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Organization of the transcortin-binding domain on placental plasma membranes

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Abstract

Complex formation between transcortin (corticosteroid-binding globulin) and 20 kDa sialoglycoprotein from human syncytiotrophoblast plasma membranes (presumably a transcortin-recognizing subunit of the transcortin membrane receptor) was studied using FPLC and cross-linking with bifunctional reagents. The action of 1,5-difluoro-2,4-dinitrobenzene (DFDNB) on a solution of the purified 20 kDa sialoglycoprotein and transcortin resulted in formation of covalently linked complexes of 95 kDa and 140 kDa consisting of one transcortin molecule and either two or four molecules of the membrane sialoglycoprotein (the molecular mass of transcortin is 55 kDa). Additionally, cross-linking resulted in the appearance of a 43 kDa species which is the cross-linked dimer of the membrane protein. The dimer was also observed during chromatography on a Superose 12 column in the absence of DFDNB treatment. Treatment of intact syncytiotrophoblast membranes with DFDNB resulted in isolation of the transcortin binding protein dimer as the major portion of total pool of the protein. Formation of the transcortin complexes with two and four molecules of the membrane protein was also observed when the membranes were incubated with ^{125}I-labeled transcortin and treated with DFDNB, but formation of the latter complexes predominated. The results obtained suggest that the recognizing and binding domain for transcortin in placental membranes is organized as dimers consisting of non-covalently linked sialoglycoprotein monomers of a 20 kDa each and that transcortin has two sites for interaction with this dimer. Apparently, binding of two dimers results in the formation of the functional form of the transcortin-receptor complex. The possible biological role of such a complex is discussed.

Keywords: Transcortin; Corticosteroid-binding globulin; Placenta; Membrane receptor

1. Introduction

Transcortin (or corticosteroid-binding globulin, CBG) – a glycoprotein of human and animal serum specifically binds corticosteroids and progesterone [1]. The main biological function ascribed to this protein is to protect steroid hormones in the extracellular environment from destruction and clearance and to deliver the hormones to target tissues. Studies of the last ten years provide evidence that transcortin is not only a passive extracellular transporter of steroids, but that it binds specifically to plasma membranes of cells in steroid hormone responsive tissues [2–9]. Further investigations revealed membrane-dependent degradation of transcortin after interaction with plasma membrane of several rat tissues [6]. Internalization of transcortin by a mammary carcinoma [10] and human syncytiotrophoblast [11] cells and activation of the adenylate cyclase system [4,10] have also been shown to occur in these cells after interaction with transcortin. In all these experiments distinct effects were observed only when steroid was bound to transcortin. These data suggest that transcortin is a prohormone which is activated by binding of steroid [10] and realizes any unknown functions at the membrane level or inside of the cell.

Attempts to isolate membrane components responsible for transcortin binding revealed a 20 kDa sialoglycoprotein in plasma membranes of human decidual endometrium [12]. This protein is clearly different from asialoglycoprotein receptor of liver [13] which participates in protein metabolism [14] and has been shown to bind asialo-

Abbreviations: DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DMS, dimethyl suberimidate; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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transcortin [15]. Further studies have shown that transcortin can form complexes with two or four molecules of the binding protein although the existence of this protein in the form of a dimer or tetramer has not been demonstrated [16]. It has been reported that two other membrane proteins are presumably involved with transcortin recognition [4], but no further evidence has been obtained that any protein except the 20 kDa sialoglycoprotein participates in transcortin recognition at the membrane surface. Recently we have shown the presence of a transcortin-binding protein in plasma membranes from human syncytiotrophoblast [17,18], that is similar to that from decidual endometrium. Both of these proteins are sialoglycoproteins, have the same molecular mass, interact with transcortin with similar affinity and these interactions are steroid dependent. In the present report we studied the chromatographic behavior of the transcortin-binding protein and its transcortin complex and used cross-linking with bifunctional reagents to elucidate the organization of the transcortin-binding site on syncytiotrophoblast plasma membranes.

2. Materials and methods

2.1. Chemicals

Sepharose CL 4B, CNBr-activated Sepharose 4B and protein standards for electrophoresis and chromatography were purchased from Pharmacia (Uppsala, Sweden), 1,5-difluoro-2,4-dinitrobenzene (DFDNB), dimethyl suberimidate (DMS), Iodo-Gen, and Iodo-Beads were purchased from Pierce (Rockford, IL, USA). Others chemicals were obtained from Serva (Heidelberg, Germany).

2.2. Transcortin

Transcortin was isolated from human postpartum serum using affinity chromatography on cortisol-Sepharose [19]. Radiolabeled transcortin was prepared using $Na^{125}I$ and Iodo-Gen as previously described [20]. Labeled transcortin was purified from the reaction mixture by chromatography on a Bio-Gel P-6 column (Bio-Rad, Richmond, CA, USA). Additional purification was performed on a Superose 12 column using an FPLC system (Pharmacia). Specific activity of the labeled protein preparations was approx. 50 μCi/μg and the radiochemical purity determined by precipitation with trichloroacetic acid was 99%. More than 95% of labeled transcortin in each preparation was bound by monospecific anti-transcortin antibodies. Both, labeled and unlabeled transcortin preparations yielded one discrete band after native and SDS-electrophoresis.

2.3. Membrane preparation

A microvesicular fraction was prepared from the syncytiotrophoblast brush border of human term placenta as earlier described [21]. The preparation displayed a high activity of the plasmalemma marker enzyme 5′-nucleotidase (EC 3.1.3.5) [22] and had a normal vesicular structure as was shown by electron microscopy. Labelling of the membranes was carried out using Iodo-Beads [23]. Membranes (about 10 mg membrane protein) were suspended in standard buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl) and incubated overnight with five Iodo-Beads and carrier-free $Na^{125}I$ (5 mCi) at 4° C with constant stirring. After that, Iodo-Beads were removed and the membranes were sedimented by centrifugation (25 000 × g, 15 min). The membrane pellet was washed with the standard buffer containing 0.15 M KI to replace non-reacted $Na^{125}I$ (three times with 50 ml buffer each) and with the standard buffer (three times with 50 ml buffer each) in order to remove non-reacted radionuclide. The labeled membrane pellet was suspended in the standard buffer (final volume of 2.5 ml) and solubilized by adding 2.5 ml of a 10% sodium cholate water solution and incubating this mixture for 3 h at 4° C with shaking. The mixture was then centrifuged (25 000 × g, 15 min), the supernatant was 10-fold diluted with the standard buffer containing $5 \cdot 10^{-6}$ M cortisol and was used for receptor isolation.

2.4. Isolation of transcortin-binding protein

Transcortin-binding protein was isolated from syncytiotrophoblast plasma membranes by affinity chromatography on transcortin-Sepharose as previously described [16]. The affinity resin was prepared by immobilization of transcortin on CNBr-activated Sepharose [24]. The transcortin content was 0.25 mg per ml settled gel volume as was determined by its capacity to bind cortisol. A column was packed with approx. 5 ml of the resin and was equilibrated with the standard buffer. The membrane solubilizate was applied to the column at a flow rate of 3 ml/h. To remove non-specifically adsorbed radioactive material, the column was extensively washed with the standard buffer containing 1 mg/ml ovalbumin and $5 \cdot 10^{-6}$ M cortisol until the radioactivity eluting from column was decreased to about 50 000 cpm per ml of eluate. Elution of the transcortin-binding protein was then performed using standard buffer containing 1 mg/ml transcortin and $5 \cdot 10^{-6}$ M cortisol. The elution rate was 2 ml/h. In another experiment the elution was done using standard buffer containing 1 mg/ml ovalbumin and $1 \cdot 10^{-5}$ M testosterone [18]. The affinity eluate was desalted by filtration through Bio-Gel P-6, freeze-dried and stored at −20° C for no longer than one week.

2.5. FPLC

A Superose 12 column and an FPLC system (Pharmacia, Uppsala, Sweden) were used. Samples (0.1 ml, about 25 000–30 000 cpm of affinity eluate) were applied to the column in 0.05 M Tris-HCl buffer (pH 7.4) with a flow

rate of 0.2 ml/min. Fractions of 0.4 ml were collected and radioactivity in the fractions was measured at 80% efficiency using an RIA Gamma counter (LKB-Wallac, Turku, Finland).

2.6. Cross-linking experiments

Cross-linking of transcortin-binding protein with DMS was carried out essentially as described by Davies and Stark [25]. To cross-link the complex of transcortin and its binding protein with DFDNB, a solution of DFDNB in dimethyl sulfoxide was added to 0.2 ml of the affinity eluate (about 50 000 cpm) in Krebs-Ringer bicarbonate buffer (pH 7.4) to a final concentration 0.25 mg/ml. The mixture was then incubated for 4 h at 4° C after which 0.025 ml of 1.0 M Tris-HCl buffer (pH 7.4) was added to the sample and the sample was incubated for 30 min at room temperature. The cross-linked sample was desalted by filtration through Bio-Gel P-6 column and was analyzed by SDS-PAGE on a 5–8% gradient gel.

To cross-link components of the transcortin-recognizing domain, syncytiotrophoblast plasma membranes (about 10 mg membrane protein) were treated with DFDNB (0.2 and 0.02 mg/ml final concentration) in Krebs-Ringer buffer overnight at 4° C with constant stirring. Excess of non-reacted DFDNB was removed by washing with 50 ml of Krebs-Ringer buffer five times. After that, membranes were labeled, solubilized and applied to the immobilized transcortin column. After elution of the column with transcortin containing buffer, eluate was analyzed by SDS-PAGE in a 12.5% gel.

To cross-link transcortin with syncytiotrophoblast plasma membranes, ^{125}I-transcortin (4 μCi) was incubated with the membrane suspension (about 2 mg membrane protein) in 1 ml of Krebs-Ringer buffer containing $5 \cdot 10^{-6}$ M cortisol. For the control sample, a 500-fold molar excess of unlabeled transcortin was added to the membranes together with labeled transcortin. The samples were incubated overnight at 4° C with shaking; then the membranes were sedimented by centrifugation for 15 min at $12\,000 \times g$ and the supernatants were discarded. The membrane pellets were twice washed with the buffer and were resuspended in 0.2 ml of the buffer. DFDNB dissolved in dimethyl sulfoxide was added to the samples up to a final concentration of 0.2 and 0.02 mg/ml. The samples were incubated 4 h at 4° C with constant stirring. The non-reacted substances were removed by washing the membranes with the buffer (three times) and with distilled water (three times). Treated membranes were suspended in 0.2 ml distilled water and were analyzed by SDS-PAGE on a 5% gel.

2.7. SDS polyacrylamide gel electrophoresis

SDS-PAGE was performed according to Laemmli [26]. After electrophoresis each lane was cut into 0.25 cm slices and radioactivity in the slices was measured at an 80% efficiency using RIA Gamma counter (LKB-Wallac, Turku, Finland).

3. Results

3.1. Chromatographic mobility of transcortin-binding protein

Chromatography on Superose 12 was used to examine the hydrodynamic properties of the transcortin-binding protein and its transcortin complex with the aim of elucidating the stoichiometry of interaction between transcortin and its binding protein in their complex. The ^{125}I-transcortin-binding protein elutes from Superose 12 as one major peak which coincides with protein of about 43–45 kDa (Fig. 1A). A second peak was observed at the void volume of the column and corresponded to substances of about 2000 kDa; this peak disappeared when chromatography was carried out in the presence of 0.5% sodium cholate (data not shown). When chromatography was conducted in the presence of transcortin, a poorly resolved biphasic peak occurred between the void volume and the transcortin-binding protein peak (Fig. 1B). Using radioimmunoassay, we examined the chromatographic fractions for the presence of transcortin. The maximum transcortin concentration corresponded with the elution of the protein itself (fractions 31–32). Transcortin was also present in the radioactive peak corresponding to fractions 25–29. These results indicate that the radioactive peak in fractions 25–29 is a complex (or complexes) of the membrane protein and transcortin. The mobilities of the complexes correspond to proteins with molecular masses of a 120–200 kDa. The heterogeneous profile of the peak corresponding to the complexes did not permit us to determine the stoichiometry of the interaction of transcortin with its binding protein. When the ^{125}I-labeled transcortin binding protein was chromatographed in the presence of 6.0 M urea, the earlier observed 45 kDa peak disappeared and a protein with a molecular mass of about 20 kDa was detected (Fig. 1C). However, high molecular weight aggregates were present at or near the void volume of the column (Fig. 1A–C).

3.2. Cross-linking of transcortin-binding protein and transcortin

For cross-linking experiments we used DFDNB, which has been successfully applied for cross-linking of proteins in solution [27], and for cross-linking to membranes [28], and DMS which has been largely used to study non-covalent protein oligomer formation [29,30]. To study the stoichiometry of the complex between transcortin and its binding protein we cross-linked a mixture of ^{125}I-labeled transcortin-binding protein and transcortin in solution. Prior

to cross-linking, the ^{125}I-transcortin-binding protein migrated on SDS-PAGE as a single band with a relative mobility corresponding to that of trypsin inhibitor (20.1 kDa, Fig. 2A). Cross-linking with DFDNB resulted in the appearance of three additional bands corresponding to species with molecular masses of 43 kDa, 95 kDa and 140 kDa (Fig. 2B). These bands correspond to transcortin-binding protein cross-linked to itself (43 kDa) and transcortin cross-linked to one (95 kDa) and two (140 kDa) binding protein dimers. The molecular mass of transcortin is 55 kDa [31]. After cross-linking with DMS, only one additional band of 43 kDa appeared (data not shown) indicating that this reagent is capable of cross-linking the binding protein to itself, but that is not able to cross-link transcortin

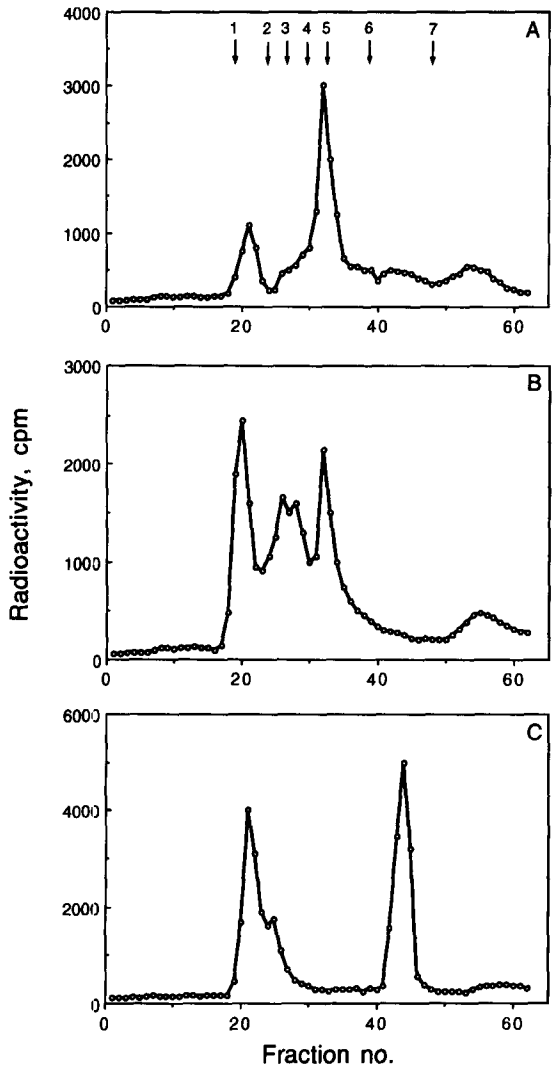

Fig. 1. FPLC of ^{125}I-labeled transcortin-binding protein on a Superose 12 column. Elution profiles of transcortin-binding protein applied in the absence of transcortin (A), together with transcortin (B) and in the presence of 6 M urea (C). Arrows indicate position of standards: dextran blue, 2000 kDa (1), catalase, 232 kDa (2), aldolase, 158 kDa (3), bovine serum albumin, 67 kDa (4), ovalbumin, 43 kDa (5), chymotrypsinogen A, 25 kDa (6), ribonuclease A, 13.7 kDa (7). During the chromatography the flow rate was 0.2 ml/min; 0.4 ml fractions were collected.

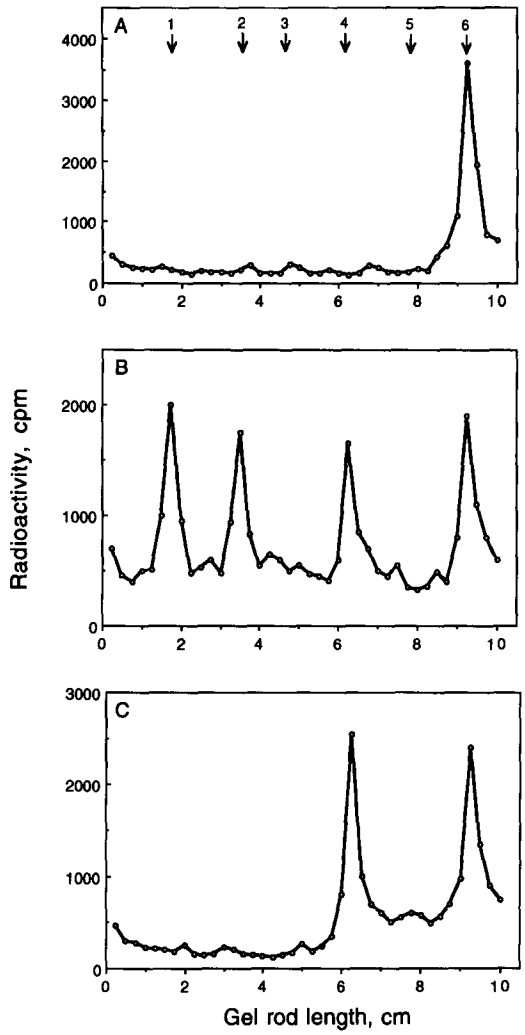

Fig. 2. SDS-PAGE of cross-linked ^{125}I-labeled transcortin-binding protein. The purified labeled protein was cross-linked in solution with DFDNB in the presence and in the absence of transcortin and the cross-linked species were analyzed by SDS-PAGE. The gel was cut into 0.25 cm slices and radioactivity of the slices was measured. (A) Purified transcortin-binding protein without cross-linking. (B) The protein after cross-linking in the presence of transcortin. (C) The protein after cross-linking in the absence of transcortin. Arrows indicate position of standards: covalently linked dimer of bovine serum albumin, 134 kDa (1), phosphorylase *b*, 94 kDa (2), bovine serum albumin, 67 kDa (3), ovalbumin, 43 kDa (4), carbonic anhydrase, 30 kDa (5), trypsin inhibitor, 20.1 kDa (6).

to the binding protein. Cross-linking of the binding protein itself (in the absence of transcortin) with DFDNB or DMS led to the appearance of one extra band corresponding to the 43 kDa binding protein dimer (Fig. 2C, shown data for DFDNB).

3.3. Cross-linking of the transcortin-binding domain on the membrane surface

To elucidate the organization of the transcortin-binding domain on syncytiotrophoblast membranes we cross-linked isolated membranes with DFDNB. After that, cross-linked

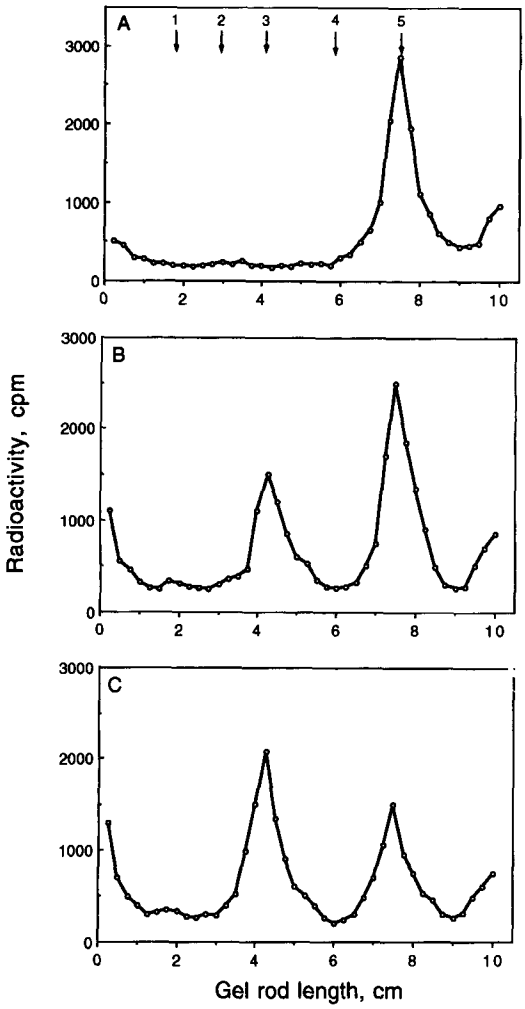

Fig. 3. SDS-PAGE of the transcortin-binding protein isolated from native and DFDNB treated membranes. (A) The protein from non-treated membranes. (B) The protein from membranes treated with 0.025 mg/ml DFDNB. (C) The protein from membranes treated with 0.25 mg/ml DFDNB. Arrows indicate position of standards: phosphorylase *b* (1), bovine serum albumin (2), ovalbumin (3), carbonic anhydrase (4), trypsin inhibitor (5).

membranes were non-specifically labeled with ^{125}I and components possessing affinity for transcortin were isolated by affinity chromatography on immobilized transcortin and analyzed by SDS-PAGE. Analysis of membrane components before cross-linking indicated a single peak (Fig. 3A) having a relative molecular mass 20 kDa which represents the transcortin-binding protein monomer. Cross-linking results in the appearance of a transcortin-binding component with a molecular mass of a 43 kDa (transcortin binding protein dimer, Fig. 3B) in addition to the 20 kDa transcortin-binding protein. Increasing the concentration of DFDNB resulted in an increase in the amount of 43 kDa dimer species and a decrease in the amount of 20 kDa species (Fig. 3C). These data indicate that transcortin-binding protein monomers can be cross-linked to each other while they are in their native position in the plasma membrane.

3.4. Cross-linking of transcortin with its membrane binding domain

To study complex formation between transcortin and membrane component(s), we used DFDNB to cross-link ^{125}I-transcortin after its binding to the membranes. To estimate non-specific interaction of transcortin with the membranes, cross-linking was performed in the presence of an excess of unlabeled transcortin. Two concentrations of DFDNB were used in these experiments −0.25 mg/ml and 0.025 mg/ml. To insure complete dissociation of membrane proteins after cross-linking, the membranes were boiled with a 2% SDS/5% 2-mercaptoethanol solution for 30 min. SDS-PAGE analysis of the membrane preparation after cross-linking with labeled transcortin revealed two bands corresponding to protein species with molecular mass of 140 kDa and 95 kDa (Fig. 4A) in addition to the

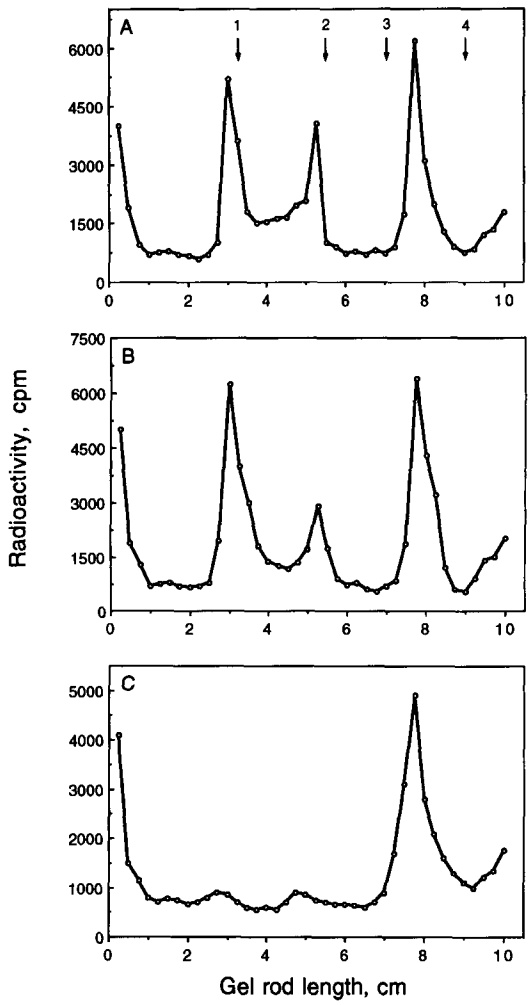

Fig. 4. SDS-PAGE of syncytiotrophoblast plasma membranes after cross-linking with labeled transcortin. (A) Cross-linking with 0.025 mg/ml DFDNB. (B) Cross-linking with 0.25 mg/ml DFDNB. (C) Cross-linking with 0.25 mg/ml DFDNB in the presence of an excess of unlabeled transcortin. Arrows indicate position of standards: covalently linked dimer of bovine serum albumin (1), phosphorylase *b* (2), bovine serum albumin (3), ovalbumin (4).

55 kDa ^{125}I-transcortin that was bound to membranes non-covalently, and was not removed during the wash. These bands would correspond to transcortin cross-linked to four and two transcortin binding proteins, respectively. Increasing the concentration of DFDNB resulted in an increase in the 140 kDa species and a decrease in the 95 kDa band (Fig. 4B). The band at the top of the gel was observed after cross-linking with labeled transcortin alone or in the presence of an excess of unlabeled transcortin (Fig. 4A–C), indicating the non-specific nature of the band. If cross-linking was performed in the presence of an excess of unlabeled transcortin (Fig. 4C), the 140 and 95 kDa bands were eliminated indicating that there are a limited number of transcortin binding sites in the membrane preparation and suggesting a specific interaction of transcortin with membrane components.

4. Discussion

We have recently demonstrated [16] that the functional form of the 20 kDa transcortin-binding protein from endometrium plasma membranes, which presumably constitutes the recognizing subunits of the transcortin membrane receptor, is a tetramer. The transcortin-binding protein which we have isolated from syncytiotrophoblast membranes has similar properties and the data suggest that it also has an oligomeric structure.

Mobility of the 20 kDa transcortin-binding protein as a 45 kDa species during gel-permeation chromatography under non-denaturing conditions indicates that the protein is organized as a dimer. Cross-linking experiments also indicate that dimerization of the protomers occurs. Homo or hetero dimers have been observed for several membrane receptors from different receptor families [32–36]. The constant of association of the protomers to form the transcortin-binding protein dimer is apparently very high since the whole pool of isolated protein is present as the dimer. Disulfide bond formation is not involved in dimerization since a single 20 kDa band is present following SDS-PAGE under both reducing and non-reducing conditions. The presence of urea which dissociates non-covalent protein complexes during gel-permeation chromatography [37,38], resulted in dissociation of the dimer into monomers thus confirming non-covalent nature of the dimer. In our experiments, formation of high molecular weight aggregates of the transcortin-binding protein has been observed. Although the tendency of membrane proteins to form aggregates is a commonly known non-specific process, the aggregation of receptors in the membrane bilayer can be the next step in receptor action after ligand binding [39]. This phenomenon is related to interactions between the aggregated protein and other cellular components [40]. We can not make definite conclusions about the nature of the aggregation in our case, but we do note, that in the presence of transcortin, higher states of aggregation occurred.

Effective cross-linking with DFDNB indicates close contact between transcortin and its binding protein and between the monomers of the binding protein, since this reagent links reactive groups located at a distance of 3–5 Å [41]. The fact that transcortin forms complexes with two and four molecules of binding protein coincides with our observation for transcortin binding protein from decidual endometrium membranes [16]. An explanation for the effectiveness of DFDNB and the ineffectiveness of DMS in cross-linking of the complexes of transcortin with its binding protein may be that DMS is able to interact only with primary amines while DFDNB has a larger spectrum of reactions [27].

When cross-linking studies were performed on the transcortin-binding protein itself, formation of tetramers did not occur. However, cross-linking studies with DFDNB in the presence of the binding protein and transcortin indicated two or four molecules of the binding protein interacted with transcortin. These observations lead us to propose that transcortin binds to two dimers of the binding protein rather than to a tetramer and that the two dimers are not in close contact to each other on the surface of the transcortin molecule. At the same time, we cannot exclude the possibility that binding protein tetramers are present when the binding protein is incorporated into the structure of plasma membrane. However, cross-linking of membranes with DFDNB also resulted in the isolation of the transcortin-binding protein primarily in the form of dimers. This observation strengthens our supposition that transcortin-binding protein interacts with transcortin as a dimer. Monomers were also observed after membrane cross-linking, this is probably due to incomplete cross-linking since increasing the concentration of DFDNB resulted in an increase in the relative content of dimer in comparison with monomer. Cross-linking of labeled transcortin with plasma membranes also provides evidence that only the dimer or two dimers, but not monomers, recognize and bind transcortin. Although on membranes transcortin can be bound to one dimer, the fact that the 140 kDa peak (Fig. 4A and B) greatly exceeds the 95 kDa peak indicates that binding to two dimers is preferable. This type of membrane receptor organization is known. For instance association of two dimers of insulin-like growth factor I receptor is required for the formation of a high-affinity binding site [42].

The above data suggest that the dimers are organized in clusters on the membrane surface. The existence of receptor clusters in the absence of ligand [39,43] or, at least the presence of adjacent receptor molecules has been observed [44]. Thus, if the dimer is the transcortin recognizing and binding unit, two dimers apparently represent the functional domain. We suggest that binding of transcortin to the two dimers initiates further processes. The other transcortin-binding protein with similar properties isolated from plasma membranes of human endometrium [12] was also demonstrated to form a complex with transcortin in

which four molecules of the protein interact with one molecule of transcortin [16]. But in endometrium membranes the protein has been found to exist as a monomer [16], while in placental membranes the transcortin-binding protein is present as non-covalent dimers. It is possible that both transcortin-binding proteins are encoded by the same gene and differences between the proteins are a result of post-translation modification. Apparently, such a difference is tissue-specific and results in formation of different transcortin-binding domains on membrane surface that may reflect differences in amount of transcortin-binding sites and/or affinity constant for transcortin binding. It could provide an additional regulatory mechanism at the tissue level. At present we have no information concerning the possible biological role of the membrane transcortin-binding proteins, but we suggest that the transcortin-binding domain is a system for transmembrane transfer of transcortin and/or is part of a signal transduction system. A number of studies [45–48] showing the presence of transcortin-like protein in the cytoplasm of several tissues provides indirect evidence for transcortin internalization mechanisms. The fact that transcortin can be taken up by syncytiotrophoblast membrane vesicles [11] also supports this suggestion. The stimulation of adenylate cyclase by transcortin [4,10] provides evidence for a signal transduction role of the transcortin-binding protein complex. Elucidation of the role of the complex of transcortin with the binding protein in the mechanism of transcortin action awaits future studies.

5. Note added in proof (received 16 February 1995)

It has been reported that, in placental membranes, there are two types of binding site for transcortin [9], which differ in affinity and binding capacity. As we have found only one type of transcortin-binding protein in these membranes, we suggest that low- and high-affinity transcortin binding reflects binding with dimers or tetramers and, apparently, binding with the latter occurs with higher affinity. If this is the case, the binding capacity of low- and high-affinity sites may indicate the amount of dimer dispersed on the membrane surface or organized in clusters.

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