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Steroid Binding Proteins—Interactions with Membranes

INTERACTION OF HUMAN CBG WITH CELL MEMBRANES

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Summary—Specific binding sites for corticosteroid-binding globulin (CBG) and its pregnancy-associated variant (pCBG), having a modified carbohydrate moiety, were found in the plasma membranes of human liver, decidual endometrium and placental syncytiotrophoblast. The membrane binding was influenced by the conformation of the glycoprotein molecules and structure of their carbohydrate chains. CBG receptor was solubilized from the endometrium membrane and partially characterized. It was found to have a subunit structure, with a homooligomeric sialoglycoprotein consisting of four 20 kDa protomeric species being involved in the recognition of the CBG molecules complexed with progesterone or cortisol. A kinetic study using membrane microvesicles derived from the syncytiotrophoblast brush border revealed that neither CBG nor pCBG restricted cortisol accumulation in the intravesicular space, whereas only normal CBG could penetrate the syncytiotrophoblast membrane. Action of the CBG–cortisol complex on trophoblast cells resulted in the activation of membrane adenylate cyclase and growth of the cAMP accumulation within these cells. Collectively, these findings suggest that both normal CBG and pCBG are involved in the guided transport of steroid hormones to the target cells and transmembrane transfer of hormones and/or hormonal signals.

INTRODUCTION

Since the specific membrane binding of human corticosteroid-binding globulin (CBG) [1] and sex hormone-binding globulin (SHBG) [2] was demonstrated in 1983–1984, the specific interaction of serum steroid-binding glycoproteins with the plasma membranes of various human and animal tissues has become a well-recognized phenomenon (for a review, see Ref. [3]). This knowledge led to a dramatic change in our views on the biological functions of these glycoproteins as well as on the mechanisms of steroid hormone action. The steroid–glycoprotein complexes and not "free" steroids seem to be the true hormonally active entities. The interaction of these complexes with the binding sites on cell membranes is likely to be the primary act in a sequence of events that gives rise to the biological response of the cell. Further progress in the field requires, on the one hand, investigation of the structure and function of the membrane components involved in the interaction with the steroid–glycoprotein complexes and, on the other hand, elucidation of the related multi-step biochemical processes of transmembrane transport and signaling.

In the present paper, we briefly summarize our data on the specific binding sites for CBG in the plasma membranes of several human tissues and report the molecular properties of CBG membrane receptor from decidual endometrium as well as the results of a kinetic study of cortisol, CBG and its pregnancy-associated variant (pCBG) interactions with the membrane microvesicles derived from the brush border of human placental syncytiotrophoblast.

MATERIALS AND METHODS

Cortisol, progesterone, testosterone, corticosterone and immobilized neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* were obtained from Sigma (St Louis, MO, U.S.A.). CBG was isolated from human retroplacental serum as described previously [4]; its molecular

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variants, normal CBG and pCBG, were prepared using lectin-affinity chromatography on Con A-Sepharose (Pharmacia, Uppsala, Sweden) [5]. Glycoproteins were radioiodinated using Iodogen (Pierce, Rockford, IL, U.S.A.) [6]. [^{3}H]Cortisol was obtained from Amersham Int. (Amersham, U.K.). RIA Gamma (LKB-Wallac, Turku, Finland) and Mark III (Tracor Europa, Bilthoven, The Netherlands) instruments were used for the measurement of ^{125}I- and ^{3}H-radioactivity, respectively.

Preparation and characterization of the plasma membranes from human liver, placental syncytiotrophoblast and decidual endometrium as well as the assays for the glycoprotein–membrane interactions at 0–4°C were described previously [1, 6, 7].

In order to isolate the components of CBG membrane receptor, the endometrium plasma membranes were labeled with ^{125}I, solubilized and applied on a CBG-Sepharose column (1.5 × 2.0 cm) as described previously [7]. After washing away the non-retained material [7], elution of the adsorbed labeled membrane components was performed by successive washing of the column with the following solutions: (i) linear sodium cholate gradient (0–5%) in 0.01 M Tris–Cl, pH 7.6; (ii) linear NaCl gradient (0–2 M) in the same buffer containing 5% sodium cholate; (iii) 1% SDS solution in water; (iv) 0.01 M sodium acetate, pH 4.0, containing 2.0 M NaCl; and (v) 1% SDS solution in water. Elution rate was 4.0 ml/h; fractions of 0.5 ml were collected. Their radioactivity was measured; fractions containing the chromatographic peaks were combined, dialyzed against a 1000-fold volume of distilled water, concentrated and analyzed by SDS–PAGE in 6 and 12% gels. Gel rods were cut into 0.25 or 0.5 cm slices, and radioactivity of these slices was measured. Protein standards obtained from Serva (Heidelberg, Germany) were used for the molecular mass determination.

To study the kinetics of cortisol uptake by the membrane microvesicles derived from human placental syncytiotrophoblast [8], the steroid (^{3}H-labeled + unlabeled; concentrations ranged from 2×10^{-10} to 7×10^{-8} M) was incubated with a suspension of the microvesicles (250–350 mg protein per ml) in 0.05 M Tris–Cl, pH 7.6, containing 0.25 M sucrose, 0.1 M NaCl, 0.01 $CaCl_2$ and 0.2 mg/ml ovalbumin (Serva) at 23 ± 2°C in the absence of steroid-binding glycoproteins or in the presence of either normal CBG or pCBG (5×10^{-7}–1×10^{-6} M). Reaction was terminated by diluting the samples with an equal volume of the chilled buffer. The membrane vesicles were then sedimented by centrifugation at 13000 *g* for 20 min. Supernatant was discarded, and the radioactivity captured by the vesicles was measured after the extraction of labeled steroids with benzene. When studying the kinetics of the binding of ^{125}I-labeled CBG variants to the microvesicles, the experimental conditions were as above and the labeled glycoprotein concentrations varied from 1×10^{-12} to 1.3×10^{-7} M, with the cortisol concentration in the medium being equal to 5×10^{-6} M in order to saturate the glycoproteins with the steroid. To study the dependence of the cortisol, normal CBG and pCBG binding on the intravesicular volume, these substances were incubated for 1 h at 23°C with the microvesicles suspended in the above buffer containing varying sucrose concentrations (from 0.25 to 1.0 M) or with the suspension of the membrane fragments obtained by incubating the microvesicles in distilled water for 30 min immediately prior the experiment.

All determinations were carried out in duplicate; analysis differing by >10% were rejected. The binding parameters were calculated as described previously [9, 10]; these are given in the text as mean ± SE of 3–7 determinations from independent experiments.

To study the complex formation between transcortin and the 20 kDa subunit of the membrane receptor, one of the bifunctional reagents, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester, dimethyl suberimidate or 1,5-difluoro-2,4-dinitrobenzene (DFDNB) (Pierce), was dissolved in dimethyl sulfoxide and added to a solution of the ^{125}I-labeled sialoglycoprotein and unlabeled transcortin to a final concentration of 0.2 mg/ml. An equal volume of pure dimethyl sulfoxide was added to the blank sample. The reaction mixture was incubated for 18 h at 4°C with continuous stirring. After that 1.0 M Tris–Cl buffer, pH 7.4, was added to the samples (one tenth of the sample volume). Samples were then incubated for 30 min at room temperature and desalted by filtration through a Bio-Gel P-6 column.

To study the transcortin complexing with the receptor components in the integral endometrium membrane, [^{125}I]transcortin was added to the membrane suspension in Krebs buffer containing 5×10^{-6} M progesterone. Into a parallel set of samples, 500-fold molar excess of unlabeled transcortin was added in order to

evaluate non-specific [^{125}I]transcortin interaction with the membrane components. All samples were incubated for 18 h at 4°C with gentle shaking. Membranes were sedimented by centrifugation for 15 min at 4000 *g*, and supernatant was discarded. The membrane pellets were washed twice with 0.01 M sodium phosphate buffer, pH 6.8, and suspended in the same buffer. DFDNB dissolved in dimethyl sulfoxide was added to the samples up to a final concentration of 0.2 mg/ml, and the samples were incubated overnight at 4°C with constant stirring. The unreacted substances were removed by washing the membranes with Krebs buffer containing 1 mg/ml ovalbumin (3 times) and distilled water (3 times). SDS–electrophoresis of the cross-linked material was performed using 6% PAGE. The gels were sliced and radioactivity of the slices was measured as above.

Trophoblasts were isolated from freshly delivered normal human placenta as described previously [11]. The obtained cell suspension contained about 1×10^6 cells per ml. Aliquots of this suspension were incubated for 5, 10 or 15 min at 37°C with various concentrations of cortisol, CBG or CBG–cortisol complex. The cells were, then, washed and homogenized in 0.6 M $HClO_4$; protein was sedimented by centrifugation. After neutralizing the supernatant with 5 M KOH, cAMP concentration was measured using a RIA kit (UVVVR, Prague, CSFR). In order to determine the adenylate cyclase activity, the washed cells were homogenized in 0.005 M Tris–Cl, pH 8.0, containing 0.25 M sucrose, 0.001 M dithiotreitol and 0.01 M GTP, and a crude membrane fraction was obtained by centrifugation at 1000 *g* for 20 min. It was resuspended in the above buffer, and the enzyme activity was measured as described previously [12].

RESULTS AND DISCUSSION

In order to study the interaction of a substance with cell membrane, two principally different experimental approaches may be used. One of them, analysis of specific membrane binding under equilibrium conditions is appropriate at the initial stages of the study, when the main goal is to detect the presence of specific binding sites for this substance in the plasma membrane of a particular tissue. When using this approach, it is important to minimize all transport and other non-equilibrium processes in order to obtain physicochemically correct values of the parameters of the binding reaction, equilibrium constant (K_a or K_d), binding capacity (B_{max}), etc. A convenient way to perform the binding assay under (quasi-)equilibrium conditions is to use simple analytical systems, consisting of a purified membrane preparation suspended and a substance under investigation dissolved in an appropriate medium, and to determine the distribution of the ligand between the membrane-bound and unbound fractions after a relatively long incubation of such systems at low temperatures (0–4°C).

Another experimental approach is based on a kinetic analysis of the ligand–membrane interaction and the biochemical processes it initiates. This approach is useful at the further stages of the study when the aim is to elucidate the biological consequences of the primary act of membrane binding of the ligand and to study the multi-step mechanisms of transmembrane transport of the ligand or ligand-mediated transmembrane signaling. Such a study usually involves short-term incubations of the ligand with a cell suspension, tissue specimens, etc, at elevated temperatures followed by the determination of the intracellular localization of the ligand or its fragments, analysis of enzyme activities and other biochemical processes modulated by the ligand-membrane interaction.

We have used both of the above experimental approaches to study the interactions of normal human CBG and pCBG with the plasma membranes of several human tissues. Presented below are the results of this work.

Table 1. Specific binding of CBG and pCBG to the plasma membranes of human tissues at 0–4°C

Tissue	B_{max} (fmol per mg membrane protein)	K_d (mol/l) CBG	K_d (mol/l) pCBG	Ref. No.
Liver	6.0 ± 1.0	$(0.9 \pm 0.2) \times 10[s-0]6$	$(4.1 \pm 0.3) \times 10^{-6}$	[1, 13]
Decidual endometrium	3.7 ± 3.2	$(1.0 \pm 0.2) \times 10^{-10}$	ND[a]	[7]
Placental syncytiotrophoblast:				
Type I sites	140 ± 60	$(1.6 \pm 0.6) \times 10^{-10}$	$(4.5 \pm 1.2) \times 10^{-9}$	
Type II sites	3.0 ± 2.2	$(2.5 \pm 0.7) \times 10^{-11}$	$(3.3 \pm 2.0) \times 10^{-12}$	

[a]Not determined.

Specific binding of normal CBG and pCBG to cell membranes

Binding assay under equilibrium conditions revealed the presence of specific binding sites for normal CBG and pCBG in plasma membranes isolated from several human tissues (Table 1) and allowed us to study selectivity and steroid dependence of the binding reactions. In all cases, none of the other human serum proteins could inhibit the interaction of the CBG variants with the membrane binding sites. This was showed both by introducing pure serum proteins, e.g. SHBG, thyroxine-binding globulin, transferrin, orosomucoid, albumin, in the analytical systems (no inhibition observed) and by inhibiting the labeled glycoprotein membrane binding by human sera with known concentrations of normal CBG or pCBG. In the latter case, inhibition was proportional to the amount of serum CBG (pCBG) introduced into the analytical samples.

The CBG binding to the liver membrane was independent on it being bound to a steroid: both CBG stripped of steroid and CBG–cortisol or CBG–progesterone complexes interacted with the membrane with the same affinity [13]. It is important, however, that heat-denatured CBG, both intact and desialylated, did not interact with the discovered binding sites. The plasma membranes of decidual endometrium displayed about 5-fold higher affinity for CBG–progesterone than for CBG–cortisol and did not bind CBG complexed with testosterone or stripped of steroid [7]. In the case of the syncytiotrophoblast membrane, the steroid dependence of the binding reaction was more complex (Fig. 1). The binding of normal CBG was almost independent on the nature of complexed steroid (cortisol, corticosterone, progesterone or testosterone) but CBG devoid of steroid could bind to the membrane only non-specifically. pCBG also did not interact with the specific binding sites in the absence of steroids. However, the binding of pCBG–cortisol complex was significantly higher than that of pCBG complexed with the other steroids. As we have previously noted [3], the physicochemical basis for the steroid dependence of the membrane binding of steroid-binding glycoproteins seems to be provided by the conformational transitions induced in the glycoprotein molecule by complexation with steroids. Furthermore, the above findings suggest that the conformation of CBG stripped of steroid is substantially different from that in the complex with a specifically bound steroid, and the CBG conformations in the complexes with different steroids are somewhat different, too.

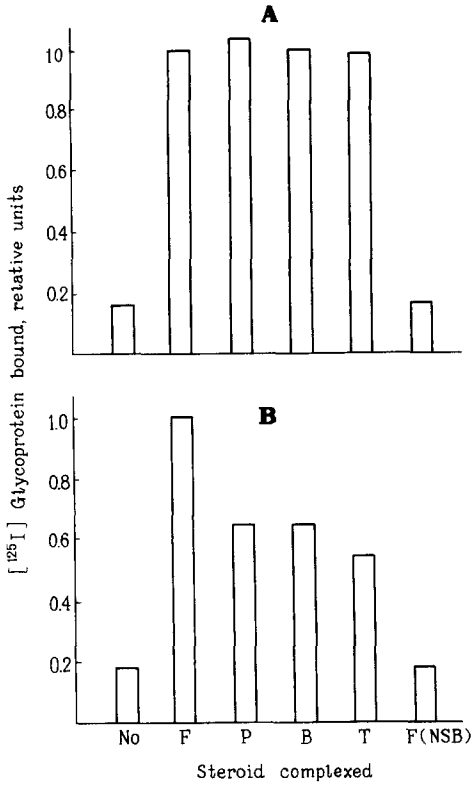

Fig. 1. Steroid dependence of the [^{125}I]CBG (A) and [^{125}I]pCBG (B) binding to the plasma membrane of human syncytiotrophoblast at 4°C. Concentrations of the labeled glycoproteins were 1×10^{-10} and 5×10^{-11} M, respectively. Cortisol (F), progesterone (P), corticosterone (B) or testosterone (T) were introduced in the analytical samples at a concentration of 5×10^{-7} M. Non-specific binding (NSB) was determined in the presence of a 200-fold molar excess of the corresponding unlabeled glycoprotein.

The binding assay under equilibrium conditions showed that the CBG carbohydrates are involved in the glycoprotein–membrane interactions. This conclusion stems from the great difference in the binding of native and desialylated CBG to the liver membrane [1] and the differential binding of normal CBG and pCBG to the liver [4] and syncytiotrophoblast [6] membranes. (Also, we have recently showed that the integrity of the carbohydrate chains of another steroid-binding glycoprotein, SHBG, is an absolute requirement for its specific interaction with the plasma membranes of human decidual endometrium [14].) Thus, the biological function of the carbohydrate components of the serum steroid-binding glycoproteins becomes clear. Though the glycoprotein carbohydrates do not participate in the

complex formation with steroids [15], they play an important role in the guided transport of steroids to/into the target cells, since they are involved in the formation of determinants for the membrane recognition of the steroid–glycoprotein complexes. It seems probable that relatively small changes of the polypeptide conformation may lead to a considerable alteration in the spatial orientation of the carbohydrate chains located on the surface of the polypeptide globule. Otherwise speaking, the involvement of the sugar chains in the recognitive processes makes possible an amplification of the conformational transitions of the polypeptide chain.

Some molecular properties of CBG membrane receptor

We have recently reported [7] isolation of a membrane sialoglycoprotein with a minimal molecular mass of 20 kDa which displayed the same steroid dependence of the CBG binding and almost the same affinity for CBG–steroid complexes as the integral endometrium plasma membrane. In the absence of detergents, this membrane component was found to form an oligomer. We have assumed that several 20 kDa protomers form a recognizing subunit of the membrane receptor for CBG. Elucidation of the structural organization of such a subunit and the existence of other subunits of the CBG receptor required further investigation.

Cross-linking experiments, using bifunctional reagents in order to fixate the complex of the purified, ^{125}I-labeled 20 kDa membrane sialoglycoprotein and CBG, revealed the formation of two electrophoretically distinct species with apparent molecular masses of 92 and 136 kDa [Fig. 2(A)]. Bearing in mind the CBG molecular mass of about 55 kDa [4], it can be calculated that the above species are likely to be the cross-linked CBG complexes with two and four 20 kDa sialoglycoprotein molecules, respectively. Effective cross-linking of CBG with the membrane sialoglycoprotein with DFDNB and ineffectiveness of the two other bifunctional reagents, *N*-hydroxysuccinimide ester of maleimidobenzoic acid and dimethyl suberimidate, suggest that the hydroxyl groups of the CBG and/or the membrane sialoglycoprotein might be involved in the cross-linking reaction along with the functional groups of the amino acid residues.

When the endometrium membrane preparations were incubated with [^{125}I]CBG and

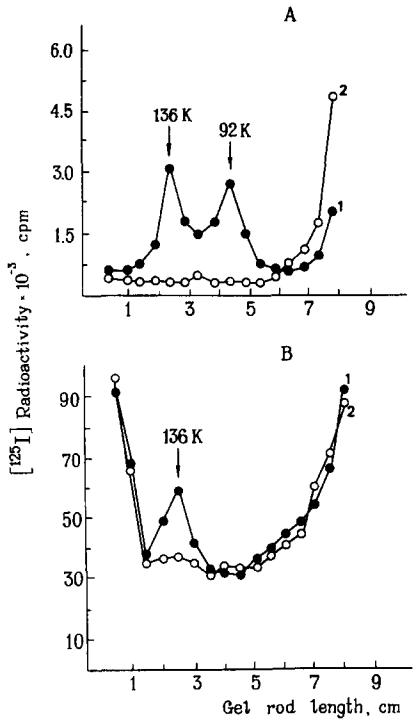

Fig. 2. (A) SDS–PAGE of the mixture of ^{125}I-labeled 20 kDa sialoglycoprotein isolated from the endometrium membrane and unlabeled CBG with (1) and without (2) the treatment with DFDNB. (B) SDS–PAGE of the endometrium plasma membrane preparations treated with DFDNB after incubation with [^{125}I]CBG in the absence (1) or in the presence (2) of a 1000-fold molar excess of unlabeled CBG. Experimental details are given in the text.

treated with DFDNB, only one distinct band appeared at SDS–PAGE [Fig. 2(B)]. It corresponded to the species with an apparent molecular mass of 136 kDa, thus indicating the formation of a complex consisting of one CBG molecule and four 20 kDa protomers. Thus, the recognition subunit of the CBG membrane receptor seems to be a homooligomer consisting of four 20 kDa sialoglycoprotein molecules.

In order to detect other possible subunits of the CBG receptor, we have altered the scheme of the elution of the ^{125}I-labeled membrane components adsorbed to CBG-Sepharose. In previous work [7], we performed a biospecific elution with CBG–progesterone or testosterone solution in detergent-free buffers. This might result in the loss of membrane components which aggregated in the column due to their hydrophobic nature. So, we attempted to elute the adsorbed membrane components, first, with a sodium cholate gradient followed by a NaCl gradient in the presence of a high sodium cholate concentration. As shown in Fig. 3(A), this resulted in the appearance of several peaks

of radioactive material. However, all of them were found to be lipids which we have previously observed [7] (data not shown). Subsequent washing of the column with an SDS solution also gave no significant amounts of labeled proteins [Fig. 3(B)]. Only denaturation of the immobilized CBG by exposing the affinity resin to strongly acidic conditions, followed by the elution of the desorbed material with a detergent solution, led to the appearance of radioactive substances [Fig. 3(B)] which migrated at SDS–PAGE as proteins with apparent molecular masses of about 20, 90 and 300 kDa (Fig. 4). All these electrophoretic bands disappeared after the treatment of the samples with pronase which proved them to be proteins.

Characterization of the two new membrane components eluted from the CBG-Sepharose column, 90 and 300 kDa proteins, is now under way. As mentioned above, the CBG binding characteristics of the 20 kDa sialoglycoprotein is virtually the same as that of the endometrium membrane itself. It seems, therefore, reasonable to assume that the two larger proteins are inserted in the membrane and interact with the CBG-recognizing subunit thus serving some function in transmembrane transfer of the components of the CBG–progesterone complex and/or in CBG-mediated transmembrane signaling.

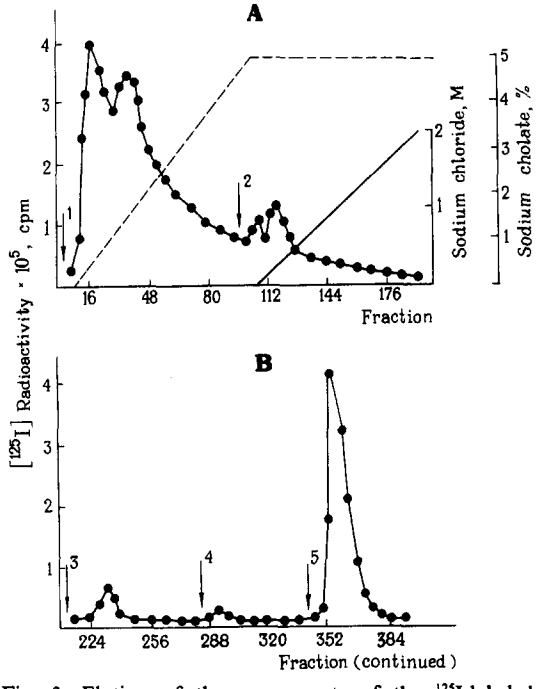

Fig. 3. Elution of the components of the ^{125}I-labeled, cholate-solubilized endometrium plasma membrane adsorbed on CBG-Sepharose: (A) with sodium cholate (1) and sodium chloride (2) gradients; (B) with SDS solution (3), sodium acetate acid buffer (4) and SDS solution again (5). Arrows indicate the beginning of elution with the corresponding solutions which are described in the Materials and Methods section.

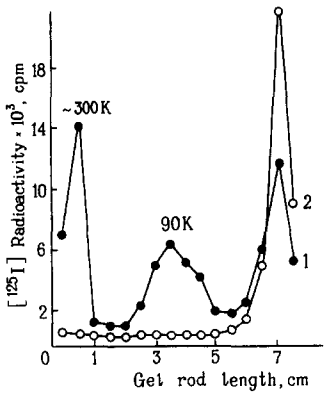

Fig. 4. SDS–PAGE of the labeled endometrium membrane components eluted from the CBG-Sepharose column by denaturing CBG with the acid buffer and washing the column with the SDS solution (fractions 352–380 in Fig. 3) before (1) and after (2) the treatment with pronase.

Kinetic analysis of the cortisol, normal CBG and pCBG interactions with membrane microvesicles

As stated above, investigation of the biochemical processes initiated by the primary act of the membrane binding of a steroid–glycoprotein complex requires the study of kinetics of the membrane binding and transmembrane transfer of the complex components as well as analysis of the concentrations of other substances which are affected by the primary binding reaction. Membrane microvesicles which can be easily prepared from human placental trophoblast [8] are a very convenient model for such a study. Unlike cultured cells or tissue specimens, these sealed, right-side-out vesicles do not contain intracellular enzymes or binding proteins which could affect the results. At the same time the microvesicles display all basic properties of the syncytiotrophoblast membrane brush border [8] and, being suspended in an appropriate medium, represent a unique example of a nearly homogeneous system consisting of two physical volumes separated by a selective membrane. Small dimensions of the membrane vesicles afford a large area of the contact surface between the intra- and intervesicular spaces.

It has been previously reported [16] that these microvesicles can accumulate glucocorticoids in the intravesicular space due to the presence of a specific transport system for these steroid hormones in the syncytiotrophoblast membrane. The transmembrane transfer of glucocorticoids

could be assumed to be an initial stage of their hormonal action on trophoblast as well as their transport to the fetal circulation. However, there are two facts arguing against this assumption. First, the transfer of free glucocorticoids was shown to be accompanied with their 11-oxidation [16]. Cortisol, the main glucocorticoid in humans, is thus transformed into cortisone which is believed to be hormonally inactive [17]. Second, fraction of "free" cortisol in human blood is very low, especially at pregnancy, while over 90% of the steroid is bound to CBG. A paradoxical situation thus emerges: syncytiotrophoblast *specifically* takes up a small portion of cortisol from the maternal circulation in order to inactivate it and to protect the fetus from the high cortisol concentration occurring in the maternal circulation [17]. But, would it not be much better not to take up the steroid at all? And how does the maternal organism protect itself from the high cortisol concentrations? These and similar questions throw doubt on the hormonal activity of free cortisol in the feto–placental unit.

As we have found [6] (see Table 1), the plasma membrane of human syncytiotrophoblast contains two types of specific binding sites for normal CBG and pCBG. Complexing with steroids is an absolute requirement for the specific glycoprotein interaction with these sites. It was interesting to follow the fates of the components of steroid–glycoprotein complexes after the binding to the membrane. First of all, we compared the kinetics of free and protein-bound cortisol uptake by the membrane

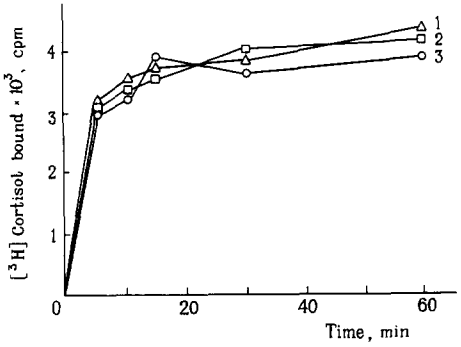

Fig. 5. Time-course of the cortisol uptake at 23 ± 2°C by the membrane microvesicles derived from the placental syncytiotrophoblast in the absence of the steroid-binding glycoproteins (1) and in the presence of CBG (2) or pCBG (3) in the incubation medium. Initial cortisol concentration in the medium was 1×10^{-8} M and about 50,000 cpm of [³H]cortisol was added into each sample as a tracer. CBG and pCBG concentrations were 5×10^{-7} M. Data of a representative experiment.

Table 2. Kinetic parameters of cortisol uptake at 23 ± 2°C by the membrane microvesicles derived from human placental syncytiotrophoblast in the absence of binding proteins and in the presence of CBG or pCBG

Parameter	No binding proteins	CBG	pCBG
K_m (nM)	35 ± 10.5	78 ± 57	52 ± 18
V_{max} (pM/s)	1.34 ± 0.50	1.68 ± 0.52	2.72 ± 1.71

See Materials and Methods for experimental details.

microvesicles. It was found that even under the conditions where the major cortisol fraction (>80–90%) was bound to normal CBG or pCBG the time-course of the steroid uptake by the microvesicles was similar to that of free cortisol (Fig. 5). The calculated values of kinetic parameters (Table 2) supported this finding. Moreover, the presence of normal CBG or pCBG decreased the oxidation of cortisol taken up by the microvesicles from 85–95% up to about 50% (detailed data will be published elsewhere). This suggests that neither normal CBG nor pCBG restrict the cortisol influx into the syncytiotrophoblast but protect the steroid, at least, partially, from inactivation. (Moreover, it could be assumed that partial oxidation of cortisol accumulated by the microvesicles, observed in these experiments, was an artifact caused by dissociation of the steroid–glycoprotein complexes in the incubation medium).

Thus, both normal CBG and pCBG can deliver hormonally active cortisol into syncytiotrophoblast. But, are the mechanisms of transmembrane steroid transport in the presence of the two glycoproteins the same? In order to answer this question, we have studied the dependence of the ^{125}I-labeled glycoprotein binding to the membrane microvesicles on the osmolarity of the incubation medium, i.e. on the intravesicular volume. Figure 6 shows that the binding of normal [^{125}I]CBG, like that of [³H]cortisol, depended on the intravesicular volume. This means that normal CBG could penetrate inside the microvesicles. This conclusion was further supported by the fact that the amount of normal [^{125}I]CBG bound to the shocked microvesicles, i.e. membrane fragments having no inner space, was similar to that obtained by extrapolation to a zero intravesicular volume. These data allow us to conclude that after the binding of the normal CBG–cortisol complex to the syncytiotrophoblast membrane it penetrates the membrane without the dissociation into the individual components.

Unlike normal CBG, the [^{125}I]pCBG binding was independent of the inner volume of the microvesicles (Fig. 6). At all the sucrose concentrations used, both intact and hypotonically shocked microvesicles bound the same amount of [^{125}I]pCBG. This means that pCBG can not penetrate the syncytiotrophoblast membrane. Since pCBG does not restrict the cortisol uptake by the microvesicles (see above), we assume that this CBG variant functions as a transport shuttle supplying trophoblast cells with the steroid. (Such a mechanism of the guided steroid transport has been offered earlier for SHBG–membrane interaction [18].) This is in line with our previous data on the absence of pCBG in fetal circulation and placental extracts [19].

Selective penetration of the CBG molecular variants through the syncytiotrophoblast membrane indicates that the carbohydrate chains are involved not only in the primary act of the glycoprotein recognition by the binding sites located on the cell membrane but in the subsequent biochemical processes as well.

Thus, at least, two different mechanisms of the steroid entry into the target cells with the involvement of the serum steroid-binding glycoproteins could be suggested: transmembrane transport of the steroid–glycoprotein complex as a whole and transfer of the steroid to the steroid membrane receptor followed by the uptake of the steroid alone. Further experiments are necessary in order to investigate the intracellular distribution and biochemical effects produced by the steroid molecules entering the cell via different routes.

The interaction of CBG–steroid complexes with cell membranes can have physiological consequences apart from the transmembrane

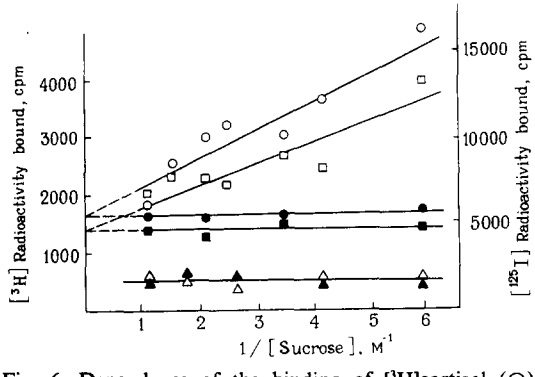

Fig. 6. Dependence of the binding of [^{3}H]cortisol (○), [^{125}I]CBG (□) and [^{125}I]pCBG (△) to the sealed microvesicles (○,□,△) and membrane fragments (●, ■, ▲) on the sucrose concentration in the incubation medium. See Materials and Methods for experimental details.

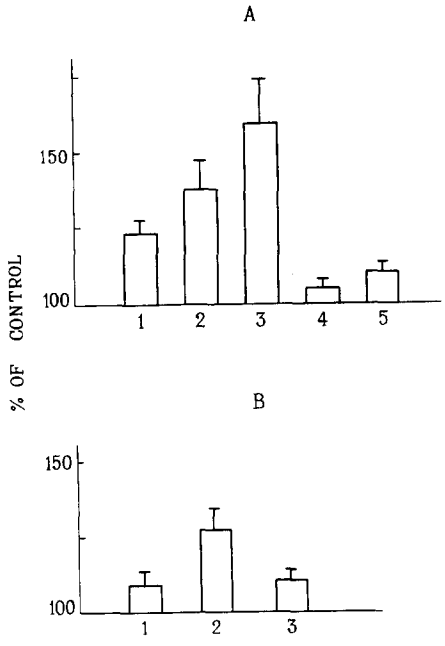

Fig. 7. (A) cAMP accumulation within the trophoblast cells incubated with CBG–cortisol (1, 1 μM for 5 min; 2, 1 μM for 15 min; 3, 2 μM for 10 min), CBG devoid of steroid (4, 1 μM for 10 min) or cortisol (5, 1 μM for 15 min). (B) Adenylate cyclase activity in the crude membrane fraction derived from trophoblasts treated with CBG–cortisol (1, 1 μM for 5 min; 2, 0.5 μM for 15 min) or cortisol (3, 1 μM for 15 min). In both cases trophoblasts incubated under the same conditions in the absence of both CBG and cortisol were used as a control.

transport of steroids. This possibility is illustrated by the modulation of membrane adenylate cyclase activity and alteration of the cAMP accumulation within the trophoblasts treated with CBG–cortisol complex. As shown in Fig. 7, both increases in the activity of the membrane enzyme and cAMP accumulation in the cells incubated with the steroid–glycoprotein complex were significantly higher than in the cells treated with individual components of the complex, with both biochemical effects being dose- and time-dependent. Nakhla *et al.* [20] have previously reported [20] similar data obtained using cultured tumor cells. This suggests that modulation of the second messenger system(s) by the action of CBG–steroid complexes on cell membranes may be a general phenomenon responsible for a part of the physiological effects which are thought to be produced by steroid hormones.

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