Biochimica et Biophysica Acta 1794 (2009) 1725–1733

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Fluorescence studies on the interaction of choline-binding domain B of the major bovine seminal plasma protein, PDC-109 with phospholipid membranes

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ARTICLE INFO

Article history:
Received 10 November 2008
Received in revised form 4 August 2009
Accepted 5 August 2009
Available online 14 August 2009

Keywords:
BSP-A1/A2
Capacitation
Cholesterol efflux
Major protein
Fluorescence quenching
Red-edge excitation shift

ABSTRACT

The microenvironment and accessibility of the tryptophan residues in domain B of PDC-109 (PDC-109/B) in the native state and upon ligand binding have been investigated by fluorescence quenching, time-resolved fluorescence and red-edge excitation shift (REES) studies. The increase in the intrinsic fluorescence emission intensity of PDC-109/B upon binding to lysophosphatidylcholine (Lyso-PC) micelles and dimyristoylphosphatidylcholine (DMPC) membranes was considerably less as compared to that observed with the whole PDC-109 protein. The degree of quenching achieved by different quenchers with PDC-109/B bound to Lyso-PC and DMPC membranes was significantly higher as compared to the full PDC-109 protein, indicating that membrane binding afforded considerably lesser protection to the tryptophan residues of domain B as compared to those in the full PDC-109 protein. Finally, changes in red-edge excitation shift (REES) seen with PDC-109/B upon binding to DMPC membranes and Lyso-PC micelles were smaller that the corresponding changes in the REES values observed for the full PDC-109. These results, taken together suggest that intact PDC-109 penetrates deeper into the hydrophobic parts of the membrane as compared to domain B alone, which could be the reason for the inability of PDC-109/B to induce cholesterol efflux, despite its ability to recognize choline phospholipids at the membrane surface.

1. Introduction

Sperm-egg fusion is the first step in the reproductive cycle of mammals. Sperm cells undergo a series of biochemical and ultrastructural changes upon ejaculation and during their residence in the female genital tract, which are collectively referred to as capacitation [1,2]. Despite its discovery over five decades ago [3,4], the molecular mechanism of capacitation is still not clear [5,6]. However, it has been clearly established that some proteinaceous factors present in the seminal plasma play a role in the maturation of the spermatozoa, which acquire fertilizing ability during their transit through the female genital tract [2]. Among the mammals, seminal plasma proteins in the bovine system have been well investigated. The bovine seminal plasma contains a group of four closely related acidic proteins, namely BSP-A1, BSP-A2, BSP-A3 and BSP-30-kDa, which bind to the spermatozoa by specific interaction with phospholipids, especially with the choline-containing lipids, phosphatidylcholine and sphingomyelin [7]. BSP-A1 and BSP-A2 have identical primary structure and differ only in glycosylation; a mixture of these two proteins is referred to as PDC-109 [8,9].

PDC-109 is present at a concentration of ca. 15–25 mg/ml in the bovine seminal plasma and is the major protein in it; hence it is also referred to as the major protein [10]. Its 109-residue polypeptide chain consists of an *N*-terminal 23 residue segment, and two tandemly repeating fibronectin type II (FnII) domains [8,9,11]. The three-dimensional structure of PDC-109 complexed with *O*-phosphorylcholine (PrC), solved by single-crystal X-ray diffraction studies has shown that each FnII domain binds one choline phospholipid molecule and that both the binding sites are on the same face of the protein [14]. Binding of PDC-109 to sperm cells leads to an efflux of choline phospholipids[1] and cholesterol (a process known as *cholesterol efflux*) [12,13], a crucial step in capacitation, which is essential for fertilization to take place. Both the binding sites of PDC-109 appear to be necessary to stimulate *cholesterol efflux* because binding of the

Abbreviations: PDC-109/B, domain B of PDC-109; PrC, phosphorylcholine; DMPC, dimyristoylphosphatidylcholine; Lyso-PC, lysophosphatidylcholine; REES, Red-edge excitation shift; FnII, fibronectin type II; ESR, electron spin resonance; FTIR, Fourier transform infrared; PC, phosphatidylcholine; Trp, tryptophan; DMPG, dimyristoylphosphatidylglycerol; DMPA, dimyristoylphosphatidic acid; EDTA, ethylenediamine tetraacetic acid; LUV, large unilamellar vesicles; TBS-I, 50 mM Tris−HCl buffer, pH 7.4, containing 0.15 M NaCl, 5 mM EDTA and 0.025% sodium azide; Gdn.HCl, guanidinium hydrochloride; β-ME, β-mercaptoethanol

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[1] The terms 'choline phospholipids' and 'choline phospholipid molecule' are used to refer to different phospholipids bearing the phosphorylcholine moiety in the head group, e.g., diacyl phosphatidyl choline, dialkyl phosphatidyl choline, lyso phosphatidylcholine, sphingomyelin, platelet activating factor etc., which can bind to PDC-109 or PDC-109/B.

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doi:10.1016/j.bbapap.2009.08.010

domain B (which corresponds to the second FnII domain and contains residues 65–109 of the full PDC-109 protein) alone to membranes does not result in lipid efflux [13].

In order to understand the role of PDC-109 in cholesterol efflux and subsequent events leading to fertilization it is imperative to understand its interaction with phospholipids and phospholipid/cholesterol mixtures and derive structure–function relationships at the molecular level. This can be of considerable relevance to different aspects of veterinary and human medicine, such as *in vitro* fertilization and birth control. ESR spectroscopic studies by us and others have investigated the specificity of PDC-109 towards different spin-labeled phospholipid and sterol probes, characterized the effect of its binding on the phase properties and dynamic order of lipid membranes, and studied the effect of cholesterol on the lipid selectivity of this protein. These studies clearly established that while PDC-109 exhibits high specificity for choline phospholipids, it also interacts with other phospholipids such as phosphatidylserine and phosphatidylglycerol with lower specificity [15–18]. Surface plasmon resonance (SPR) studies aimed at delineating the mechanism and energetics that characterize the interaction of this protein with various phospholipids indicate that the higher affinity of PDC-109 for phosphatidylcholine (PC) results from a faster association rate constant and a slower dissociation rate constant as compared to other phospholipids [19]. Thermodynamic studies have shown that lysophosphatidylcholine (Lyso-PC) binds to PDC-109 with a 250-fold higher affinity over PrC, clearly demonstrating that although recognition of the choline moiety by the protein is an essential step for binding to occur, the glycerol moiety and acyl chains of the lipid also make positive contributions to the binding process [20]. Recently, it has been shown that PDC-109 extracts phospholipids with a phosphorylcholine head group mainly from the outer leaflet of the plasma membrane of human erythrocytes and bovine spermatozoa [21].

Despite the significant progress made in understanding the interaction of PDC-109 with phosphorylcholine and choline phospholipids, molecular features of the interaction with phospholipid membranes or assemblies such as micelles are still not very clear. Thus while FTIR studies have shown that PDC-109 undergoes conformational changes upon binding to PC membranes and ESR studies show that upon binding to lipid membranes some segments of PDC-109 get inserted into the hydrophobic interior of the membrane [15,16,22,23], it is not clear as to which regions of the protein are embedded into the membrane interior. In a very recent study employing different fluorescence approaches, we have provided evidence which suggests that the segment containing Trp-90 may penetrate into the hydrophobic interior of the membrane [24]. In the present study, these studies are extended to domain B of PDC-109 (PDC-109/B), which contains three tryptophan residues, whose fluorescence properties can be monitored to investigate changes in its conformation induced by its interaction with lipid membranes and soluble ligands. The results obtained indicate that PDC-109/B does not penetrate into the hydrophobic interior of the membrane to the same extent as the whole protein, but most likely interacts with it at regions close to the interface between the polar head group region and the hydrophobic tails.

2. Materials and methods

2.1. Materials

Acrylamide, guanidine hydrochloride, β-mercaptoethanol, phosphorylcholine (PrC), choline chloride, succinimide, cesium chloride, trypsin, citraconic anhydride and Sephadex G-50 were purchased from Sigma (St. Louis, MO, USA). Potassium iodide (KI) was purchased from Qualigens (Mumbai, India). Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), dimyristoylphosphatidic acid (DMPA) and lysophosphatidylcholine (Lyso-PC) were from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals used were of analytical grade and were obtained from local suppliers.

2.2. Purification of PDC-109

PDC-109 was purified from bovine seminal plasma from healthy and reproductively active Ongole bulls by gel filtration on Sephadex G-50 followed by affinity chromatography on DEAE Sephadex A-25, as reported earlier [15]. The purified protein was dialyzed extensively against 50 mM Tris–HCl buffer, pH 7.4, containing 0.15 M NaCl, 5 mM EDTA and 0.025% sodium azide (TBS-I) to remove the choline chloride used for elution. Concentration of PDC-109 was estimated from its extinction coefficient of 2.5 for 1 mg/ml concentration at 280 nm for 1.0 cm pathlength [25].

2.3. Isolation of domain B of PDC-109

Domain B of PDC-109 was obtained by limited proteolysis of PDC-109 as described by Bányai et al. [26]. Briefly, 100 mg of PDC-109 was dialyzed against 0.25 M borate buffer (pH 9.0) and was citraconylated with 250 µl of citraconic anhydride at 25 ºC. The pH of the reaction mixture was maintained at 9.0 by the addition of small aliquots of 1 M NaOH as required. The reaction mixture was then dialyzed thoroughly against 0.1 M ammonium carbonate buffer, pH 8.0. The citraconylated protein was digested with 2 mg of trypsin for 15 min at 25 ºC. The reaction was stopped by adding acetic acid (10% final concentration) and kept for incubation for 24 h at 37 °C to decitraconylate the protein. The decitraconylated protein was subjected to gel filtration on a Sephadex G-50 (fine) column, pre-equilibrated with 10% acetic acid. Fractions were collected and absorbance was monitored at 280 nm. The elution profile yielded a broad peak with a shoulder corresponding to ca. 10 kDa and a second sharper peak corresponding to 5 kDa, consistent with the report by Bányai et al. [26]. This 5 kDa fragment corresponds to the second fibronectin type II domain of PDC-109 and is referred to as domain B (PDC-109/B) [26,27]. Purity of domain B was assessed by SDS-PAGE on 16% gels and by reverse phase HPLC on a C-18 analytical column using a Shimadzu LC 1080 VP HPLC system (Shimadzu Corporation, Tokyo, Japan) using a gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid. The HPLC purified domain B was further characterized by ESI mass spectrometry on an Agilent 6520 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) to determine its exact mass and that of the peptides obtained from its tryptic digest.

2.4. Preparation of liposomes

Lipids dissolved in dichloromethane in a glass tube were dried under a gentle stream of nitrogen gas. After removing the traces of solvent by vacuum desiccation for over 3 h, the lipid was hydrated with TBS-I to give the desired lipid concentration. Large unilamellar vesicles (LUV) were prepared in TBS-I by sonication of the lipid suspension using a bath sonicator for 30 min at room temperature. These vesicles are likely to be heterogeneous in their size, yet they were optically clear to the naked eye and exhibited very low light scattering in the concentrations used in the fluorescence studies presented here.

2.5. Steady-state fluorescence measurements

All steady state fluorescence measurements were performed using a Spex Fluoromax-3 spectrofluorimeter at 25 °C, with both the excitation and emission band pass filters set at 3 nm. Experiments were carried out using solutions of PDC-109/B samples with $OD_{280\,nm} < 0.1$ in TBS-I. Samples were irradiated at 295 nm to selectively excite the indole side chains of Trp residues and emission spectra were recorded