Biochemistry & Analytical Biochemistry
ISSN: 2161-1009

Burkova et al., Biochem Anal Biochem 2018, 7:1
DOI: 10.4172/2161-1009.1000351

Research Article Open Access

Identification of Major Proteins of a Very Stable High Molecular Mass Multi-Protein Complex of Human Placental Tissue Possessing Nine Different Catalytic Activities

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Abstract

Human placenta is an organ protecting, feeding, and regulating the grooving of the embryo. Therefore, identification and characterization of placental components including proteins and their multi-protein complexes is an important step to understanding the placenta function.

Here we analyzed for the first time human placentas extremely stable multi-protein complex (SPC, ~1000 kDa) by MALDI MS and MS/MS spectrometry using proteins tryptic hydrolyzates after proteins separation by SDS-PAGE and 2-D electrophoresis. The formation of such a very stable complex due to the random association of several various proteins is very unlikely. It was shown that SPCs contain twelve proteins: hemoglobin, alkaline phosphatase, cytoplasmic actin, human serum albumin, chorionic somatomammotropin hormone, heat shock protein beta-1, peroxiredoxin-1, 78 kDa glucose-regulated protein, protein disulfide isomerase A3, serotransferrin, annexin A5, and IgGs. These twelve proteins have in themselves many different and important biological functions, which can be inherent for these proteins in the complex. In addition, the complex demonstrated nine different enzymatic activities: DNase, RNase, ATPase, phosphatase, protease, amylase, catalase, peroxidase (H_2O_2-dependent) and oxidoreductase (H_2O_2-independent). The efficiency of the catalysis of each of these reactions by SPC preparations from three placentas was comparable. It was shown that hydrolysis of r(pU)23, r(pA)23, and r(pC)23 leads to the formation of 1-22-mer oligonucleotides, while digestion of microRNA mirR137 is a site-specific (3A-4U > 9U-10A > 8U-9U ≥ 15U-16A) resulting in the formation of only four major products. A large number of potentially possible functions of the complex in accordance with the functions of its individual proteins are considered. Progress in the study of placental proteins complexes can promote understanding of their biological functions.

Keywords: Human placenta; Multi-protein complex; Protein complex; Catalytic activities

Abbreviations: DAB: 3,3'-Diaminobenzidine; HS: Human Serum Albumin; sTR: Serotransferrin; MALDI: Matrix-Assisted Laser Desorption/Ionization; MM: Molecular Mass; m/z and Δm/z: Ratio of Molecular Mass and Molecule Charge and Difference between these Values, respectively; MHS: α,D-maltoheptaose; pNPP: Para-Nitrophenylphosphate; RA: Relative Activity; RON: Ribooligonucleatide; SDS-PAGE: SDS Polyacrylamide Gel Electrophoresis; SPC: Stable Soluble High Molecular Mass Multi-Protein Complex; WB: Western Blotting

Introduction

The placenta of mothers is much more than a filter: it is an organ protecting, feeding, and regulating the grooving of the embryo [1,2]. Progress in the study of the placenta and its components functioning can promote the development of transplantation methods. In spite of countless data obtained, placenta study still requires clarification of many important questions and some controversial results. Approximately 15% of all pregnancies are high-risk, resulting in the birth of premature babies, to increase the number of births by cesarean section as well as to prolonged maternal hospital stay, among others [3]. Identification and characterization of placentas proteins and their complexes are is important step for understanding the placenta functions.

It was argued that most biological processes are performed by many complexes of proteins [4]. For example, many cellular processes require several proteins and enzymes, which are association form larger stable or temporary protein complexes for the increase of the efficiency, new specificity and rate of metabolic pathways [4].

Soluble or solubilized proteins of placental extracts may be divided into three categories [1]: 1) proteins associated with pregnancy; 2) placental soluble proteins; 3) placental proteins associated with membranes. Soluble placental proteins circulate in the placenta fetal, and bloodstream and they are scarcely secreted into the blood of mothers.

During the last 30 years, more than 60 soluble placental enzymes and proteins and more than 100 various solubilized antigens of the placental membranes were identified by immunochemical methods [1-12]. Some of these proteins were analyzed using different physicochemical methods including MALDI mass spectrometry and/or sequencing of the full-length cDNA [7-11]. To date, most of the described proteomic analyses were focused on the comparison of placenta protein expression profiles of normal or diseased mothers [9,11,12]. Such data do not

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Received: February 02, 2017; **Accepted:** February 28, 2018; **Published** March 09, 2018

Citation: Burkova EE, Dmitrenok PS, Bulgakov DV, Ermakov EA, Buneva VN, et al. (2018) Identification of Major Proteins of a Very Stable High Molecular Mass Multi-Protein Complex of Human Placental Tissue Possessing Nine Different Catalytic Activities. Biochem Anal Biochem 7: 351. doi: 10.4172/2161-1009.1000351

provide information concerning possible protein complexes existing in placentas and their possible biological functions.

Placental membrane-associated proteins were analyzed by SDS-PAGE and MALDI mass spectrometry [12]. 733 unique proteins and 34 known and novel heterooligomeric protein complexes were identified.

Using different methods, we have recently analyzed a possibility of an existence of multi-protein complexes in the soluble fraction of homogenates of human placentas [13]. Using gel filtration on Sepharose-4B, the extremely stable multi-protein complex (SPC, ~1000 ± 100 kDa) was separated from other placenta proteins. It was shown, that the SPC is stable in the presence of guanidinium chloride, acetonitrile, Triton X100, NaCl, and $MgCl_2$ in high concentrations. This complex dissociates only in the presence of 8 M urea, 0.5-1.0 M NaCl, and 50 mM EDTA. According to SDS-PAGE SPC contains several major and minor proteins with high, moderate, and low molecular masses, 14-79.3 kDa [13].

Using different methods including MALDI mass MS and MS/MS spectrometry after SDS-PAGE, 2-D electrophoresis, here we analyzed and identified for the first time proteins of this very stable complex. In addition, it was shown that this very stable multi-protein complex is capable of catalyzing nine different chemical reactions.

Materials and Methods

Chemicals and donors

High purity reagents (Tris, NaCl, SDS, EDTA, Bromphenol blue, glycerol, DTT, urea, Nonidet P-40, NH_4HCO_3, trifluoroacetic acid, and some other compounds) were obtained from Sigma. Sepharose 4B was bought from Pharmacia (Sweden). Ethical statement: The placenta sampling protocol was approved by the local human ethics committee guidelines (Ethics committee of Novosibirsk State Medical University, Novosibirsk, Russia), which approved this study in accordance with Helsinki ethics committee guidelines. All healthy mothers gave written consent to present of their placentas for scientific purposes. Doctors provided us that placenta samples are from donors having no history of rheumatologic, respiratory, gastrointestinal, reproductive, cardiovascular, nervous system, or autoimmune pathologies.

Stable placental multi-protein complex purification and analysis Purification of the placental multi-protein complex was performed [13]. The homogenates of placentas after removal of insoluble compounds were subjected to gel filtration on Sepharose 4B columns (50 ml) equilibrated in TBS buffer (20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl. The proteins were eluted using the same TBS buffer. Fractions (3-4 ml) were collected and used for different type of analysis. The well separated stable protein complexes from three placentas have molecular masses (MMs) ~1000 ± 100 kDa. Very high stability of SPC was confirmed using its gel filtration in drastic conditions and light scattering approach [13].

SDS-PAGE assay

SDS-PAGE analysis of the intact complex proteins before and after SPC treatment with 50 mM DTT was performed using Laemmli system: a 5-16% gradient gel containing 0.1% SDS. Before SDS-PAGE SPC preparations (40-80 μg) were incubated for 20 min at 100°C in a buffer containing Tris-HCl (50 mM; pH 6.8), 1% SDS, 0.001% bromophenol blue, 10% glycerol, 10 mM EDTA in the presence or without 50 mM DTT. Proteins were stained with Coomassie R-250. After SDS-PAGE, proteins transfer on nitrocellulose membrane was carried out [13].

Trypsinolysis of proteins after electrophoresis

Identification of SPC proteins was performed using MS and MS/MS data from MALDI-TOF mass spectrometric analysis of their tryptic hydrolyzates after standard SDS-PAGE or after 2-D electrophoresis. In the case of 2-D electrophoresis, the separation of proteins was first carried out using devices for isoelectrofocusing of proteins (Protean IEF Cell, Bio-Rad, USA). SPC and solubilizing buffer containing 8 M urea, 2% Nonidet P-40, 0.2% ampholine pH 3-10, and 50 mM dithiothreitol were added in the IEF cell (0.315 ml) according to manufacturer's procedure.

Then special linear IPG strips (pH 3-10, 18 cm, Bio-Rad, USA) were used, which tops were layered with mineral oil. Strips were dehydrated passively for 1 hour, then actively for 12 hours. Isoelectrofocusing was performed at 250 V for 15 min, then for 7 hours at 104 V. IEF strips were then incubated for 30 min in buffer containing 0.38 M Tris-HCI (pH 8.8), 6 M urea, 20% glycerol, 2% SDS, and 0.001% Bromphenol blue. Additional incubation was carried out using the same buffer, containing 100 mM iodoacetamide without DTT. After the incubation, the strips were used for standard SDS-PAGE, and protein spots on the gels were revealed by Coomassie R-250 staining.

The Coomassie-stained gel fragments after SDS-PAGE were consequently washed twice with 100 μl of water by shaking for 15 min, and for removing of the dye were twice washed for 30 min with 50 μl 50 mM NH_4HCO_3 containing 50% acetonitrile, and for removing of H_2O gel fragments were washed with 100 μl of 100% CH_3CN for 20 min. Then fragments of the gels were dried for 10 min at 30°C using vacuum evaporator. For the hydrolysis of the proteins, 20 μl of 25 mM NH_4HCO_3 containing 12.5 μg/ml of sequencing grade trypsin (Promega) was added and after mixture incubation for 45 min at 0°C the solution was removed. The gel fragments were incubated additionally with 20 μl 25 mM NH_4HCO_3 for 18 hours at 37°and solution was removed. For additional extraction of the peptides, the gel fragments were washed twice with 25 μl of 50 mM NH_4HCO_3 containing 50% acetonitrile with shaking for 15 min. Fractions obtained after three treatments of the gel were pooled, lyophilized, dissolved in 20 μl water and used for subsequent MALDI-TOF mass spectrometric analysis.

Analysis by MALDI-TOF mass spectrometry of the SPC proteins

All mass spectra were acquired with an Autoflex (Bruker Daltonics, Bremen, Germany) MALDI-TOF mass spectrometer with a nitrogen laser operated in the positive reflector mode (standard method RP 700-3500 Da.par) under the control of Flex Control software (version 3.4; Bruker Daltonics). Saturated solution α-cyano-4-hydroxycinnamic acid was used as the matrix; the acid was solved in 0.1% trifluoroacetic acid and acetonitrile (1:2). To 2 μl of the reaction mixture containing analyzed component, 2 μl of a mixture of 0.2% trifluoroacetic acid and matrix were added; 1 μl of the final mixtures were spotted on the MALDI standard steel plates, air-dried, and used for the analysis.

The analysis was performed in the automatic mode (AutoXecute - automatic Run). The spectra were externally calibrated using the Calibrate Peptide Standards; FAMS Method and a standard calibration mixture (Protein Calibration Standard I, Bruker Daltonics). The data files were transferred to Flexanalysis software version 3.4 (Bruker Daltonics) for automated peak extraction. Assignment of the first monoisotopic signals in the spectra was performed automatically using the signal detection algorithm SNAP (Bruker Daltonics).

For MS and MS/MS analyses, we used the PMF. FAMS Method and SNAP_full_process, FALIFT Method, respectively. Each spectrum

was obtained by averaging 1500-5000 laser shots (300 shots in a step) acquired at the minimum laser power. The data were analysed using BioTools (version 3.2; Bruker Daltonics). A peptide mass tolerance of 0.5 Da and a fragment mass tolerance of 0.5 Da were adopted for database searches. The m/z spectra were searched against the NCBInr and SwissProt databases using the Mascot search engine. Threshold score was 40. Protein identifications were accepted if they were established at score significantly greater than 40 (3-5 repeats) and contained at least three identified peptides, using the Mascot search engine. Further data were analyzed using 2016 SwissProt program and UniProt (http://www.uniprot.org/uniprot/).

DNase activity assay

DNase activity of SPCs was analyzed using supercoiled (sc) DNA pBluescript. The reaction mixture (20 μl) contained 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, 20 μg/ml (or 6.7 nM) supercoiled (sc) DNA, (pH 7.5), and 25 μg/ml one of three SPCs, and was incubated for 2 h at 37°C [14]. The products of cleavage were analyzed using electrophoresis in 0.8% agarose gel with the subsequent coloring of the DNA by ethidium bromide. The pictures of ethidium bromide-stained gels were captured using Sony DSC-F717 camera. The hydrolysis of scDNA leads to forming its relaxed form, which has a lower electrophoretic mobility. The initial native scDNA always contains small amount of hydrolyzed relaxed DNA. The relative intensity of DNA in different bands was analyzed by ImageQuant v5.2 (Molecular Dynamics). The SPCs activities were first determined as a decrease in the percent of scDNA converted from the initial supercoiled form to its relaxed form. The data were corrected for the distribution of DNA between these two bands in control after incubation of the plasmid in the absence of the SPCs. All initial rates were estimated within the linear regions of the time courses (15-40% of DNA hydrolysis). Taking into account the DNA content in the reaction mixture (6.7 nM), the percentage of the hydrolysis for 2 h was recalculated into the amount of hydrolyzed DNA (nM/1 h). Using the concentration of SPCs (16-25 mg/l), the specific activity of SPCs was calculated from three independent experiments as pmole DNA/h/mg of SPC.

RNase activity assay

As the substrate for analysis of RNase activity, we have used four substrates: three 5'-fluorescently labeled 23-mer homo-ribooligonucleotides (RONs): Flu-r$(pA)_{23}$, Flu-r$(pU)_{23}$ Flu-r$(pC)_{23}$ and one 23-mer microRNA (Flu-miR-137; 5'-Flu-UUAUUGCUUAAGAAUACGCGUAG) [15]. All these RONs contained fluorescent residue fluorescein (Flu) on their 5'-terminus. The reaction mixtures (10 μl) contained 50 mM Tris-HCl pH 7.5, 0.01 mg/ml one of RONs and $2 \times 10^{-5\text{-}6} \times 10^{-3}$ mg/ml one of three different SPCs. The reaction mixtures were incubated for 1 h at 37°C. After incubation, 10 μl of a denaturing buffer consisting of 8 M urea and 0.025% xylenicanol was added. To obtain markers of the oligonucleotide length, the limited alkaline hydrolysis of RONs was performed. The reaction mixture under alkaline hydrolysis contained 0.05 M $NaHCO_3$-Na_2CO_3 buffer, pH 9.5, and 0.02 mg/ml RNA. After incubation for 15 minutes at 90°C, the reaction mixture was cooled, and a volume of denaturing buffer equal to the volume of the reaction mixture was added thereto. The hydrolysis products were analyzed by electrophoresis. Electrophoresis (10 μl of mixture) was performed under denaturing conditions (20% acrylamide (30:1), 8 M urea, TBE buffer, pH 8.3 (89 mM Tris, 89 mM H_3BO_3 and 2 mM EDTA) at 800 V and 40 mA for 3 hours. The results of electrophoresis were recorded on a Typhoon FLA 9500 laser scanner (GE Healthcare). First the activity of the complexes was calculated from a decrease (%) in the amount of intact RONs in comparison control experiments without SPCs (100%) at their fixed concentrations ($2 \times 10^{-5\text{-}6} \times 10^{-3}$ mg/ml). Considering the molecular weight of each ORN, the percentage of its hydrolysis was recalculated into the amount of hydrolyzed substrate, mM ORN/h using the concentration of SPCs (6.0 mg/l), the specific activity of RNase activity of SPCs was calculated from three independent experiments as mM ORN/h/mg of SPC.

ATPase and phosphatase activities of the SPCs

For estimation of ATPase activity reaction mixture (20 μl) contained 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.3 mM EDTA, 1.0 mM ATP, and 0.05 mg/ml one of SPCs by analogy [14] with some modifications. The mixtures were incubated for 0-2 hours at 37°C; 3 μl aliquots were applied to PEI-cellulose plates, and thin-layer chromatography was performed using 0.25 M potassium phosphate buffer pH 7.0. The plates were dried and photographed. The level of ATP hydrolysis (%) was determined from the ratio of relative fluorescence of unhydrolyzed ATP and products of its hydrolysis estimated by Image Quant 5.2. The specific ATPase activity of SPCs was calculated from a decrease in the amount of intact ATP (%) at its fixed concentration, and then specific activity was expressed as M ATP/h/mg of SPC.

For analysis of phosphatase activity the reaction mixture (80 μl) contained 20 mM Tris HCl, pH 9.0, 10 mM $MgCl_2$, 5 mM para-nitrophenylphosphate (pNPP), and 0.05 mg/ml SPC. The accumulation of the colored product was measured at a wavelength of 400 nm for 0-30 s [16]. The specific activities of SPCs were calculated from an increase in optical density at 400 nm (A_{400}), and specific activity was expressed as M pNPP/h/mg of SPC using extinction coefficient equal to 18300 M^{-1} sm^{-1} [16].

Protease activity of the SPCs

Protease activity of the SPCs was measured by the standard method using azocasein as substrate [17]. The reaction mixture (30 μl) contained 50 mM Tris-HCl (pH 7.5), 3.3 mg/ml azocasein, and 0.017 mg/ml SPC. It was incubated for 20 hours at 37°C and then was stopped by adding of 24 μl of 20% trichloroacetic acid and centrifuged at 13,000 rpm for 1 min. An equal volume of 1 M NaOH was added to the supernatant and allowed mixture to stand at room temperature for 30 min.

Then the mixture was centrifuged at 13,000 rpm for 3 minutes, the supernatant was collected and the absorbance measured at a wavelength of 436 nm (A_{436}) against the buffer (50 mM Tris-HCl, pH 7.5). The optical density increase due to the elimination of azo dye from casein was measured. The specific activity was expressed as A_{436} of azocasein/h/mg of SPC.

Amylase activity of SPCs

To analyze the amylase activity of SPCs, the reaction mixture (15 μl) contained 30 mM Tris-HCl, pH 7.5, 5 mM α,D-maltoheptaose (MHS) and 0.05 mg/ml one of three SPCs. The mixtures were incubated for 24 hours at 30°C by analogy [18] with some modifications. The hydrolysis products were analyzed by ascending thin layer chromatography on Kieselgel F_{254} (Merck) plates with aluminum base in the system: acetic acid: butanol-1: water (4:12:4) [18]. The plates were dried, treated with a solution containing 12.5% concentrated H_2SO_4 in 87.5% isopropyl alcohol, dried over a heater to visualize the hydrolysis products. The specific activity of SPCs was estimated from the decrease of the initial oligosaccharide (%) taking into account distribution of its hydrolyzed forms after reaction mixture incubation without protein complexes and expressed as mM MHS/h/mg SPC.

Catalase activity assay

Measurement of the catalase activity was carried out [19]. For an accurate estimation of peroxide concentration, we have used its manganometric determination [20]. Found by us concentration of hydrogen peroxide within the error of the method (±3%) was as indicated on the manufacturer's packaging. Reaction mixture contains 50 mM K-phosphate (pH 7.0), 30 mM H_2O_2, and 0.01 mg/ml one of SPCs. Catalase activity was estimated from a decrease in A_{240} absorbance for 1-10 min at 25°C caused by the disproportionation of H_2O_2 using Varian Cary 50 UV-VIS (Agilent). Initial rates were determined from the linear regions of the kinetic curves using SPCs concentrations corresponding to linear part of the dependences upon their concentrations. Like in the case of determination of the specific activity of enzymes, this approach allowed normalization of the relative activity to any standard condition. For the calculation of the activity, the molar extinction coefficient of hydrogen peroxide (ε=81 $M^{-1}cm^{-1}$) was used [19]. The measured relative activity of IgGs was normalized to standard conditions: mM H_2O_2/min/mg of SPC.

Peroxidase and oxidoreductase activities assays

The reaction mixture (100 µl) for analysis of peroxidase (H_2O_2-dependent oxidation) and oxidoreductase (H_2O_2-independent oxidation) activities consisted of 25 mM K-phosphate (pH 6.8), 0.2 mg/ml 3,3'-diaminobenzidine (DAB), and 0.01 mg/ml one of SPCs [21]. Reaction mixtures for analysis of peroxidase activity contain 10 mM H_2O_2, while oxidoreductase activity was analyzed using the same mixture containing no H_2O_2. The reaction mixtures were incubated in cells of immunological plates in the dark at 22°C for 1-20 minutes, measuring the amount of colored product formed every 30 to 120 seconds. The optical density of the solutions (A_{450}) was determined using a Labsystems Uniskan II spectrophotometer. The reaction mixtures containing no SPCs were used as controls. Initial reaction rates were determined using the Origin 8.5 program from the slopes of the linear parts of the kinetic curves; SPC concentration corresponds to the linear sections of the reaction rate dependence on the concentration of SPC. The activity was first expressed in units of A_{450}/min/mg SPC and then as mM DAB/min/mg SPC using molar extinction coefficient of DAB oxidized product, ε=27850 $M^{-1}cm^{-1}$ [21].

Statistical Analysis

The results are reported as mean ± S.E. from at least two-three independent experiments for each sample of the complex and every catalytic activity.

Results

Isolation and analysis of placental protein complex

We have purified soluble SPCs from fresh human extracts of placentas by gel filtration on Sepharose 4B [13]. Figure 1 demonstrates a typical profile of gel filtration of concentrated extract of one fresh placenta. Similarly [13], it was shown that this complex efficiently dissociates only in the presence of 8 M urea containing 0.5-1.0 M NaCl, 50 mM EDTA.

Analysis of SPC proteins by MALDI mass spectrometry after2-D electrophoresis

The SPC with MMs about 1000 ± 100 kDa was first analyzed by standard SDS-PAGE [13]. Several minor, average, and major protein bands were revealed. Here for identification of SPC proteins we have used 2-D electrophoresis and observed 32 visible protein spots in the case of the complex from the first (SPC-1; Figure 2) and 44 protein spots

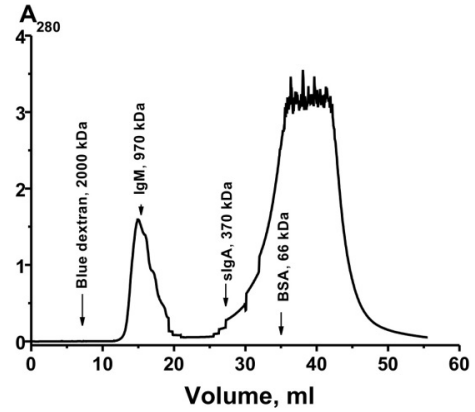

Figure 1: Gel filtration of proteins corresponding to the extract of one placenta donor on a Sepharose 4B column. The placenta extract before gel filtration was concentrated: (—), absorbance at 280 nm (A_{280}). For ddetails, see Materials and methods.

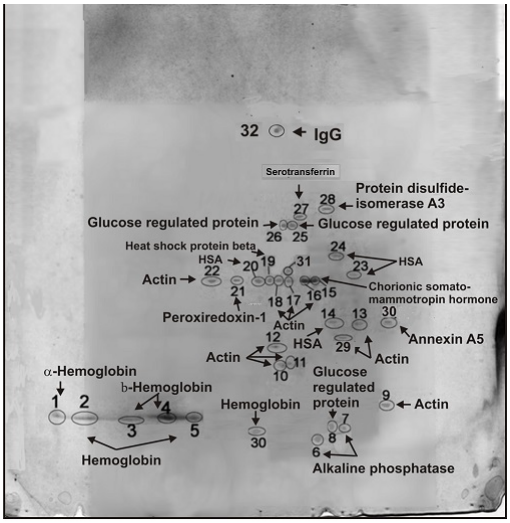

Figure 2: Two-dimensional gel electrophoresis of the SPC-1. The proteins were first separated by isoelectrofocusing and then SDS-PAGE in denaturing conditions. The spots were stained with Coomassie R-250, then cut, proteins we subjected to proteolysis for their identification using MALDI mass MS and MS/MS spectrometry. All identified proteins and their numbers are shown in the figure.

of the complex from the second placenta (SPC-2; Figure 3). Only 12 visible major and moderate proteins and their different isoforms were identified using MALDI mass MS/MS data of proteins hydrolysates corresponding to the protein sports after 2-D electrophoresis (Table 1). The data of protein identification using MS and MS/MS analysis of tryptic hydrolysates are given in Supplementary Table 1.

Interestingly, only one protein spot corresponded to heat shock protein beta-1, peroxiredoxin-1, and serotransferrin (sTR) in SPC-1 and SPC-2. Several proteins were represented by one spot in the case of SPC-1, but 2-4 spots for SPC-2: chorionic somatomammotropin hormone, IgG, annexin A5, protein disulfide isomerase A3. All other spots correspond to different isoforms of placenta proteins (number of spots for SPC-1 and SPC-2, respectively): hemoglobin (6 and 6), alkaline phosphatase (2 and 2), 78 kDa glucose regulate protein (3 and 2), cytoplasmic actin (11 and 14), HSA (4 and 6) (Table 1). Four of these proteins (sTR, annexin A5, IgG, and HSA) were identified not only using MALDI spectrometry but also additionally by Western

blotting (WB) after SDS-PAGE (Figure 4 and Table 1). The fact that stable complexes from different placentas contain mainly a limited number of the same proteins may be indicative of their non-random but specific association.

Enzymatic activities of the SPCs

The SPCs could contain not only proteins but also different

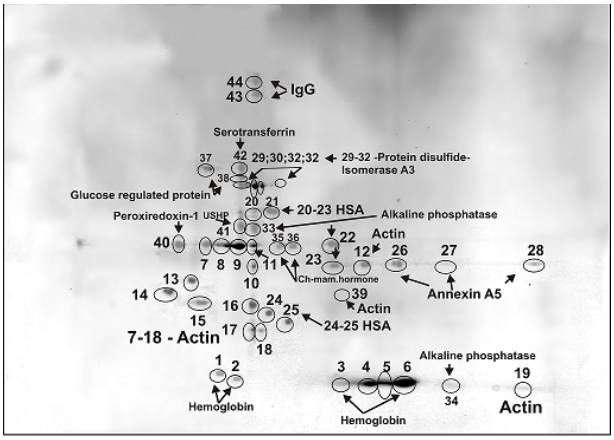

Figure 3: Two-dimensional gel electrophoresis of the SPC-2. The proteins were first separated by isoelectrofocusing and then SDS-PAGE in denaturing conditions. The spots were stained with Coomassie R-250, then cut, proteins we subjected to proteolysis for their identification using MALDI mass MS and MS/MS spectrometry. All identified proteins and their numbers are shown in the figure.

Number of protein	Identified protein	Numbers of protein bands (the amount of spots)		Methods of identification		
		Placenta 1	Placenta 2			
1	Hemoglobin subunit beta+alpha+gamma	1-5, 30 (6)	1-6 (6)	MS[a]	MS/MS[b]	
2	Alkaline phosphatase	6, 7 (2)	33, 34 (2)	MS[a]	MS/MS[b]	
3	78 kDa glucose regulate	8, 25, 26 (3)	37, 38 (2)	MS	MS/MS	
4	Protein Actin, cytoplasmic	9-13, 16-18, 22, 29, 31 (11)	7-19, 39(14)	MS	MS/MS	
5	Human serum albumin	14, 20, 23, 24 (4)	20-25 (6)	MS	MS/MS	IB[c]
6	Chorionic somato-mammotropin hormone	15 (1)	35, 36 (2)	MS	MS/MS	
7	Heart shock protein beta-1	19 (1)	41 (1)	MS	MS/MS	
8	Peroxiredoxin-1	21 (1)	40 (1)	MS	MS/MS	
9	Serotransferrin	27 (1)	42 (1)	MS	MS/MS	IB
10	Protein disulphide isomerase A3	28 (1)	29-32 (4)	MS	MS/MS	
11	Annexin A5	30 (1)	26-28 (3)	MS	MS/MS	IB
12	IgG	32 (1)	43, 44 (2)	MS	MS/MS	IB

[a]MS – identification on the basis of a set of different peptides of proteins tryptic hydrolysates.
[b]MS/MS according to the sequences of the peptides (from three to eleven peptides).
[c]Analyzed not only my by MS/MS, but also using immunoblotting (IB).

Table 1: Proteins of the stable complexes purified from placentas 1 and 2 (Figures 2 and 3).

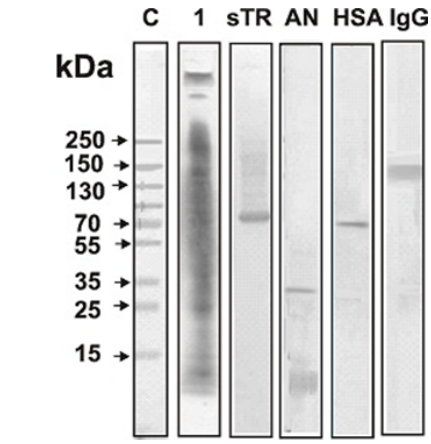

Figure 4: Immunoblotting analysis of SPC-2 proteins. After SDS-PAGE, proteins transfer were transferred on nitrocellulose membrane. The membrane was colored with silver (lane 1) or treated with monoclonal mouse Abs (conjugated with horseradish peroxidase) against human serotransferrin (LF), annexin A5 (AN), IgG, and HSA. The arrows (lane C) show the positions of molecular mass markers.

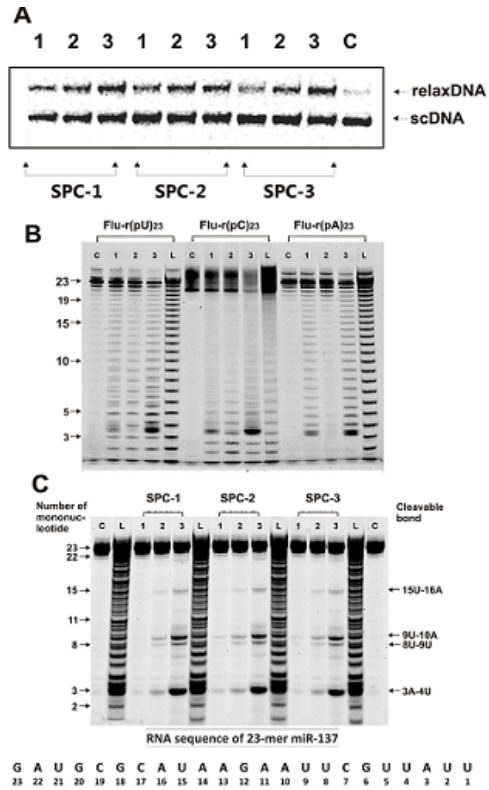

Figure 5: Analysis of DNase activity of three SPCs (lanes 1–3) in the hydrolysis of supercoiled (sc) DNA plasmid resulting in a formation of relaxed plasmid (relaxDNA) (A). scDNA was incubated at 37°C for 2 h with the SPCs; lanes 1-3 correspond to 1.6×10^{-2}, 2.1×10^{-2}, and 2.5×10^{-2} mg/ml of three SPCs, respectively. Lane C corresponds to scDNA incubated in the absence of the complex. The patterns of Flu-r(pU)$_{23}$, Flu-r(pC)$_{23}$, and Flu-r(pA)$_{23}$ hydrolysis for 1 h by 6×10^{-3} mg/ml SPC-1 (lanes 1), SPC-2 (lanes 2), SPC-3 (lanes 3) (B).The patterns of Flu-miR-137 hydrolysis for 1 h by SPC-1, SPC-2, SPC-3 in different concentrations: lane 1-2 × 10^{-5}, lane 2-2 × 10^{-4}, and lane 3-2 × 10^{-3} mg/ml (C). Lanes C correspond to RONs incubated without SPCs, while lanes L - to the mixture of RON after its statistical alkaline hydrolysis. The products of hydrolysis were detected by their fluorescence due to the fluorescent residue (Flu) on their 5'-ends. The lengths of the products of RONs hydrolysis by each preparation are indicated in the panels.

enzymes. In addition, the active sites catalyzing different reactions can sometimes be formed at the interface of proteins possessing no catalytic activities in their free states. Sometimes some proteins in an individual state may have an incompletely formed active center with a very weak activity. The formation of a complete centre with increased activity can occur at the junction of subunits of different enzymes or proteins [4]. Here it was shown that three SPCs possess nine different activities: DNase, RNase, ATPase, phosphatase, protease, amylase, catalase, peroxidase (H_2O_2-dependent oxidation of substrate) and oxidoreductase (H_2O_2-independent oxidation of substrate) activities.

The relative activity (RA) in scDNA hydrolysis of three SPCs was estimated (Figure 5A). These values were comparable in the case of all three preparations (Table 2). Figure 5B demonstrates the patterns of Flu-r(pU)$_{23}$, Flu-r(pC)$_{23}$, and Flu-r(pA)$_{23}$ hydrolysis by three SPC preparations. It can be seen that the hydrolysis of these 23-mer homo-ribooligonucleotides (homo-RONs) by all three preparations occurs almost statistically with the formation of products having a length from 1 to 22 nucleotide units. Only the cleavage products at one of all sites corresponding to the 3N-4N sequences from the 5'-end of all three Flu-r(pN)$_{23}$ are major. The relative RNase activities of all three SPCs are to some extent comparable, but still, SPC-3 more efficiently hydrolyzes all three homo-RONs. Interestingly, unlike homo-RONs, hydrolysis of hetero-microRNA Flu-miR-137 is a site-specific in the case of all three SPCs (Figure 5C). Hydrolysis of Flu-miR-137 occurs mainly on four sites: 3A-4U > 9U-10A > 8U-9U ≥ 15U-16A. It should be mentioned, that all SPCs hydrolyze Flu-r(pC)$_{23}$ less efficiently than Flu-r(pU)$_{23}$ and Flu-r(pA)$_{23}$ (Figure 5B), while the major cleavage sites of Flu-miR-137 correspond only after A or U nucleotides. One cannot exclude, that hydrolysis of RONs before and after C-base is thermodynamically less favorable. The relative activities of the SPC preparations in the hydrolysis of RONs are summarized in Table 2.

SPCs efficiently hydrolyze ATP (Figure 6A) and p-nitrophenylphosphate (Figure 6B). Figure 6C shows kinetic curves of optical density changes in the reaction of proteolytic hydrolysis of azocasein by three SPCs. All three SPCs possess amylase activity (Figure 6D). The RAs of the SPCs in the hydrolysis of ATP, p-nitrophenylphosphate, azocasein, and maltoheptaose are summarized in Table 2.

	Type of reaction	SPC-1	SPC-2	SPC-3
1	DNase (pmole DNA/h/mg	88.0 ± 7.0	62.0 ± 3.0	71.0 ± 5.0
	Flu-r(pU)23 (mM ORN/h/mg)	93.3 ± 9.0	98.3 ± 9.2	121 ± 11.4
2	RNase Flu-r(pA)23 (mM ORN/h/mg)	58.2 ± 5.0	25.3 ± 2.1	88.5 ± 8.0
	Flu-r(pC)23 (mM ORN/h/ mg)	37.3 ± 3.1	39.7 ± 3.6	79.3 ± 6.9
3	Flu-miR-137 (mM ORN/h/ mg)	193.8 ± 18.1	201 ± 17.0	201 ± 17.2
	ATPase activity (M ATP/h/mg)	0.12 ± 0.01	0.33 ± 0.01	0.09 ± 0.01
4	Phosphatase activity (M pNPP/h/mg)	0.16 ± 0.01	0.14 ± 0.01	0.1 ± 0.01
5	Protease activity (A436 azocasein /h/mg)	2.8 ± 0.2	4.9 ± 0.2	2.2 ± 0.17
6	Amylase (mM MHS/h/mg)	16.7 ± 1.3	38.9 ± 2.1	30.6 ± 2.5
7	Catalase activity (mM H_2O_2/min/mg)	1.2 ± 0.10	6.5 ± 0.5	16.0 ± 1.4
8	Peroxidase activity (mM DAB/min/mg)	11.2 ± 1.0	11.1 ± 0.7	11.5 ± 0.8
9	Oxidoreductase activity(mM DAB/min/mg)	22.8 ± 1.6	2.9 ± 0.17	9.2 ± 0.7

[a]For all specific activities of three SPCs a mean value and deviation of three independent measurements is reported.

Table 2: Specific activities of three SPCs in the catalysis of nine different reactions[a].

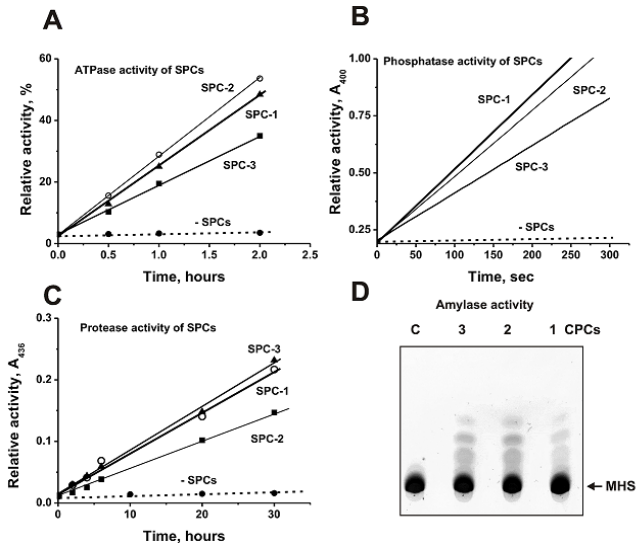

Figure 6: Typical kinetic curves characterizing ATPase (A), phosphatase (B), protease (C) and amylase (D) activities of three SPCs. SPCs (0.05 mg/ml (A and B), 0.017 mg/ml SPC (C) and (D) and substrates (1.0 mM ATP and *para*-nitrophenylphosphate; 3.3 mg/ml azocasein, and 5 mM maltoheptaose) were used in different concentrations.

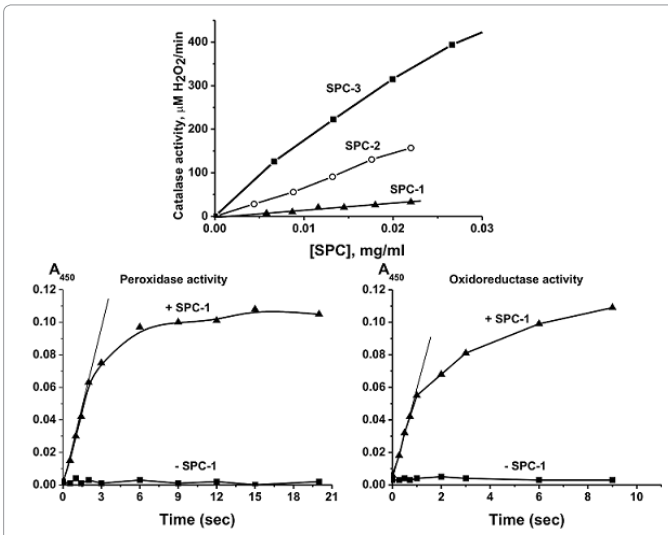

Figure 7: Dependence of the relative catalase activity on the concentration of three SPCs at fixed 30 mM concentration of H_2O_2 (A). Typical time-dependent changes in the formation of the product of DAB (0.55 mM) oxidation by SPC-1 in the presence (peroxidase activity) (B) and in the absence (C) of H_2O_2 (peroxidase activity). The A_{450} changes in the presence and the absence of SPC-1 are shown.

All three SPCs possess catalase (Figure 7A), peroxidase (H_2O_2-dependent oxidation of 3,3'-diaminobenzidine) (Figure 7B), and oxidoreductase (H_2O_2-independent oxidation of the substrate) activities (Figure 7C), the RAs of which are given in Table 2.

Discussion

Recently we have shown, that human placentas contain a very stable complex of proteins (~1000 kDa), which can be destroyed only using very drastic conditions. In this article using 2-D electrophoresis, MS and MS/MS MALDI mass spectrometry data we have established for the first time that this complex consists of twelve proteins: hemoglobin subunits, alkaline phosphatase, cytoplasmic actin, human

serum albumin, chorionic somatomammotropin hormone, heat shock protein beta-1, peroxiredoxin-1, 78 kDa glucose-regulated protein, protein disulfide isomerase A3, serotransferrin, annexin A5, and IgGs (Table 1). Each of these proteins in themselves possesses multiple very different biological functions (see below).

In this article, it was shown, that the stable complex possesses nine different enzymatic activities: DNase, RNase, ATPase, phosphatase, protease, amylase, catalase, peroxidase (H_2O_2-dependent oxidation of substrate) and oxidoreductase (H_2O_2-independent oxidation of substrate) activities (Table 2).

It is known that some proteins of biological fluids and cells in themselves do not possess any enzymatic activities. At the same time, they can exhibit certain properties of enzymes or acquire other new biological functions after the formation of oligomeric complexes with other proteins and/or enzymes [4]. In addition, new active centers of enzymes may often be formed at the interface of different protein globules having in the individual state only partial fragments of final active centers of oligomers. Sometimes some proteins after the formation of specific complexes with others ones can acquire a function of effectors that enhance the efficiency of the catalysis or binding with specific ligands [4]. Some of the revealed nine enzymatic activities of the SPC can be provided by proteins located directly on the surface of the complex or in its deeper layers, which are accessible for enzyme substrates.

What possible biological function of detected enzymes and proteins of SPCs is not yet clear. However, the inclusion in a stable complex of twelve different proteins powerfully expands its possible polyfunctional biological functions including specific functions of individual proteins and their possible associates. Considering this, it is reasonable to note potentially presumable biological functions of the SPC in accordance with the functions of its individual proteins.

Thus, the stable complexes contain alkaline phosphatase, which very effectively catalyzes the hydrolysis of p-nitrophenylphosphate (Figure 6B). Phosphatase plays an integral role in metabolism within the liver and development within the skeleton [22].

The complex contains several proteins that are themselves active in the hydrolysis of DNA and RNA. It was shown, that, human serum albumin has RNase activity [23-25], while IgGs possess relatively low DNase and RNase activities [26-29]. However, it is yet not clear which of these proteins or any combination of the complex proteins can hydrolyze specifically Flu-miR-137 only on four sites (Figure 5C). It cannot be ruled out, that some associates of these two proteins or some of their associates with other ones entering the complex can form active sites for specific hydrolysis of Flu- miR-137. HSA is an important major protein of human blood having very important physiological and biochemical functions [30]. It is responsible for maintaining osmotic pressure, and it may influence on microvascular integrity as well as aspects of the inflammatory pathway, including neutrophil adhesion and the activity of cell signaling moieties; it possesses general antioxidant functions and protection from lipid peroxidation. IgGs have four major effector functions: 1) neutralization of antigens, 2) antibody opsonization, 3) complement fixation, and 4) antibody dependent cell-mediated cytotoxicity [30].

In addition to DNase and RNase activities, human IgGs with catalytic activities (abzymes) are also possess very well detectable ATPase, amylase, and protease activities [26-29]. Thus, these proteins of the SPCs can catalyze the hydrolysis of ATP, oligosaccharides, and azocasein. However, one cannot exclude, that any associates of these proteins or their complexes with other enzymes or proteins can also catalyze these reactions.

The specific ATPase and phosphatase activities of three SPCs are given in Table 2. Classical ATPases are enzymes of the plasma membrane of all animal cells, which specifically accumulates potassium ions in the cell and pumps sodium ions outward using ATP energy for this work [31,32]. In known specific protein complex containing ATPase, this enzyme powers lipopolysaccharide transport from the cytoplasmic membrane across the cell envelope [33]. Classical amylases have at least three distinct biological functions including digestion of carbohydrates, may contribute to bacterial clearance and nutrition and an important role in the adhesion of alpha-amylase-binding bacteria [34]. Typical proteases mediate different processes including blood coagulation, an effect on immune function, bone formation, maturation of prohormones, apoptosis, antigen presentation and leukocyte migration, and the recycling of cellular proteins that are no longer needed [35].

Like horseradish peroxidase, some other proteins can also efficiently decompose hydrogen peroxide demonstrating not only peroxidase but also catalase activity [36-38]. It can be assumed that in the catalysis of degradation of hydrogen peroxide, as well as the oxidation of 3,3'-diaminobenzidine (DAB) in the presence and absence of H_2O_2 can participate several proteins that enter the stable complex: hemoglobin, peroxiredoxin-1, HSA, and IgGs. It was shown, that hemoglobin possesses peroxidase activity [39-41]. Peroxiredoxins have catalase and peroxidase activities [42-44]. In the presence of H_2O_2 human serum albumin is capable with low efficiency to oxidize DAB [45,46]. It was revealed, that IgGs (abzymes) from healthy humans possess catalase, peroxidase, and oxidoreductase activities [26-29].

Hemoglobin releases the oxygen to permit an aerobic providing energy to power the functions of the organism metabolism [39]. Perodixins are antioxidant enzymes, which reduce H_2O_2 and alkyl hydroperoxides and may play an antioxidant protective function in cells, and may contribute to the antiviral activity of CD8 (+) T-cells [47]. 78 kDa glucose regulating protein is a heat shock protein of endoplasmic reticulum of cells involved in protein folding; it plays a role in cancer cell proliferation [48]. Cytoplasmic actin participates in several important cellular processes, including cell signaling, cell motility, cell division and cytokinesismuscle contraction, organelle and vesicle movement, and the establishment and maintenance of cell shape and cell junctions [49]. Chorionic somatomammotropin hormone (or human placental lactogen or chorionic growth hormone prolactin) affects metabolic homeostasis by regulating key enzymes and transporters associated with glucose and lipid metabolism in several target organs [50]. In the lactating mammary gland, it increases the production of milk proteins, lactose, and lipids. High-mobility group box 1 is a nuclear non-histone DNA binding protein with key roles in maintaining nuclear homeostasis [51]. Heat shock protein beta 1 is a member of the small heat shock protein family, and it is involved in a wide variety of cellular processes. It was originally described as an intracellular chaperone able to stabilize the actin cytoskeleton in response to various stresses. Annexin A5 is commonly used for detection of apoptotic cells by its ability to interact with phosphatidylserine, a known marker of cell apoptosis when it is on the outer leaflet of the plasma membrane. All functions of the protein are unknown exactly, but, annexin A5 has been proposed to play a role in the inhibition of blood coagulation due to competing for phosphotidylserine binding sites with as well as to inhibit the activity of phospholipase A1 [52]. Protein disulfide isomerase catalyzes the formation and breakage of disulfide within proteins as they fold [53]. Serotransferrin is iron binding transport protein also having a role in stimulating cell proliferation [54].

One cannot exclude that all or at least part of these plenty different functions including enzymatic ones of the twelve complex proteins can be important for providing a set of SPC multifunctional properties in the female placenta. In addition, these different functions of SPC and its proteins may be important for the development of protective functions of the mother's organisms, as well as well for protecting the embrio from any harmful factors.

In overall, we have here shown for the first time that soluble fraction of placental proteins contained an extremely stable complex containing twelve proteins and analyzed the SPC catalytic functions.

Acknowledgments

This research was supported by grants from Russian Foundation for Basic Research (16-04-00609), the Presidium of the Russian Academy of Sciences (Molecular and Cellular Biology Program, 6.2), and funds from the Siberian Division of the Russian Academy of Sciences (1.7.15).

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