C with three Iodobeads (Pierce, Rockford, IL, U.S.A.) and carrier-free $Na^{125}I$ (3 mCi) [21]. After that, Iodobeads were taken out and the radioiodinated membranes were sedimented by centrifugation (25,000xg, 15 min) and washed with the standard buffer containing 0.15 M KI (50 ml, three times) and with the standard buffer (50 ml, three times) in order to remove non-reacted radionuclide. The labeled membrane pellets were then suspended in the standard buffer (final volume of 2.5 ml) and solubilized by adding 2.5 ml of 10% sodium cholate solution in water and incubating this mixture for 3 h at 4°C with shaking. The mixture was then centrifuged, the supernatant was 10-fold diluted with the standard buffer containing $5x10^{-6}$ M cortisol and applied on a column packed with transcortin-Sepharose.

Affinity chromatography. The affinity resin was prepared by immobilization of transcortin on CNBr-activated Sepharose [22]. The transcortin content was 0.25 mg per ml settled gel volume as determined by its capacity to bind cortisol. A column packed with approx. 5 ml of the resin and equilibrated with the standard buffer was used for affinity chromatography. The membrane solubilizate was applied on the column at a flow rate of 3 ml/h. To remove nonspecifically adsorbed radioactive material, the column was extensively washed with the standard buffer containing 1 mg/ml ovalbumin and $5x10^{-6}$ M cortisol until the radioactivity was decreased to about 50,000 cpm per ml eluate. After this the column was washed with the standard buffer containing 1 mg/ml ovalbumin for the removal of cortisol. Elution of transcortin-binding membrane components was then performed by washing the column with the standard buffer containing 1 mg/ml ovalbumin and $1x10^{-5}$ M testosterone. The elution rate was 2 ml/h. After that additional elution was performed with the standard buffer containing 1 mg/ml transcortin and $5x10^{-6}$ M cortisol, i.e. with transcortin complexed with cortisol. In another experiment the elution was performed with the standard buffer containing 1 mg/ml ovalbumin, $1x10^{-5}$ M testosterone and $5x10^{-6}$ M cortisol, and after that with the same solution without cortisol. Fractions of 0.4 ml were collected and their radioactivity was measured. The content of the protein-bound radioactivity in the chromatographic peaks was determined by precipitation of aliquots from pooled fractions with 10% solution of trichloroacetic acid (TCA). Affinity eluate was desalted by filtration through Bio-Gel

P-6 (Bio-Rad, Richmond, CA, U.S.A.), freeze-dried and stored at 18 °C for no longer than one week.

Analytical methods. The obtained affinity eluate was analyzed using SDS-PAGE [23] and by electrophoresis under non-denaturing conditions [24]. Isoelectrofocusing was performed in 7.5% polyacrylamide gel using Servalyts 3-10 and 3-7 [25]. The gel rods were cut out into 0.5-cm (after electrophoresis) or 0.25-cm (after isoelectrofocusing) slices, and ^{125}I-radioactivity of these slices was measured using a RIA Gamma counter (LKB-Wallac, Turku, Finland). After the isoelectrofocusing experiments the control gel rod was also cut out into 0.25-cm slices and the pH was measured after elution of Servalyts with 1.0 ml of distilled water.

RESULTS AND DISCUSSION

For the isolation of the transcortin-binding protein from placental syncytiotrophoblast plasma membranes we have used affinity chromatography of the solubilized non-selectively labeled membrane components on transcortin-Sepharose [18]. During the affinity chromatography immobilized transcortin was complexed with cortisol as this complex had been shown to bind with the syncytiotrophoblast membranes [11]. The elution from the affinity column was carried out with the transcortin-cortisol containing buffer. In the present study we have used testosterone containing buffer for the elution instead of the buffer containing transcortin-cortisol complex.

Similar to the elution with transcortin-cortisol complex, elution with the testosterone buffer gave a peak of radioactivity (Fig. 1A). Approximately 35% of radioactivity of this peak has been associated with the protein species as was evidenced by the precipitation with TCA (Table1). The possible nature of the non-precipitated radioactivity was discussed in the previous communication [14]. The following elution from the affinity column with transcortin-cortisol containing buffer gave another peak of radioactivity (Fig. 1A) 2.5-fold poorer in the protein components (Table 1). When the sequence of the elution steps have been changed (elution with transcortin-cortisol complex performed before elution with testosterone), a single peak of radioactivity was obtained (Fig. 1B). In our opinion, the incomplete elution of the transcortin-binding components achieved by substitution of cortisol, bound to immobilized transcortin, for testosterone was due to hindered conformation transitions of the immobilized glycoprotein molecules.

To elucidate whether the elution have occured as a result of non-specific hydrophobic influence of testosterone we have carried out the testosterone elution in the presence of equivalent amount of cortisol. In this case considerable decrease in the amount of the eluted protein components was observed. After removal of cortisol the main quantities of the protein components have been eluted by testosterone (Fig. 1C).

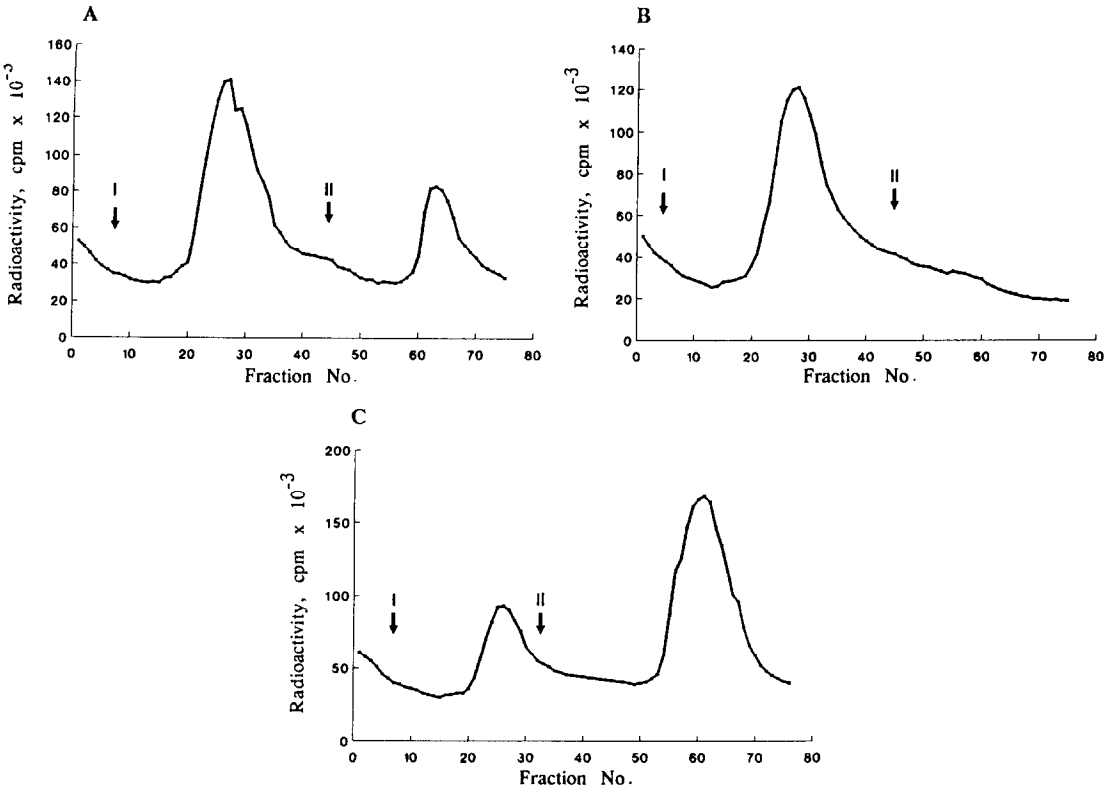

Fig. 1. Affinity chromatography of the labelled membrane components on transcortin-Sepharose with the elution by the different agents. (A) Elution with testosterone containing buffer (1) followed by the elution with transcortin/cortisol containing buffer (i.e. with transcortin-cortisol complex) (2); (B) elution with transcortin/cortisol containing buffer (1) followed by the elution with testosterone containing buffer (2); (C) elution with testosterone/cortisol containing buffer (1) followed by the elution with the same buffer in the absence of cortisol (2). Arrows indicate the beginning of the elution with a given agent.

We have compared the characteristics of the affinity eluates obtained by testosterone and transcortin elutions. In both cases SDS-electrophoresis has shown the presence of a single protein band with apparent M_r 20 kDa. This protein is inclined to aggregation as confirmed by its low mobility during electrophoresis in non-denaturing (without detergents) conditions. While low electrophoretic mobility of the membrane protein preparation obtained by transcortin elution might be explained by its complexation with transcortin, the present study gives evidence of the aggregation of the membrane protein itself.

Isoelectrofocusing of the testosterone eluate revealed a single discrete band corresponding pH 4.4 (Fig. 2A). This band coincides with one of those obtained during isoelectrofocusing of transcortin eluate [18]. Pretreatment with neuraminidase resulted in a shift of the isoelectric point to 5.0 (Fig. 2A). When the testosterone

Table 1. TCA precipitation of ^{125}I-radioactivity in the chromatography peaks (see Fig. 1)

Fraction no.	Radioactivity		
	Total, cpm	Precipitated	
		cpm*	% of total
21-37 (Fig. 1A)	1147000	402000	35
60-70 (Fig. 1A)	410000	183000	45
21-37 (Fig. 1B)	845000	389000	46
21-32 (Fig. 1C)	730000	95000	13
56-71 (Fig. 1C)	1297000	411000	32

*Amounts of the precipitated radioactivity have been calculated from total radioactivity and experimentally determined percent of precipitated radioactivity (see "Materials and Methods").

eluted receptor preparation have been incubated with transcortin-cortisol (but not transcortin-testosterone) complex before isoelectrofocusing, two additional bands at pH 3.7 and 4.0 were observed (Fig. 2B). These results confirm our earlier supposition that two additional bands represent the complexes of membrane protein with transcortin but were not caused by the membrane sialoglycoprotein microgeterogenity. Thus, it should be emphasized that both elution procedures produced the same labeled membrane protein.

The approach used in this study has been based on the fact that binding of the steroid induce conformational changes in transcortin molecule dependent on the steroid structure [26,27]. The conformation of the transcortin-cortisol complex is sufficiently different from that of transcortin-testosterone complex [27], this difference may be reflected on transcortin affinity for the receptor. Indeed, the substitution of transcortin bound cortisol for testosterone results in dissociation of transcortin-receptor complex indicating the receptor to be inefficient in binding of transcortin in the conformation of the transcortin-testosterone complex. Similar results concerning the influence of steroid on SHBG-membrane interaction have been obtained [5,7,17].

Equimolar concentration of cortisol which interferes the transcortin-testosterone complexation (since K_a of transcortin with cortisol is twenty fold higher than with testosterone [28]) considerably decreases the elution of the receptor. Therefore the effect of testosterone cannot be explained by its non-specific hydrophobic influence.

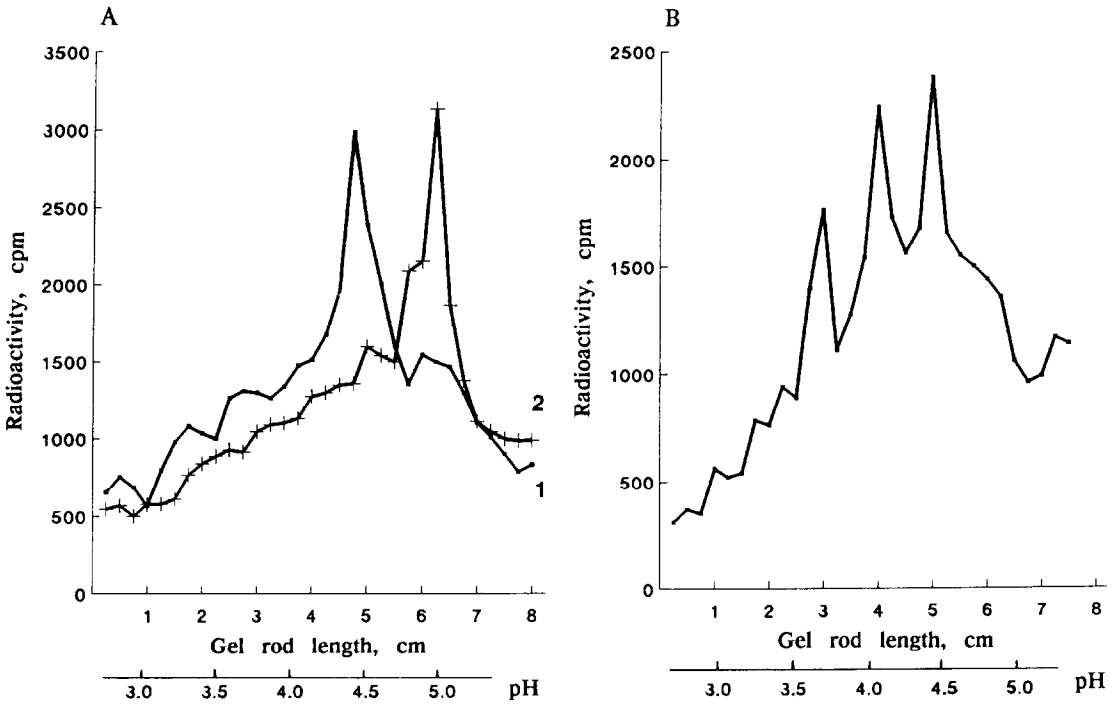

Fig. 2. Isoelectrofocusing of the affinity eluate obtained by the testosterone elution. (A) Intact eluate (curve 1), the same after neuraminidase treatment (curve 2); (B) intact eluate after incubation with the transcortin-cortisol complex.

So, the results of this investigation suggest the interaction of transcortin with its membrane receptor to be steroid-dependent.

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