Molecular Cloning and Characterization of New Placental Proteins

PP17 family - PP17a; PP17b / TIP47; PP17d
and
PP13 - human placental galectin / lysophospholipase

Doctoral Dissertation
&
PhD Thesis

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CONTENTS

CHAPTER 1: INTRODUCTION

1.1. Pregnancy-related protein research

"Pregnancy-related proteins" are more or less specific for pregnancy. They normally do not occur in the non-pregnant state or only in much lower concentrations. Part of these proteins are secreted into the fetal or maternal blood or amniotic fluid, or appear in the maternal urine. Others are mainly found in fetal or placental tissues without secretion. Most of the pregnancy-related proteins originate from the placenta, the others are synthesized by the mother or by the fetus. Their physiological roles are: in assisting at the blastocyst's embedding, in regulating the growth of the fetus and placenta, in preventing rejection of the fetal allograft, and in regulating the balance between coagulation and fibrinolysis and thus maintaining pregnancy. The physiological function of pregnancy-related proteins is in most cases unclear. Some are grouped as hormones, growth-factors, enzymes, activators and inhibitors, receptor proteins, binding and storage proteins, or structural proteins.

The pregnancy-related proteins were discovered through comparative examinations of pregnant and non-pregnant samples. As early as 1958 Thornes (using anti-placenta immunoserum) observed four components of the serum of pregnant women which were not present in the blood of healthy non-pregnant controls (Thornes, 1958). The improvement of separation techniques during the last 40 years provided considerable opportunities for the identification of protein components found only in pregnant serum. A good example of this is the "pregnancy-zone protein" discovered by Smithies using Starch gel electrophoresis, which protein is now known as pregnancy-associated α_2-glycoprotein (Smithies, 1959; Than et al., 1974; Berne et al., 1975).

Of the placental hormones and enzymes, human chorionic gonadotropin (hCG) (Ascheim and Zondek, 1927; Morgan et al., 1975), human placental lactogen (hPL) (Ehrhart, 1936; Ito and Higashi, 1961; Josimovich and MacLaren, 1962; Li et al., 1971; Sherwood et al., 1971) and heat-stable alkaline phosphatase (Ahmed and King, 1960; Fishman et al., 1972; Lehmann, 1975; Nozawa and Fishman, 1982; Stigbrand et al., 1982) have been known for several decades. Since the '70's the number of these pregnancy-related proteins has grown steadily. In addition to three fetal and seven pregnancy proteins, Hans Bohn has isolated 20 solubilized or membrane associated placental proteins (MPs) and 26 soluble placental tissue proteins (PPs) (Bohn, 1985, 1991). He also performed detailed physico-chemical characterization of the pure antigens, generating the antisera in rabbits. Since that time 56 different pregnancy-related proteins have been discovered and classified in our book *Advances in Pregnancy Related Protein Research* (Than et al., 1993). These proteins were divided into different categories according to their occurrence: fetal-, pregnancy-, soluble placental tissue- and membrane-associated placental proteins were found, and many were also investigated by our research groups **(Table 1)**.

Table 1.

FETAL PROTEINS

1	AFP	α-Fetoprotein
2	FA-1	Fetal Antigen 1
3	FA-2	Fetal Antigen 2

PREGNANCY PROTEINS

1	SP1	Pregnancy-Specific β_1-Glycoprotein (PSβG)
2	SP2	Sex-Hormone-Binding Globulin (SHBG)
3	SP3	Pregnancy-Associated α_2-Glycoprotein (α_2PAG) or Pregnancy Zone Protein (PZP)
4	PAPP-A	Pregnancy-Associated Plasma Protein A
5	PAPP-B	Pregnancy-Associated Plasma Protein B
6	β_1-PAM	Pregnancy-Associated β_1-Macroglobulin
7	α_2-PAM	Pregnancy-Associated α_2-Macroglobulin

SOLUBLE PLACENTAL TISSUE PROTEINS

1	PP1	
2	PP2	Ferritin
3	PP3	Flavin-Containing Placental Protein
4	PP4	Placental Coagulation Inhibitor (Annexin V)
5	PP5	Serine Protease Inhibitor
6	PP6	19 S-α_1-Glycoprotein
7	PP7	Placental Glutathione S-Transferase
8	PP8	
9	PP9	Placental Aldose Reductase
10	PP10	Plasminogen Activator Inhibitor 2 (PAI-2)
11	PP11	Placental Serine Protease
12	PP12	Insuline-Like Growth Factor Binding Protein 1 (IGFBP-1)
13	**PP13**	**Placental Lysophospholipase**
14	PP14	β_1-Lactoglobulin Homologue (Glycodelin-A)
15	PP15	
16	PP16	
17	**PP17**	**Cargo Selection Device for Mannose-6-Phosphate Trafficking**
18	PP18	
19	PP19	
20	PP20	
21	PP21	Membrane Cofactor Protein (MCP)
22	PP22	Cellular Thyroid Hormone Binding Protein
23	PP23	
24	PP24	Human Sphingolipid Activator Protein 1 (SAP-1)
25	PP25	
26	PP26	

Table 1.

SOLUBILIZED OR MEMBRANE-ASSOCIATED PLACENTAL PROTEINS

1	MP_1	Heat-Stable Alkaline Phosphatase (HSAP)
2	MP_2A	
3	MP_2B	
4	MP_2C	Membrane Cofactor Protein
5	MP_2D	
6	MP_2E	
7	MP_2F	
8	MP_2G	
9	MP_2H	
10	MP_2I	
11	MP_2K	
12	MP_2L	
13	MP_3	Laminin
14	MP_4	
15	MP_5	
16	MP_6	
17	MP_7	
18	MP_8	
19	MP_9	
20	MP_{10}	

1.2. Aims of the study

Of the 26 soluble placental tissue proteins previously identified in the literature, the amino acid sequence and physiological function had been identified for only fourteen. Science had had only patchwork information on these proteins, which are produced in the placenta during pregnancy and appear only in trace amounts after delivery. Similarly, knowledge is fragmentary of pregnancy proteins in cancer patients and their reexpression observable in body fluids; probably even less is known of the physiological function and significance of these proteins.

Since 1958 the Women's Clinic of the University Medical School of Pécs has been involved in the examination of pregnancy-, placental-, and endometrial proteins, in the form of inter-institutional and international collaborations. Jointly with the Biochemical Institute, basic and applied research was conducted, identifying 9 proteins and elaborating assay methods, as well as examining the behavior of the proteins in healthy and diseased cases (Szabó et al., 1975; Than et al., 1979; Szabó et al., 1980; Than et al., 1983; Than et al., 1986; Szabó et al., 1988; Than et al., 1988; Than, 1989). Given that our molecular biology workgroup had already isolated the cDNAs of 5 proteins and defined their nucleotide sequence (Kispál et al., 1986; Evans et al., 1988; Song et al., 1989; Kispál et al., 1991; Kispál et al., 1993), and that in 1993 a high-sensitivity chemiluminescence assay was introduced, my PhD research was aimed at continuing this research through molecular-biological means. Of these placental proteins, of which still little is known, molecular biological examinations were planned primarily for proteins PP13 and PP17.

Proteins PP13 and PP17 have been known for only slightly more than a decade. Their molecular weights are 16 and 30 kDa respectively. The former consists of two identical subunits; the latter of a single peptide chain. They are

glycoproteids, with known amino acid and carbohydrate compositions. The present work is aimed at a more in-depth study of these two placental proteins, including:

1) Isolation of the cDNAs of PP13 and PP17, and analysis of the nucleotide and deduced amino acid sequences.

2) Simultaneous delineation of the protein families with homology search, thereby providing a good starting point for function analysis, which had not yet begun for PP13 and PP17 when this research was begun.

3) Functional analysis of the isolated proteins.

4) Examination of the mRNA expression of PP13 and PP17 through Northern-blot analysis of tissue from healthy individuals and cancer patients.

5) Western-blot examination of the expression of the two proteins in tissue from healthy individuals and cancer patients.

6) Development of high-sensitivity measurement methods, and the introduction of more sensitive chemiluminescence assay for the examination of PP13 and PP17, in place of the radioimmunological assays on which our research team relied previously.

7) Clarification of the physiological characteristics of PP13 and PP17 during pregnancy, similar to work done earlier by our team on nine other pregnancy and placental proteins.

8) Research of new diagnostic potential: of the pregnancy serum proteins, SP1 has already been found useful for judging the intrauterine condition of the fetus and for early biochemical confirmation of atrophy during pregnancy; as the result of a suggestion we made some years ago, SP1 has become internationally accepted, along with hCG, as one of the markers for trophoblastic tumors (Than et al., 1988). It can be used in examinations similarly to PP12/IGFBP-1 and is currently used in the guise of the PROM test for identifying amniotic fluid

leakage. Elaboration of assay methods for PP13 and PP17 may be hoped to expand the repertoire of biochemical methods for examining the function of the placenta or analogous to the case of other oncofetal antigens, detection of the expression of these proteins may prove of diagnostic value in the monitoring of various tumorous diseases.

1.3. Investigated soluble placental tissue proteins

1.3.1. Placental Protein 17 (PP17)

The isolation and characterization of PP17 was first reported in 1983 (Bohn et al., 1983). The biological function of PP17 was not known. The purified protein was found to have an electrophoretic mobility between α_2-globulins and β_1-globulins. Its molecular mass was determined to be 30.300 kDa by ultracentrifugation. PP17 apparently consisted of a single polypeptide chain. The carbohydrate content was found to be 2.1% by weight. From one human term placenta, an average of 2.5 mg PP17 could be extracted. In concentrated extracts of other human tissues, PP7 could not be detected. Localization of PP17 was investigated by Inaba with the use of an avidin-biotin immunoperoxidase technique (Inaba et al., 1983). In human early placentas, PP17 was localized mainly in the cytoplasm of villous syncytiotrophoblast, whereas in human term placentas the staining in the syncytiotrophoblastic cells was not so strong. On the other hand, in human term placentas positive staining for PP17 was obtained in the cytoplasm of both basal and reflected chorionic trophoblastic cells. In addition, PP17 was found to be localized in the cytoplasm of polymorphonuclear neutrophils of the intervillous space as well as in the cytoplasm of decidual large cells. With the use of an electroimmunoassay (detection limit 1 mg/l), PP17 could neither be detected

in normal male or female sera, nor in pregnancy sera, cord blood sera, or in the amniotic fluid. A radioimmunoassay developed by Than et al. (1986) showed PP17 to be slightly increased in serum samples of pregnant women. With an enzyme immunoassay developed by Maekawa et al. (1993), it was found that in non-pregnant women the serum levels of PP17 are slightly higher in the follicular phase than in the luteal phase.

1.3.2. Placental Protein 13 (PP13)

The soluble placental tissue protein 13 (PP13) was isolated and characterized physicochemically in 1983 (Bohn et al., 1983). The biological function of this protein was unknown. Its molecular mass was determined to be 30.300 kDa by ultracentrifugation and 29.000 kDa by SDS-polyacrilamide gel electrophoresis. After reduction with mercaptoethanol, PP13 was reduced to about half of its original size. PP13 apparently is composed of two identical subunits (16.000 kDa) which are held together by disulfide bonds. The electrophoretic mobility of PP13 corresponds to that of albumin. The carbohydrate content of PP13 was low (0.6%) For measuring the concentration of PP13 in extracts of human term placentas, an electroimmunoassay was used. The results showed that one human term placenta contains an average amount of 3.7 mg PP13. By the same method (detection limit 1mg/l) the occurrence of PP13 in different body fluids was investigated; PP13 could not be detected in normal male or female sera, in pregnancy sera, cord blood sera, or in the amniotic fluid. The localization of PP13 in placental tissues has not yet been investigated. The occurrence of PP13 in concentrated extracts of other human tissues was studied by Ouchterlony's gel-diffusion test. PP13 could not be detected in a number of fetal or adult human tissues investigated.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

Pure PP13 (Op. 234/266) and PP17 (Op. 169/195) antigens and monospecific anti-PP13 (160 ZB) and anti-PP17 (54ZB) rabbit sera were prepared by Dr. Hans Bohn, Behringwerke AG, Germany. The soluble placental tissue proteins, PP13 and PP17, have been isolated from extracts of human term placentae by using immunoadsorption techniques. The extract was first fractionated with 2-ethoxy-6,9-diaminoacridine lactate and ammonium sulphate to give six (fractions I-VI) or three (fractions 1-3) protein fractions. Placental fraction I served as the starting material for the purification of PP13, whereas placental fraction 2 was used as the starting material for the isolation of PP17. These placental fractions then were subfractionated by gel filtration and ion-exchange chromatography, respectively, and ethanol precipitation to enrich further specifically the wanted proteins before immunoadsorption. The final purifications were done by using gel filtration and inverse immunoadsorption. For PP17, chromatography on DEAE-Sephadex was also used. The proteins thus obtained were more than 99% pure. Antibodies to PP13 and PP17 were obtained by immunizing rabbits with soluble protein fractions of human term placentae. The antisera prepared in this way against crude placental fractions usually were polyspecific, i.e., they contained antibodies to a number of different proteins. They were first absorbed with normal human serum to remove all the antibodies directed against serum proteins. Then they were made more or less specific to a certain protein by absorption with protein fractions from the placenta which did not contain the particular protein. With antisera prepared in this way it was possible to trace the particular protein through various purification processes by immunochemical methods

and also to prepare an immunosorbent which often considerably facilitated the isolation of the corresponding antigen. With the help of the purified proteins, highly specific antisera could be obtained (Bohn et al., 1983).

Escherichia coli XL1-Blue MRF strain, human placental Uni-ZAP™ XR expression cDNA library and *in vitro* packaging system were purchased from Stratagene (La Jolla, CA, USA), restriction endonucleases and T4 DNA ligase from Fermentas (Vilnius, Lithuania), T7 DNA Sequenase kit Version 2.0 from Amersham Life Science (Cleveland, Ohio, USA). We used Taq DNA Polimerase and Ultrapure dNTP-Set (Pharmacia Biotech, Uppsala, Sweden), [α-^{35}S] dATP and [α-^{32}P] dCTP (Izotóp Intézet Kft, Budapest, Hungary) and Random Primers DNA Labelling System (Sigma Chemical Co. St Louis, MO, USA).

2.2. Cell line

Human epitheloid cervical carcinoma cell line (HeLa) was grown on Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf sera (FCS), 100 U/ml Penicillin and 100 mg/ml Streptomycin and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h before use.

2.3. Western-blot analyses

100 tissue specimens were collected from 21 types of normal human adult and fetal tissue including term placenta, as well as various types of tumorous tissues (uterine cervical carcinoma (n=5), adenocarcinoma of the colon (n=4), adenocarcinoma of the liver (n=3), neurogen tumor (n=3), kidney tumor (n=2) and malignant melanoma (n=3). Tissues were homogenized by ultraturrax in standard Laemmli sample solution (Laemmli, 1970) containing 1 mM PMSF, and

ultracentrifuged at 10.000g for 10 minutes. All supernatants were then measured by BCA reagent (Pierce Chemical Company, Rockford, IL, USA) and equalized for 1 mg/ml protein content (Smith et al., 1985).

Blood was collected weekly from healthy primiparous women (n=75) between the 7th and 40th weeks of pregnancy; as follow-up from cervical carcinoma (FIGO stage Ib-IIa, n=20) and ovarium carcinoma (FIGO stage Ia-Ic, n=15) patients before treatment and then ten days, again at two months and finally six months after radical operations; from cervical carcinoma patients (FIGO stage III-IV, n=10) before and two months after irradiation and from gestational choriocarcinoma cases (n=5) before and two months after the start of chemotherapy. Sera were separated by ultracentrifugation and diluted equally in standard Laemmli sample solution. All samples were stored at -20°C until assay. For the conducting of human examinations, our bank of serum and other samples already contained several tens of thousands of samples.

We used standard sample preparation conditions for dispersion of proteins before electrophoresis (heating for 5 min at 100°C). PP13 and PP17 antigen, serum and tissue samples were subjected to 12 % (w/v) SDS/PAGE and electrophoresed for 2 h. The separated proteins were electroblotted on nitrocellulose membranes (Sigma) with a semi-dry blotter (BioRad). Membranes were then washed once with TBST (Tris/HCl, pH 7.4, containing 150 mM NaCl and 0/1% Tween-20) and then blocked for 1 h in TBST containing 3% skim milk. After the membranes had been washed with TBST, anti-PP13 or anti-PP17 sera were added at a dilution of 1:2000 in TBST and incubated for 1h. After three washes with TBST, membranes were treated for 1h with horseradish peroxidase conjugated goat anti-rabbit IgG (BioRad) diluted 1:5000 in TBST. After five

washes the protein bands were revealed with an enhanced chemiluminescence analysis system (Amersham). Quantitative densitometric analysis was performed using Scion Image for Windows.

2.4. Cloning and sequence analyses of cDNAs

For identification of cDNAs encoding either PP13 or PP17, placental cDNA library was plated both cases on 150-mm Petri dishes at 100.000 plaques/plate, and replicas were loaded onto nitrocellulose membrane (Sigma) from all twenty plates. Filters were screened using monospecific anti-PP13 or anti-PP17 serum. The primary immunoreactions were detected by alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) and developed by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase reaction system. Positive plaques were isolated and plaque-purified by rescreening with the same procedure three more times. λ phages were converted into pBluescript SK- phagemids with R408 helper phage (Russel et al., 1986) by the method of Short and Sorge (1992). Cultivation of bacteria, preparation of plasmid DNA, characterization of cDNA inserts by restriction enzyme analysis and subcloning of insert fragments were done as described in Sambrook et al. (1989). The nucleotide sequence of the inserts was determined from both strands by the dideoxy sequencing method (Sanger et al., 1977).

2.5. Computer analyses of nucleotide and deduced amino acid sequences

We scanned the PROSITE database (Bairoch et al., 1997) for biologically significant patterns and profiles, and we also looked for putative glycosilation sites on all proteins by the program O-glycobase version 2.0 (Hansen et al., 1997).

Secondary structural characteristics of the deduced amino acid sequences have been predicted by different methods (Kyte and Doolittle, 1992; Rost and Sander, 1993; Rost et al., 1995). Nucleotide and derived amino acid sequences of the cDNAs were compared with all non-redundant sequences and databases by the BLAST algorithm (NCBI, Bethesda, MD, USA) (Altschul et al., 1997) and by the ProDom domain search (Corpet et al., 1998).

2.6. Northern-blot analyses

1., Total RNA was isolated from 10^7 HeLa (human epitheloid cervical carcinoma) cells by the method described in Sambrook et al. (1989), and from 0.5 g of term placenta (n=3) and cervical carcinoma (n=5) tissues were prepared by the acid guanidium-phenol-chloroform (AGPC) extraction procedure (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987; Ausubel et al., 1994). 10 μg of RNA was separated by denaturing gel electrophoresis and blotted onto Hybond-N^+ nylon filters (Amersham).

2., We purchased two Northern Territory™ Human Normal Blots (Invitrogen, De Schelp, The Netherlands). 20 μg of total RNA from 8-8 human tissues were loaded on each blot (Blot I: heart, brain, kidney, liver, lung, pancreas, spleen, skeletal muscle; Blot II: esophagus, stomach, intestine, colon, uterus, placenta, bladder and adipose tissues).

An 0.5 kb coding segment of $PP17a_2$ cDNA was amplified by PCR, the 0.6 kb insert of PP13 cDNA was cut off by restriction endonucleases and both fragments were then labeled by [α-^{32}P] dCTP using Random Primers DNA Labelling System. RNA was hybridized by the labeled DNA probes as described previously (Sambrook et al., 1989). Analysis was performed by Packard

Cyclone™ Storage Phosphor System (Packard Instrument Company, Inc., Meriden, CT, USA).

2.7. NMR spectroscopic measurements

We monitored the lysophospholipase activity of PP13 by ^{31}P NMR measurements similar to those described in Loo et al. (1997). Two 2.5 mg portions of L-α-1-lysophosphatidylcholine (Sigma Chemical Co. St Louis, MO, USA) was dissolved in 1-1 ml of 7:3 mixture of 200 mM Hepes (pH 7, with 10 mM $CaCl_2$ and 150 mM NaCl) and D_2O. 20 μg of pure PP13 antigen was added to one of these mixtures and 700 μl of them was transferred to NMR tubes. The samples were stored at 37°C and their NMR spectra were recorded at varying time intervals.

^{31}P NMR spectra were obtained on a Varian ^{UNITY}INOVA 400 WB spectrometer at 161.90 MHz in a 5 mm switchable broad-band probe. Typically 128 transients were acquired using 30° flip angle pulses with 3.5 s delays and 0.5 s acquisition time, in order to have peak integrals represent the relative concentrations of the phosphorus-containing species. WALTZ-16 proton decoupling was utilized during the acquisition. The FIDs were apodized by exponential multiplication using 3 Hz line broadening. The chemical shifts were referred to 90% phosphoric acid in an insert tube.

CHAPTER 3: RESULTS

3.1. PP17 family - PP17a; PP17b / TIP47- cargo selection device for mannose 6-phosphate receptor trafficking; PP17d

3.1.1. Western-blot analysis

The originally highly purified PP17 antigen migrated in two bands with molecular masses of 31.500 and 60.900 kDa on 12 % (w/v) SDS/PAGE. When we dissolved PP17 antigen in 8M urea and repeated SDS/PAGE, PP17 antigen moved in only one band with a molecular mass of 31.500 kDa. In term placental tissue extract we discovered a total of four PP17 immunoreactive proteins of different molecular weights, which we designated PP17a (31.500 kDa), PP17b (48.000 kDa), PP17c (60.900 kDa) and PP17d (74.000 kDa) (Than NG et al., 1997; Than NG et al., 1998). In addition to term placenta, in normal human tissues (women and men, adult and fetus) PP17 immunreactive proteins were found to be expressed tissue-dependently. PP17a and PP17c were found mainly in steroidogenic tissues (uterus, ovarium, adrenal gland). PP17b seems to be ubiquitous, while PP17d is expressed only in term placenta **(Table 2)**.

An oncodevelopmental significance of PP17b has been strongly postulated: in cervical carcinoma tissues (n=5) all PP17 proteins - with two other immunoreactive bands - could be detected. Compared to normal conditions of the cervix, these proteins - especially PP17b - were overexpressed (Than NG et al., 1998; Than NG et al., 1999a) **(Figure 1)**. We could not find similar changes in PP17b expression in adenocarcinoma of the colon, adenocarcinoma of the liver, neurogen tumor, kidney tumor or malignant melanoma specimens compared to healthy tissue samples (Than NG et al., 1999b).

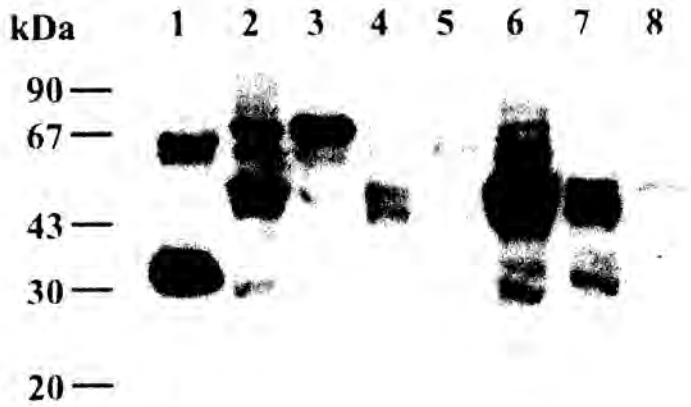
Table 2.

Quantity of PP17 variants in human normal adult/fetal and tumor tissue extracts and adult serum samples

Equally 10 μg protein in each sample were subjected to 12 % (w/v) SDS/PAGE. After Western- blotting, protein bands were revealed with an ECL enhanced chemiluminescence analysis system. Quantitative densitometric measurements were done by Scion Image for Windows. Amount of PP17 variants are shown semiquantitatively. Tissues taken only from adults are asterisked.

	PP17a	PP17b	PP17c	PP17d
<u>tissue samples</u>				
placenta (terminus)*	+	+++	+	+++
ovarium*	+	++		
corpus uteri*		++	+	
cervix uteri*	+	+	+	
cervix carcinoma*	++	+++	++	+
mammary gland*		+		
gall bladder*		+		
muscle		++		
heart		++		
adrenal gland	+	+++	+	
thyroid gland	++	+	+	
brain		++		
lung			+	
stomach		+		
pancreas		+		
liver		+		
spleen			+	
sigma		+		
skin		+		
fat	+	+	+	
kidney		+		
bladder		+		
<u>serum samples</u>				
non-pregnant woman		+		
pregnant woman (terminus)	+/-	++	+	+++
cervix carcinoma (stage Ib-IIa.)		+++		
ovarium carcinoma		+		
choriocarcinoma		+		

Figure 1.



Western-blot analysis of PP17 variants

1 ng of purified PP17 antigen (lane 1), 10 μg protein from term placenta (lane 2), pregnant serum - week 40th (lane 3), uterus corpus (lane 4), uterus cervix (lane 5), cervical carcinoma (lane 6), ovarium (lane 7) and mammary gland (lane 8) were electrophoresed on 12 % SDS/PAGE and Western-blotted using anti-PP17 serum and horseradish peroxidase conjugated secondary IgG. Protein bands were revealed with ECL enhanced chemiluminescence analysis system. The positions of molecular mass markers are signed at the left in kDa.

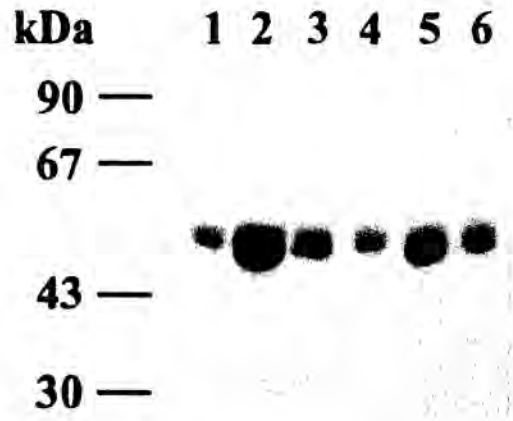
Serum levels of PP17b, unlike that of other PP17 proteins, were significantly elevated (mean values were five times higher than healthy controls) in sera of 20 untreated cervical carcinoma patients (FIGO stage Ib-IIa), and after radical operations PP17b values dropped with time and in proportion to the success of operation **(Figure 2)**. In 10 cases of inoperable cervical carcinoma patients (FIGO stage III-IV) serum PP17b levels were also above the controls, but there was no decline after radiotherapy. We detected no elevation in serum levels of PP17 proteins 5 chorio- or 15 ovarium carcinoma cases.

We did find notable changes during healthy pregnancy: beginning in the 7th week of gestation, the PP17b level increases continuously until its peak in the 33rd or 34th week, followed by a decline until term. PP17d also enters into the circulation in the third trimester only, and reaches its peak parallel to PP17b, while only trace amounts of PP17a and PP17c can be detected **(Figure 3).**

3.1.2. Cloning and sequence analysis of PP17 cDNAs

Immunoscreening $2x10^6$ recombinant plaques of the human placental cDNA library yielded 13 positive recombinant clones with different insert lengths. The shortest clones ($PP17a_1$ and $PP17a_2$) carried cDNA inserts of 1.4 kb encoding PP17a variant. There were ten clones ($PP17b_1$-$PP17b_{10}$) with insert-size of 2 kb encoding PP17b variant. One clone ($PP17c_1$) had a 2.7 kb insert also encoding PP17b variant: compared to clone $PP17b_1$, the insert of clone $PP17c_1$ turned out to contain additionally a 0.7 kb fragment of human nucleophosmin cDNA in its 3' end, between PP17b cDNA and vector sequence, and therefore it is assumed to be a cDNA synthesis artifact. By restriction endonuclease analysis the same-sized clones showed similar restriction patterns. All cDNA inserts and their restriction endonuclease-digested smaller fragments were sequence-analyzed in order to determine entire nucleotide sequences on both strands **(Figure 4)**.

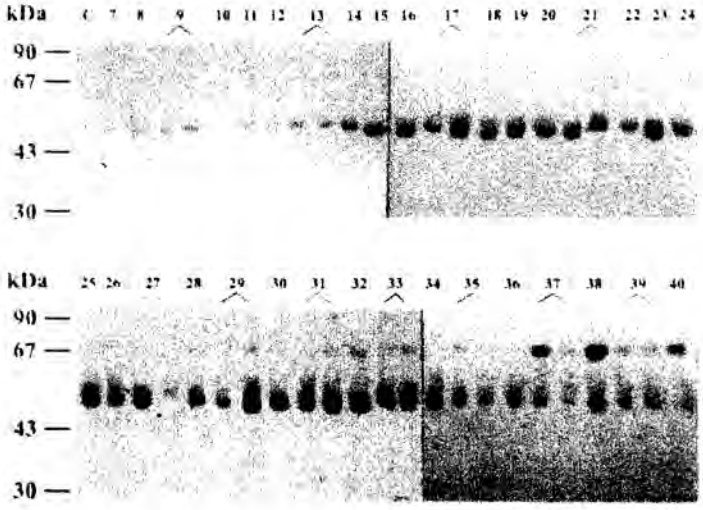
Figure 2.



Detection of PP17 variants in sera of cervical carcinoma patients

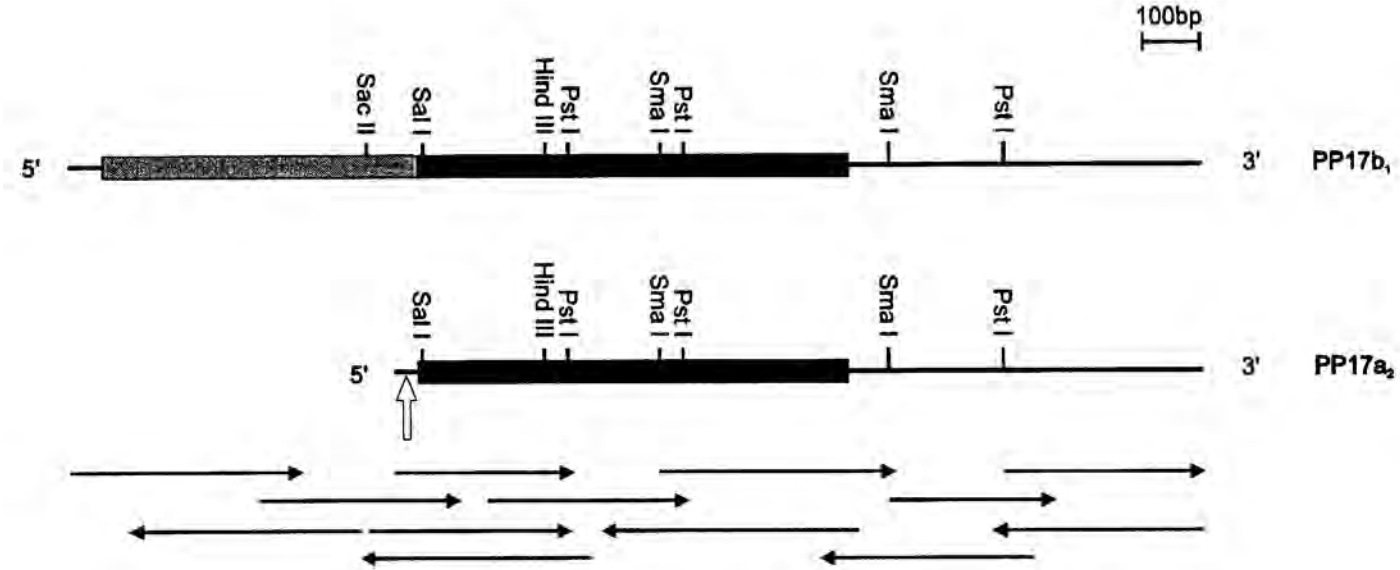
10 μg protein of serum samples from healthy non-pregnant woman (lane 1); cervical carcinoma patient (FIGO stage IIa) before treatment (lane 2), 10 days (lane 3) and 2 months after radical operation (lane 4); cervical carcinoma patient (FIGO stage Ib) before treatment (lane 5) and 10 days after radical operation (lane 6) were analyzed for PP17 variant content by SDS/PAGE - chemiluminescence Western-blotting. The positions of molecular mass markers are signed at the left in kDa.

Figure 3.



Detection of PP17 variants in sera of pregnant women

Serum samples were collected weekly from healthy primiparous women between the 7th and 40th weeks of pregnancy and analyzed for PP17 variant content by SDS/PAGE - chemiluminescence Western-blotting. Control serum sample of a healthy non-pregnant woman is indicated by "C", weeks of gestation is indicated by lane numbering. The positions of molecular mass markers are signed at the left in kDa.



Restriction endonuclease map and sequencing strategy of PP17 cDNAs

PP17a_2 and PP17b_1 cDNAs represent full length clones encoding for PP17a and PP17b variants. The 5' and 3' untranslated regions are displayed as solid lines, coding regions as thick lines, identical sequences are shown with black. Empty arrow indicates the region of divergence from where the two cDNAs contain different sequences in 5' direction. Sequence analysis was carried out with T3, T7 and clone specific internal primers on full length clones as well as on their restriction endonuclease digested smaller fragments. Strategy is indicated with arrows representing the direction and extent of sequencing runs.

Figure 4.

Nucleotide and deduced amino acid sequences of $PP17a_1$, $PP17a_2$ and $PP17b_1$ cDNAs have been submitted to the GenBank database and are available under accession numbers AF051314, AF051315 and AF055574.

Clone $PP17a_1$ (Than NG et al., 1999a) is identical to $PP17a_2$ (Than NG et al., 1998) except for 24 bp missing from the end of a 5' untranslated region. Clone $PP17a_2$ carries a 1411 bp insert which contains a 38 bp 5' untranslated region, a coding region of 753 bp followed by a 3' untranslated region of 620 bp including a proposed polyadenylation signal - 5' AATACA 3' - and a consensus sequence - 5' CATTG 3' - described for the 3' end of polyadenylated mRNAs (Benoist et al., 1980). Assuming that the first AUG is the translation initiator codon (located in an optimal context - 5' GCCAG**ATG**G 3' - typical for vertebrate mRNAs recognized by the 40S subunit of ribosome (Kozak, 1987) and also preceded 30 bases upstream by a sequence - 5' CCGCCGG 3' - partially complementary to the 3' region of eucaryotic 18S rRNA (Chan et al., 1984), therefore a putative ribosome binding site), the open reading frame encodes a single polypeptide chain of 251 residues with a predicted molecular mass of 28.129 kDa. **(Figure 5).**

Clone $PP17b_1$ (Than NG et al., 1998) carries a 1974 bp insert with a 53 bp 5' untranslated region, a coding region of 1302 bp followed by a 3' untranslated region of 619 bp. In clones $PP17b_{2\text{-}10}$ the 5' untranslated region is shorter than in clone $PP17b_1$ by up to 2-18 bases. Clone $PP17b_1$ contains almost the entire nucleotide sequence of $PP17a_2$. There is only one base in the 3' noncoding region of $PP17a_2$ which is not in $PP17b_1$, and - from the point of divergence of the two cDNAs into 5' direction - $PP17a_2$ contains an additional 19 bases and $PP17b_1$ another 583 bases which are different or lacking in the other cDNA. $PP17b_1$ has the same proposed polyadenylation signal and consensus sequence (Benoist et al., 1980) at the 3' end as $PP17a_2$. The first AUG in $PP17b_1$ is in a typical context

Figure 5.

```
                                                  *
1                          ATTGGACGCCGCCGGTCACGGGCTCCCGCTTGGGCCAG
39   ATGGTGCTGAGTGGGGTCGACACGGTGCTGGGGAAGTCGGAGGAGTGGGCGGACAACCAC
     M  V  L  S  G  V  D  T  V  L  G  K  S  E  E  W  A  D  N  H     20
99   CTGCCCCTTACGGATGCCGAACTGGCCCGCATCGCCACATCCCTGGATGGCTTCGACGTC
     L  P  L  T  D  A  E  L  A  R  I  A  T  S  L  D  G  F  D  V     40
159  GCGTCCGTGCAGCAGCAGCGGCAGGAACAGAGCTACTTCGTACGTCTGGGCTCCCTGTCG
     A  S  V  Q  Q  Q  R  Q  E  Q  S  Y  F  V  R  L  G  S  L  S     60
219  GAGAGGCTGCGGCAGCACGCCTATGAGCACTCGCTGGGCAAGCTTCGAGCCACCAAGCAG
     E  R  L  R  Q  H  A  Y  E  H  S  L  G  K  L  R  A  T  K  Q     80
279  AGGGCACAGGAGGCTCTGCTGCAGCTGTCGCAGGCCCTAAGCCTGATGGAAACTGTCAAG
     R  A  Q  E  A  L  L  Q  L  S  Q  A  L  S  L  M  E  T  V  K    100
339  CAAGGCGTTGATCAGAAGCTGGTGGAAGGCCAGGAGAAGCTGCACCAGATGTGGCTCAGC
     Q  G  V  D  Q  K  L  V  E  G  Q  E  K  L  H  Q  M  W  L  S    120
399  TGGAACCAGAAGCAGCTCCAGGGCCCCGAGAAGGAGCCGCCCAAGCCAGAGCAGGTCGAG
     W  N  Q  K  Q  L  Q  G  P  E  K  E  P  P  K  P  E  Q  V  E    140
459  TCCCGGGCGCTCACCATGTTCCGGGACATTGCCCAGCAACTGCAGGCCACCTGTACCTCC
     S  R  A  L  T  M  F  R  D  I  A  Q  Q  L  Q  A  T  C  T  S    160
519  CTGGGGTCCAGCATTCAGGGCCTCCCCACCAATGTGAAGGACCAGGTGCAGCAGGCCCGC
     L  G  S  S  I  Q  G  L  P  T  N  V  K  D  Q  V  Q  Q  A  R    180
579  CGCCAGGTGGAGGACCTCCAGGCCACGTTTTCCAGCATCCACTCCTTCCAGGACCTGTCC
     R  Q  V  E  D  L  Q  A  T  F  S  S  I  H  S  F  Q  D  L  S    200
639  AGCAGCATTCTGGCCCAGAGCCGTGAGCGTGTCGCCAGCGCCCGCGAGGCCCTGGACCAC
     S  S  I  L  A  Q  S  R  E  R  V  A  S  A  R  E  A  L  D  H    220
699  ATGGTGGAATATGTGGCCCAGAACACACCTGTCACGTGGCTCGTGGGACCCTTTGCCCCT
     M  V  E  Y  V  A  Q  N  T  P  V  T  W  L  V  G  P  F  A  P    240
759  GGAATCACTGAGAAAGCCCCGGAGGAGAAGAAGTAGGGGGAGAGGAGAGGACTCAGCGGG
     G  I  T  E  K  A  P  E  E  K  K  ---                          251
819  CCCCGTCTCTATAATGCAGCTGTGCTCTGGAGTCCTCAACCCGGGGCTCATTTCAAACTT
879  ATTTTCTAGCCACTCCTCCCAGCTCTTCTGTGCTGTCCACTTGGGAAGCTAAGGCTCTCA
939  AAACGGGCATCACCCAGTTGACCCATCTCTCAGCCTCTCTGAGCTTGGAAGAAGCCTGTT
999  CTGAGCCTCACCCGTATCAGTCAGTAGAGAGAGATGTCCAGAAAAAAATATCTTTCAGGAA
1059 AGTTCTCCCCTGCAGAATTTTTTTTCCTTGTTAAATATCAGGAATATAGGCCGGGTGCGG
1119 TGGCTCACACCTGTAATCCCAGCACTTTGGGAGGCTGAGGCGGGCGGAACACCTGAGGTC
1179 AGGTGTTCGAGACCAGCCAGGCCAACATGGTGAAACCCCGTCTCTACTAAAAATACAAAA
1239 AAAAATGAGCCGGGCATGGTAGCAGGTGTCTGTTATCCCAGTTAGGAGGCTGAGGCAAGA
1299 GAATCTCTTGAACCTGAGAGGCGGAGGTTGCAGTGAGCCAAGATCGCGCCATTGCACTCC
1359 AGCCTGGGGGACAAGAGTGAGACTTAGTCTCAAAAAAAAAAAAAAAAAAAAAA
```

Complete nucleotide and deduced amino acid sequences of PP17a_2 cDNA

Numbers at the left and right indicate nucleotide and amino acid positions, respectively. Putative ribosome binding sites, proposed polyadenylational signal and consensus sequence for the 3' end of polyadenylated mRNAs are underlined. Region of divergence is shown with an asterisk, nucleotides lacking from PP17b_1 cDNA are typed with bold.

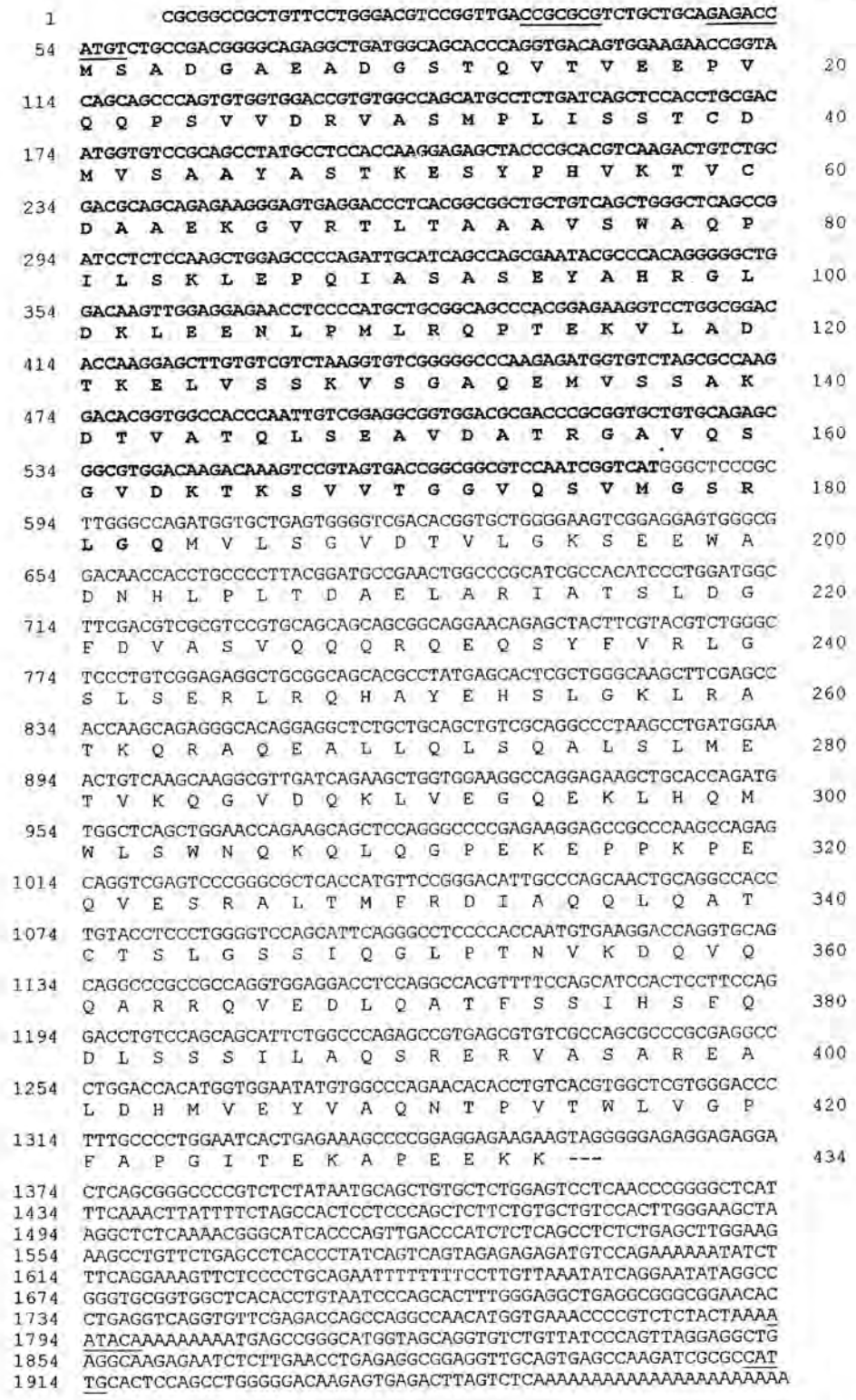
- 5' GAGACCATGT 3' - for vertebrate mRNAs (Kozak, 1987) and preceded 22 bases upstream by a putative ribosome binding site - 5' CCGCGCG 3' - (Chan et al., 1984). The open reading frame encodes a 434 residue long polypeptide with a predicted molecular mass of 47.208 kDa **(Figure 6).**

3.1.3. Computer analysis of nucleotide and amino acid sequences of PP17 variants

Computer analysis of PP17a and PP17b variant amino acid sequences using the Multicoil program (Wolf et al., 1997) showed that both PP17 variants contain the same region in their C-terminal part that displays a high probability (≥0.9) of forming an α-helical dimeric coiled coil. Searching the PROSITE database by the EXPASY Molecular Biology Server for biologically significant patterns and profiles of the deduced amino acid sequences, we found 4 putative protein kinase C and 7 casein kinase 2 phosphorylation sites in PP17a variant, 8 PKC and 14 CK2 phosphorylation sites in PP17b variant. In PP17a and PP17b variants some possible glycosilation sites were detected, and though some probable N-myristoylation sites were also found in both variants, they are believed not to be processed in a way as required for acyl modification, since the molecular masses are very similar to those predicted from the cDNAs.

The nucleotide and derived amino acid sequences of the cDNAs were compared to all non-redundant sequences and databases using the BLAST algorithm. PP17a and PP17b variants have the highest identity to human adipophilin (*PP17a:* 37.5%, *PP17b:* 40.5%) and the mouse adipose differentiation-related protein /ADRP/ (*PP17a:* 35%, *PP17b:* 38.5%), which are the earliest markers of adipocyte differentiation (Jiang et al., 1992; Jiang and Serrero, 1992; Steiner et al., 1996; Heid et al., 1996; Brasaemle et al., 1997).

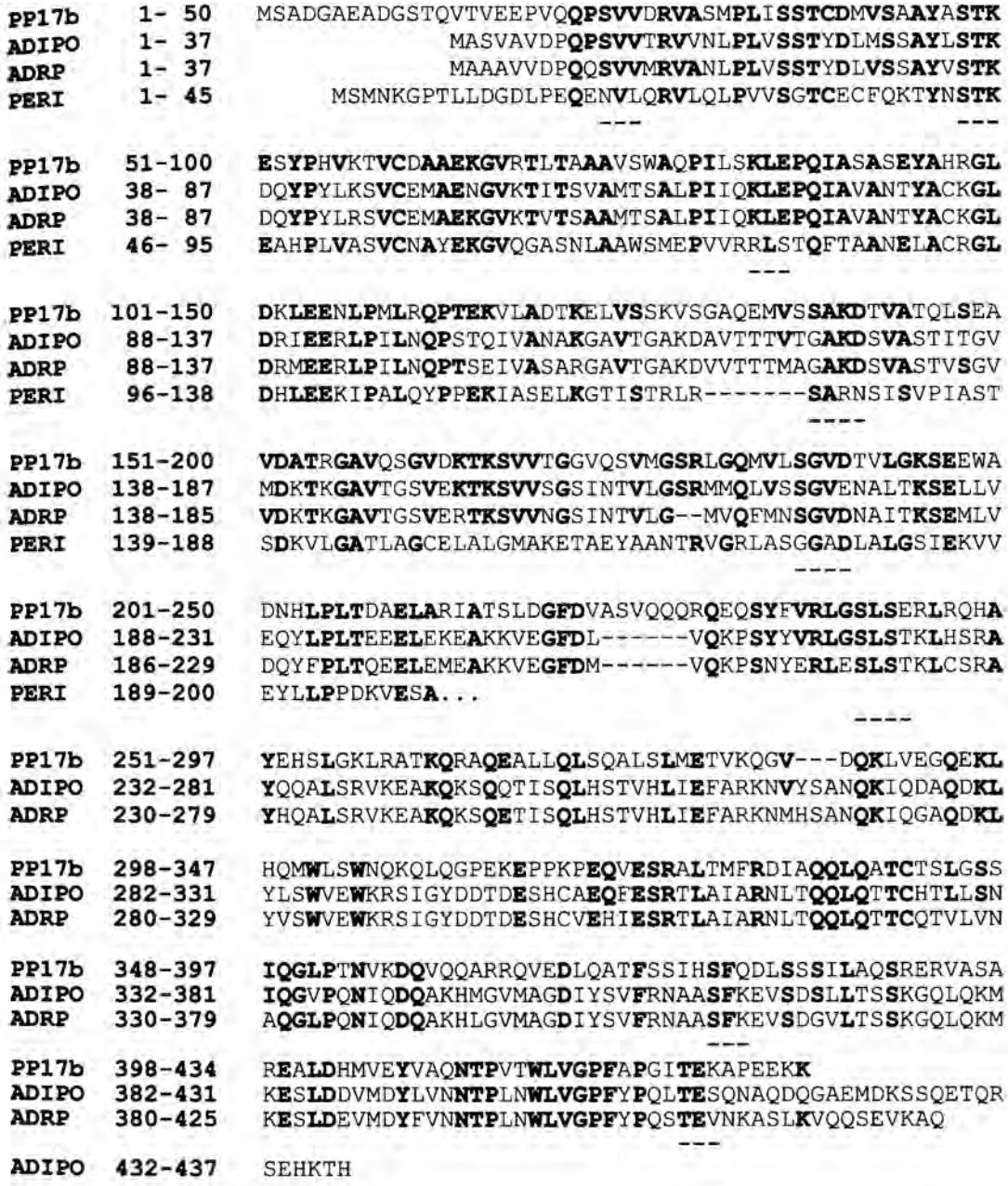
Figure 6.



Complete nucleotide and deduced amino acid sequences of PP17b$_1$ cDNA

Numbers at the left and right indicate nucleotide and amino acid positions, respectively. Putative ribosome binding sites, proposed polyadenylational signal and consensus sequence for the 3' end of polyadenylated mRNAs are underlined. Region of divergence is shown with an asterisk, nucleotides and deduced amino acids lacking from PP17a$_2$ cDNA and PP17a variant respectively, are typed with bold.

Figure 7.



Sequence alignment between PP17b variant and its homologues

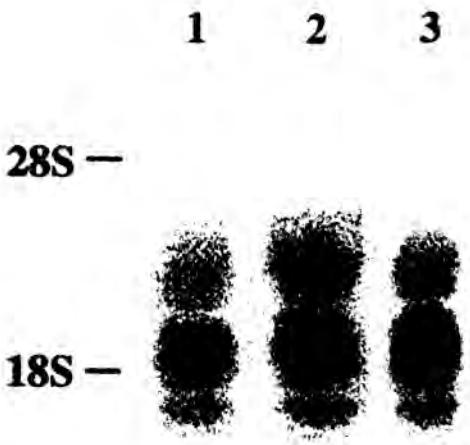
Entire amino acid sequences of PP17b variant, human adipophilin (ADIPO), mouse adipose differentiation-related protein (ADRP) and N-terminal sequence of rat perilipin A/B (PERI) were aligned. Numbers at the left denote amino acid positions. Identical residues to PP17b variant are indicated with blue, dashes (-) are added to maximize alignment of the sequences. Putative phosphorylation sites in conserved regions are underlined.

A 200-residue long segment in the N-terminal of PP17b variant has 30 % identity to rat perilipin A and B(Greenberg et al., 1991, 1993), the closest homologues of ADRP, which are the major cAMP-dependent protein kinase substrates in adipocytes. Alignment shows that the most conserved portions of these proteins contain several phosphorylation sites which were preserved **(Figure 7)**. Just a few months after our findings, the amino acid sequence of TIP47, a 434 residue long protein functioning in mannose 6-phosphate receptor (MPR) trafficking, turned out to be entirely identical to the previously determined 434 amino-acid long PP17b variant of the PP17 family (Díaz and Pfeffer, 1998).

3.1.4. Northern-blot analysis

We identified three different size mRNAs (1.4, 2.0 and 2.9 kb) in placental and cervical carcinoma tissues and in HeLa cells corresponding well to isolated PP17 cDNA sizes **(Figure 8)**. In 15 other different healthy human tissues only the 1.4 and 2.0 kb. PP17 mRNAs were expressed in a tissue-specific pattern **(Figure 9)**.

Figure 8.



Northern-blot analysis of PP17 mRNAs

RNA (10-10 μg) was separated on a 1 % formaldehyde/agarose gel. Northern-blot hybridization was performed using [α-^{32}P] dCTP labeled PCR amplified 525 bp segment of PP17a_2 cDNA. Lane 1: placental tissue; Lane 2: HeLa cells. Lane 3: cervical carcinoma tissue. The positions of 18 S and 28 S rRNAs are marked at the left.

Figure 9.

Northern-blot analysis of PP17 mRNAs

Expression of alternatively spliced PP17 mRNAs in heart (1), brain (2), kidney (3), liver (4), lung (5), pancreas (6), spleen (7), sceletal muscle (8), esophagus (9), stomach (10), intestine (11), colon (12), uterus (13), placenta (14), bladder (15) and adipose (16) tissues. Commercial filters contained 20 μg total RNAs per lane and were hybridized with ^{32}P labeled cDNA probe. The positions of single strand RNA markers are indicated at the left margin.

3.2. PP13 - human placental galectin / lysophospholipase

3.2.1. Western-blot analysis

The originally highly purified PP13 antigen migrated as a single 16 kDa protein band on SDS/PAGE. We detected the immunologically same protein in human term placental tissue **(Figure 10)**. From other 25 types of tissue our highly specific anti-PP13 serum gave immunoreaction with a 16 kDa protein in certain normal (human fetal/adult spleen, fetal kidney and adult bladder) and in some tumorous tissues (adenocarcinoma of the liver, malignant melanoma and neurogen tumor) **(Table 3)**. PP13 is not secreted into circulation as we could not find PP13 neither in sera of healthy controls, nor in sera of pregnant women (Than NG et al., 1999c).

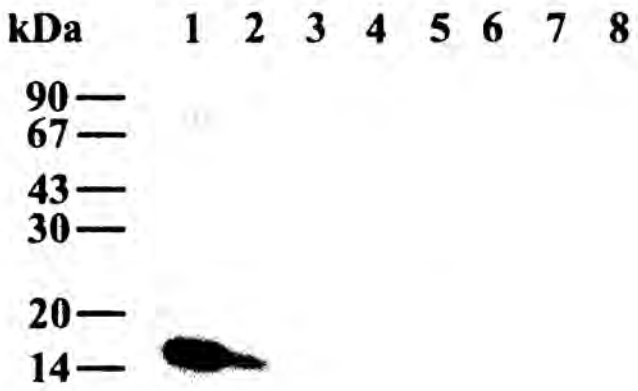
3.2.2. Cloning and sequence analysis of PP13 cDNA

Immunoscreening 10^6 recombinant plaques of the human placental cDNA library with anti-PP13 serum yielded one positive recombinant clone. cDNA insert and its restriction endonuclease-digested smaller fragments were sequence-analyzed in order to determine the entire nucleotide sequences on both strands **(Figure 11)**.

The novel nucleotide and amino acid sequence has been submitted to the GenBank database and is available under accession number AF117383.

The 578 bp full-length cDNA insert contains a 14 bp 5' untranslated region, a coding region of 417 bp followed by a 3' untranslated region of 147 bp including a proposed polyadenylation signal - 5' ATTAAA 3' - and a consensus sequence - 5' CACTT 3' - described for the 3' end of polyadenylated mRNAs (Benoist et al., 1980). Assuming that the first AUG is the translation initiator codon, located

Figure 10.



Chemiluminescence Western-blot analysis of PP13

1 ng of purified PP13 antigen (lane 1), 10 μg protein from term placenta (lane 2), ovarium (lane 3), uterus corpus (lane 4), uterus cervix (lane 5), mammary gland (lane 6) brain (lane 7) and muscle (lane 8) were electrophoresed on 12 per cent (w/v) SDS/PAGE and Western-blotted using anti-PP13 serum and horseradish peroxidase conjugated secondary IgG. Protein bands were revealed with an enhanced chemiluminescence analysis. The positions of molecular mass markers are signed at the left.

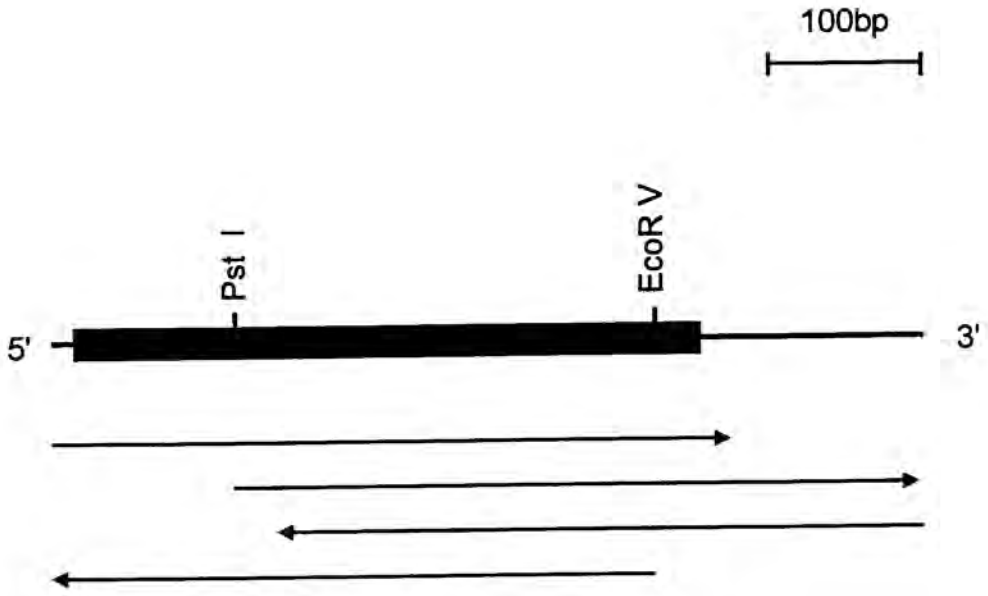
Table 3.

Quantity of PP13 in human healthy adult/fetal and tumorous tissue extracts and adult serum samples

Equally 10 μg protein in each sample were subjected to 12 per cent (w/v) SDS/PAGE. After Western-blotting protein bands were revealed with ECL chemiluminescence analysis system followed by quantitative densitometric measurements performed by Scion Image for Windows. Amount of PP13 is shown semiquantitatively.

<u>healthy tissues</u>	
placenta (terminus)	+
ovarium (adult)	-
corpus uteri (adult)	-
cervix uteri (adult)	-
mammary gland (adult)	-
gall bladder (adult)	-
muscle (adult/fetal)	- / -
heart (adult/fetal)	- / -
adrenal gland (adult/fetal)	- / -
thyroid gland (adult/fetal)	- / -
brain (adult/fetal)	- / -
lung (adult/fetal)	- / -
stomach (adult/fetal)	- / -
pancreas (adult/fetal)	- / -
liver (adult/fetal)	- / -
spleen (adult/fetal)	++ / +
sigma (adult/fetal)	- / -
skin (adult/fetal)	- / -
fat (adult/fetal)	- / -
kidney (adult/fetal)	- / +++
bladder (adult/fetal)	+++ / -
<u>tumorous tissues</u>	
adenocarcinoma of the liver	+
adenocarcinoma of the colon	-
tumor of kidney	-
malignant melanoma	+
neurogen tumor	+
<u>serum samples</u>	
non-pregnant women	-
pregnant women (7-40th week)	-

Figure 11.



Restriction endonuclease map and sequencing strategy of PP13 cDNA

cDNA represent full length clone encoding for PP13. The 5' and 3' untranslated regions are displayed as solid lines, coding region as thick line. Sequence analysis was carried out with T3 and T7 primers on full length clone as well as on its restriction endonuclease digested smaller fragments. Strategy is indicated with arrows representing the direction and extent of sequencing runs.

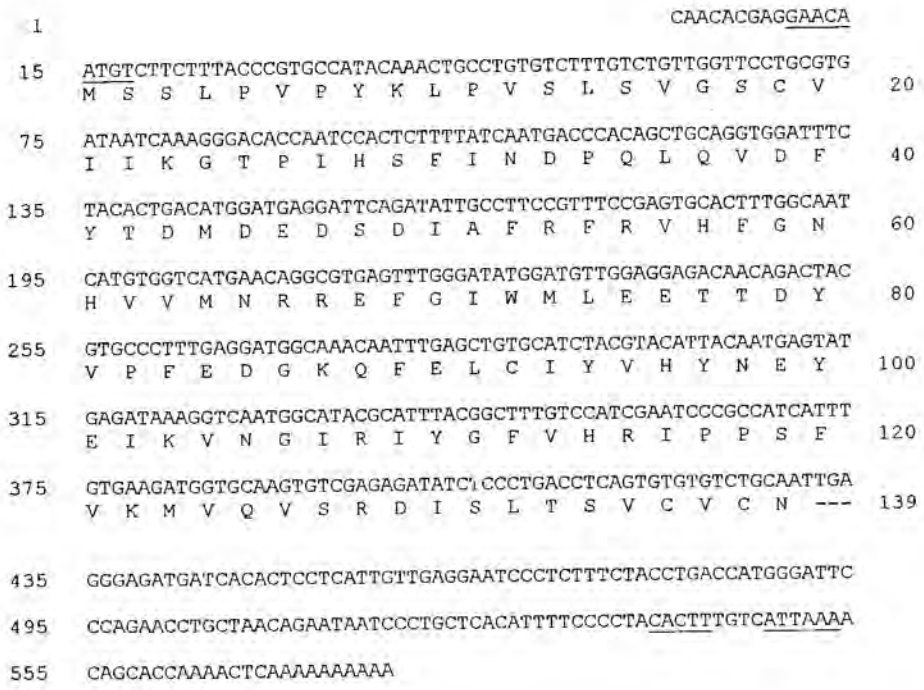
in a context - 5' GAACA**ATG**T 3' - typical for vertebrate mRNAs recognized by the ribosome (Kozak, 1987), the open reading frame encodes for a 139 residue long protein with a predicted molecular mass of 16,118 kDa (Than NG et al., 1999c) **(Figure 12)**. The amino acid composition and molecular mass of the deduced protein is basically identical to that of PP13 antigen, isolated and determined physico-chemically by Bohn et al. in 1983.

3.2.3. Computer analysis of nucleotide and deduced amino acid sequences of PP13

Comparing the deduced amino acid sequence to protein sequences in PROSITE database, one putative casein kinase II phosphorylation site, one tyrosine kinase phosphorylation site and one ATP-dependent DNA ligase AMP-binding site were detected starting at residues 42, 87 and 101, respectively. In concordance with previous data we found one potential glycosilation-site at the N-terminus of PP13 containing serine at residue 3.

By the alignment search, PP13 has the highest homology (56% identity and 69% similarity) to the 16,5 kDa human eosinophil Charcot-Leyden Crystal (CLC) protein (Weller et al., 1980; Ackerman et al., 1993), a unique dual-function lysophospholipase. CLC protein is a member of the beta-galactoside binding S-type animal lectin (galectin) superfamily (Barondes, 1984; Leffler et al., 1989), which consists of smaller molecular mass, highly conserved proteins isolated from human and animal tissues. Members of the galectin family show conservation of 16 invariant residues comprising the carbohydrate recognition/binding domains (Drickamer, 1988). Sequence of PP13 is conserved at 8 of these 16 residues together with tryptophan at position 72, which is in the closest vicinity to the carbohydrate binding site (Paroutaud et al., 1987). PP13 is highly homologous (50%) to several galectins (human, mouse, orangutan, rat, pig, rabbit, hamster)

Figure 12.



Complete nucleotide and deduced amino acid sequences of PP13 cDNA

Numbers at the left and right indicate nucleotide and amino acid positions, respectively. Putative ribosome binding site, proposed polyadenylational signal and consensus sequence for the 3' end of polyadenylated mRNAs are underlined.

and also shows up to 50% homology to the carboxyl-terminal domains of four IgE binding proteins (35 kDa mouse carbohydrate binding protein (Laing et al., 1989), Mac-2 murine macrophage cell surface protein (Cherayil et al., 1989), 31 kDa rat (Liu et al., 1985) and human (Robertson et al., 1990) IgE binding proteins). Alignment of the closest galectin homologues are shown on **Figure 13**.

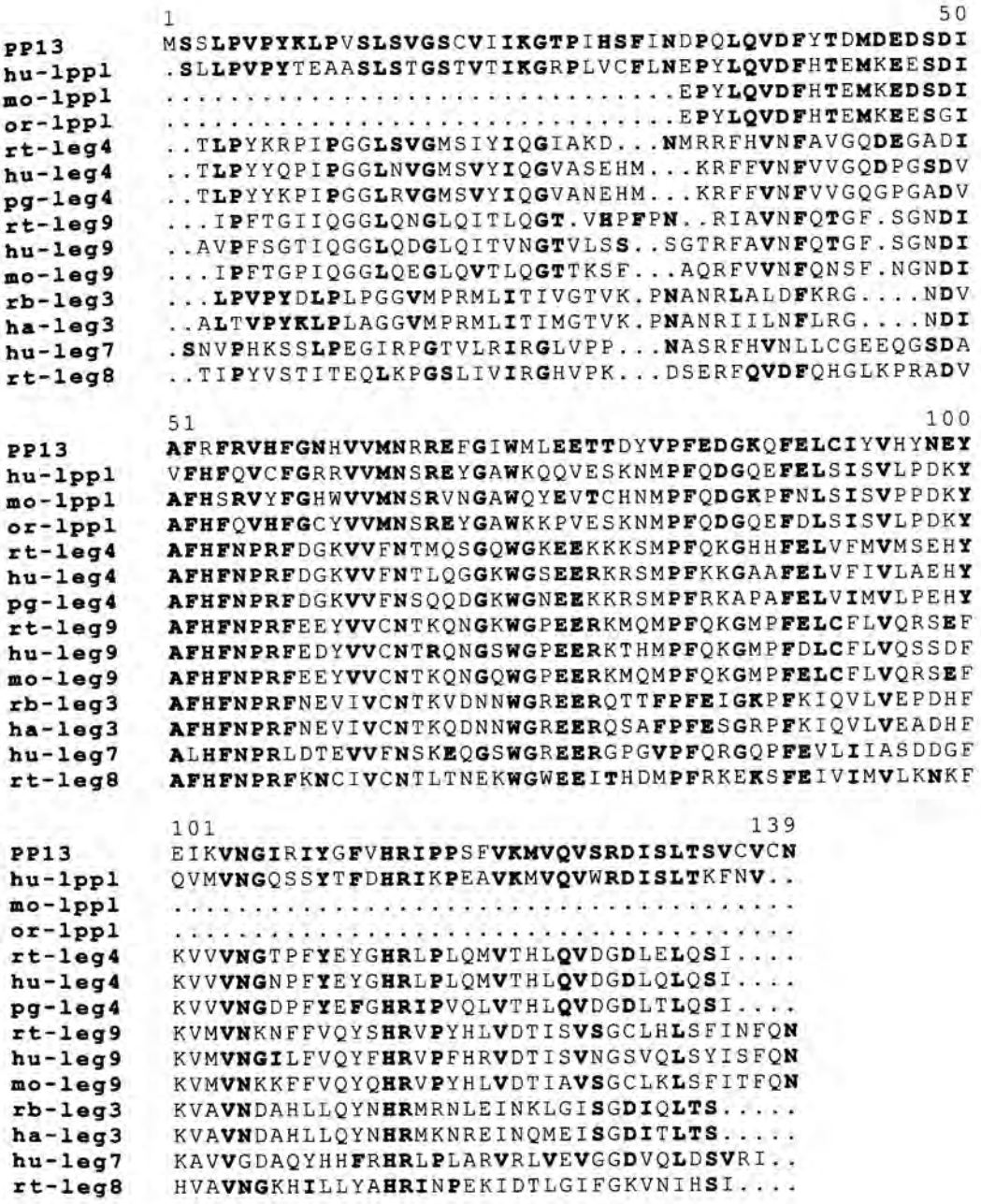
3.2.4. Northern-blot hybridization

Labeled insert of PP13 cDNA hybridizes to a 600 bp messenger RNA extracted from human placental tissue, its length is identical to the size of the cloned cDNA. There was no signal for PP13 mRNA in 15 other types of human adult tissue investigated parallel **(Figure 14)**.

3.2.5.NMR spectroscopic functional analysis of PP13

Lysophospholipase activity of PP13 was detected by ^{31}P NMR, by monitoring the mixture of PP13 and L-α-1-lysophosphatidylcholine (LPC). Under our experimental conditions, in the presence of PP13 90 % of LPC was transformed into phosphatidylcholine, as identified by its ^{31}P NMR chemical shift, and by the presence of fatty acids in the endreaction-mixture **(Figure 15)**. In a parallel experiment, involving only LPC in the same mixture without PP13, no such change could be observed.

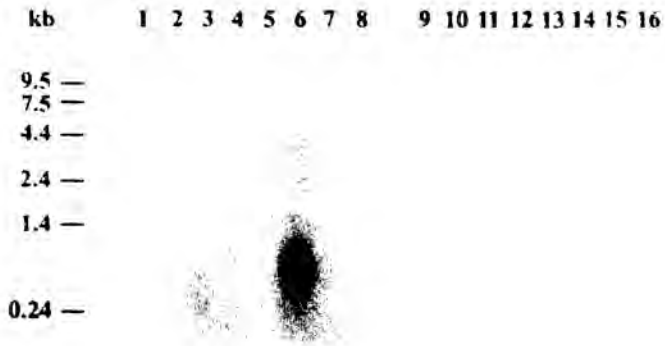
Figure 13.



Sequence alignment between PP13 and its closest homologues

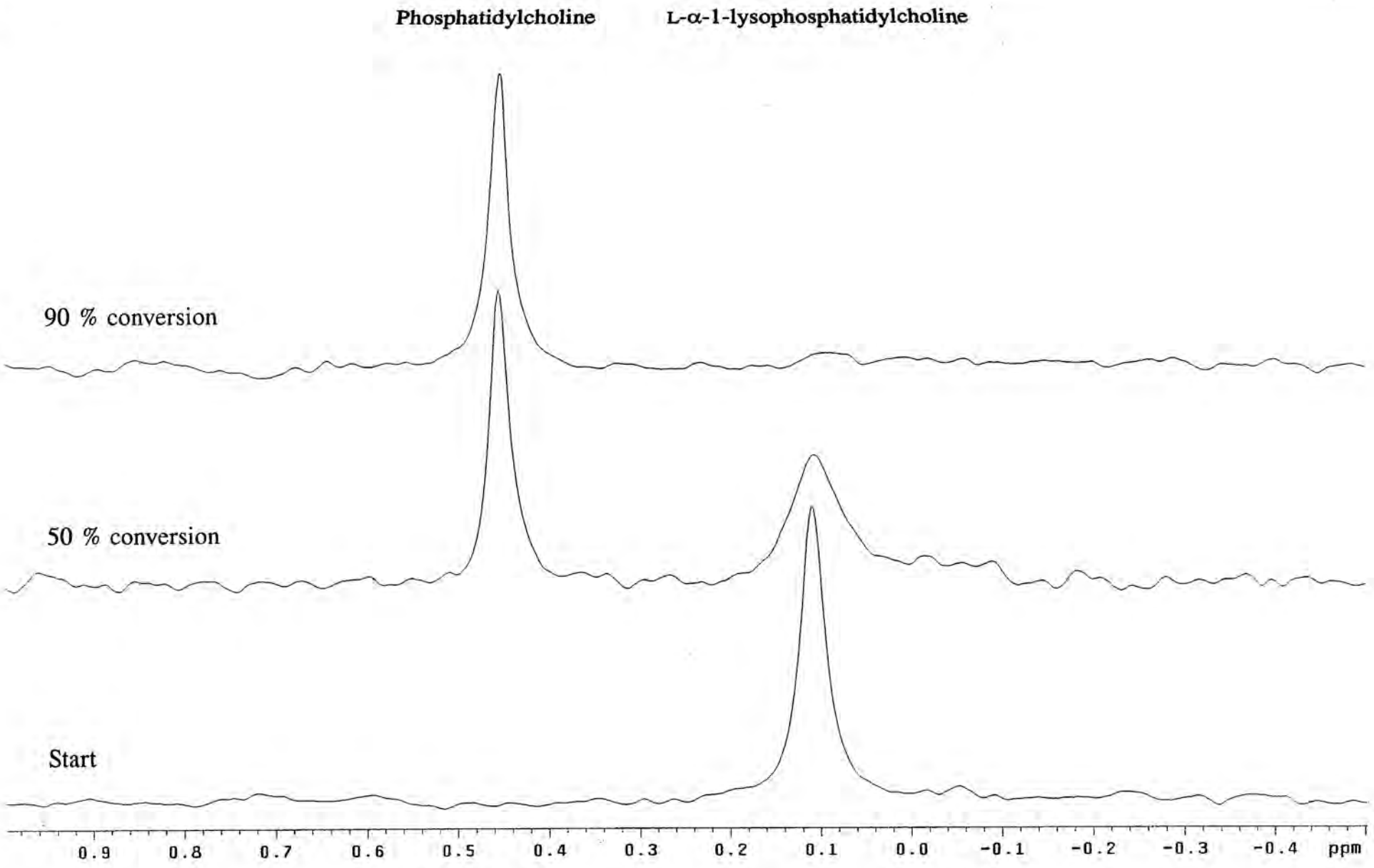
Amino acid sequences of PP13, human- (hu-lppl), mouse- (mo-lppl) and orangutan (or-lppl) eosinophil lysophospholipase; rat- (rt-leg4), human- (hu-leg4) and pig (pg-leg4) galectin-4; rat- (rt-leg9), human- (hu-leg9) and mouse (mo-leg9) galectin-9; rabbit- (rb-leg3) and hamster (ha-leg3) galectin-3; human (hu-leg7) galectin-7 and rat (rt-leg8) galectin-8 are aligned. Identical residues to PP13 are indicated with blue, dots (.) are added to maximise alignment of the sequences. The highly conserved residues comprising the carbohydrate / beta-galactoside binding domain of galectins - including tryptophan in the closest vicinity to the carbohydrate binding site - are indicated with magenta. Amino acid positions in PP13 are shown above the sequences.

Figure 14.



Northern-blot analysis of PP13 mRNA

Filters contained 20 μg total RNAs per lane and were hybridized with [α-^{32}P] dCTP labeled insert of PP13 cDNA. Lane 1: esophagus; Lane 2: stomach; Lane 3: intestine; Lane 4: colon; Lane 5: uterus; Lane 6: placenta; Lane 7: bladder; Lane 8: adipose tissue; Lane 9: heart; Lane 10: brain; Lane 11: kidney; Lane 12: liver; Lane 13: lung; Lane 14: pancreas; Lane 15: spleen; Lane 16: skeletal muscle. The positions of single strand RNA markers are indicated at the left.



^{31}P NMR spectroscopic functional analysis of PP13

Lysophospholipase activity of PP13 was verified as L-α-1-lysophosphatidylcholine (LPC) was transformed into phosphatidylcholine in the presence of PP13. In control experiment, involving only LPC in the same mixture without PP13, no such change could be observed.

Figure 15.

CHAPTER 4: DISCUSSION OF THE MAIN RESULTS

Through the molecular-biological investigations we began in 1997, we have made significant achievements in the examination of placental proteins. The next two subchapters will discuss the structural and functional characteristics identified by our examinations for two previously unidentified proteins and protein families.

4.1. PP17 family - PP17a; PP17b / TIP47- cargo selection device for mannose 6-phosphate receptor trafficking; PP17d

In earlier studies the highly purified PP17 antigen was found to have two variants (31.500 and 60.900 kDa) on SDS/PAGE (Bohn et al., 1983). Now we clarified that the 60.900 kDa band corresponds to a PP17 antigen dimer, as when we dissociated PP17 antigen with 8M urea it moved with one band (31.500 kDa) on SDS/PAGE. In our recent investigations, in term placental tissue extract four different molecular mass PP17 immunoreactive proteins were detected: PP17a (31.500 kDa), PP17b (48.000 kDa), PP17c (60.900 kDa) and PP17d (74.000 kDa). PP17a and PP17b have been cloned and sequence analyzed by our group; PP17c is dimer of PP17a verified by SDS/PAGE containing 8M urea; the cDNA of PP17d has not yet been isolated (Than NG et al., 1998). Expression patterns of the four PP17 immunoreactive bands in other healthy human tissues is the following: PP17a and PP17b variants are expressed tissue dependently, while PP17d is placenta specific.

In cervical carcinoma tissues we detected overexpression of PP17b at the protein level; in other tumors there were no significant PP17b changes compared to healthy controls. Overexpression of PP17b variant in cervical carcinoma tissues is verified by detecting large amounts of PP17 mRNAs in HeLa cells and in cervical carcinoma tissues.

PP17b variant is normally released/secreted into circulation, amounts of PP17b and PP17d elevate during pregnancies. Serum levels of PP17b are increased in operable (FIGO stage Ib-IIa) as well as in inoperable (FIGO stage III-IV) cervical carcinoma patients compared to healthy controls, but not in other tumor cases investigated. During operative treatment PP17b serum levels dropped, which offers the possibility of seromonitoring, but only for operable cervical carcinoma patients.

The protein encoded by $PP17a_{1-2}$ cDNAs is identical to the earlier isolated PP17 antigen described by Bohn *et al.* in 1983 and PP17a determined currently: *(1)* The calculated molecular mass of the deduced amino acid sequence (28.129 kDa) is similar to the molecular mass of PP17 glycoprotein obtained by ultracentrifugal determination (30.300 kDa) and by SDS/PAGE - Western-blot (31.500 kDa). Observations concerning molecular mass differences are in good agreement with the results of molecular mass determinations of other placental proteins like PP9 or pregnancy-specific β1-glycoprotein (Grundmann et al., 1990, Watanabe and Chou, 1988). *(2)* The deduced amino acid composition of PP17a variant also shows a good correlation with previously published data (Bohn et al., 1983).

The open reading frame of $PP17b_{1-10}$ cDNAs encodes a 434 residue long polypeptide with a predicted molecular mass of 47.208 kDa, which is identical to PP17b variant determined currently: the calculated molecular mass is very similar to that obtained by SDS/PAGE - Western blot analysis (48.000 kDa).

Because of the special difference in the 5' region of $PP17a_2$ and $PP17b_1$ cDNAs containing a putative splice-site (Smith et al., 1989), we supposed that different size PP17 variants detected by Western-blot analyses might be the products of alternatively spliced PP17 mRNAs. Our latest results by Northern-blot

investigations in 16 healthy human tissues confirmed this hypothesis. The 1.4 kb mRNA encodes PP17a variant and the 2.0 kb mRNA encodes PP17b variant. PP17d detected by Western-blots is either the product of the 2.9 kb. mRNA detected on Northern-blots, or can possibly be originated by post-translational modification (e.g. glycosilation, acylation) of the two shorter PP17 variants (PP17a and PP17b).

All these findings suggest that the heterogeneity detected immunochemically can be attributed to the existence of a new protein family, similarly to the case of the pregnancy-specific β_1-glycoprotein, where a number of different protein bands were detected by immunoblot analysis and heterogeneity of mRNAs was also observed (Streydio et al., 1988; Watanabe and Chou, 1988; Streydio and Vassart, 1990). $PP17b_{1\text{-}10}$ cDNAs contain the whole coding region of $PP17a_{1\text{-}2}$ cDNAs in their 3' end, therefore PP17a and PP17b variants share a common C-terminal. The fact that anti-PP17 antiserum was produced by immunizing rabbits with PP17a variant may explain why our antiserum is reactive with these different proteins.

Homology search of nucleotide and protein databases revealed that very recently, simultaneously with our latest results, a PP17b identical protein - TIP47 - was discovered. Díaz and Pfeffer (1998) stated that the newly isolated TIP47 binds to the cytoplasmic domains of cation-dependent and cation-independent MPRs facilitating their transport from late endosomes to *trans*-Golgi network. The depletion of the cargo selection device TIP47 using either anti-TIP47 antibodies or TIP47 antisense oligonucleotides led to a defect in MPR recycling and a decrease in the half-life of MPRs.

Since TIP47 functions in MPR trafficking, involvement of MPRs in herpes simplex virus-2 (HSV-2) infections was discovered (Brunetti et al., 1994, 1995), and the connection of HSV-2 infection to cervical cancer was recently reinforced

(Southern and Herrington, 1998; DiPaolo et al., 1993, 1998), the functional findings regarding TIP47 may explain our observations for PP17b in cervical cancer tissues and in sera of patients.

Hypothetically: Brunetti et al. (1994, 1995) verified that HSV-2 glycoprotein D (gD) is essential for virus entry into host cells and also required for the efficient transmission between cells: HSV-2 gD is extensively modified by mannose 6-phosphate, binds to both cation-dependent and cation-independent MPRs and localizes into endosomes that contain MPRs. Since PP17b/TIP47 performs the transport of MPRs, it can also facilitate HSV-2 infection. Southern and Herrington (1998) emphasize that - contrary to earlier controversial studies on the etiopathogenic agents of cervical cancer - it has now become clear that most uterine cervical neoplasia are the result of a multistage carcinogenic process. Human papilloma virus (HPV) infection, which was earlier considered to be the main cause of cervical cancer, by itself is not sufficient for tumorgenesis, and HSV-2 involvement might also play an important role. Current findings by DiPaolo et al. (1993, 1998) emphasize that HPV integration and expression result in immortalization of human genital epithelial cells but are insufficient to induce malignancy. Transfection of the immortalized keratinocytes with HSV-2 resulted in invasive and noninvasive indolent cystic squamous cervical carcinomas, although HSV-2 DNA was not retained. HSV-2 infection itself or following the malignant transformation of cells possibly involves induction of altered gene expression, e.g. overexpression of its transporter PP17b/TIP47. This data may open a new trend in the research of pathological processes in human uterine cervical cancer.

4.2. PP13 - human placental galectin / lysophospholipase

In concordance with the isolation data (Bohn et al., 1983), we found that the highly purified PP13 antigen has a molecular mass of 16 kDa on SDS/PAGE. In human term placental tissues we detected the same protein, but it turned out not be entirely placenta specific, contrary to previous studies (Than et al., 1993), as variable quantities of PP13 was found in some other human tissues from 26 types of normal adult and fetal, and tumorous tissues investigated (human fetal/adult spleen, fetal kidney and adult bladder, adenocarcinoma of the liver, malignant melanoma and neurogen tumor). This data is in good agreement with data on other oncodevelopmental placental proteins of which genes are repressed after pregnancy, but can be reexpressed in various, benign and malignant tumorous cases.

We confirmed the results of previous radioimmune measurements of our research team (Than et al., 1986): PP13 is not secreted into circulation in cases of healthy controls nor in pregnant women.

PP13 protein encoded by the 578 bp full-length cDNA is essentially identical to PP13 antigen isolated by Bohn et al. in 1983: *(1)* The calculated molecular mass of the deduced amino acid sequence (16,118 kDa) is matching to the molecular mass of PP13 obtained by SDS-polyacrilamide gel electrophoresis after reduction with mercaptoethanol (16.000 kDa); *(2)* The deduced amino acid composition of PP13 shows similarity to the amino acid composition of PP13 antigen determined by physico-chemical methods previously; *(3)* Putative glycosilation site was found on the N-terminus of PP13 which is in accordance the data that PP13 is a glycoprotein.

Northern-blot analysis of PP13 mRNA in 16 healthy human adult tissues including placental tissue revealed different results from Western-blot analysis:

we found PP13 mRNA only in placental tissue sample but not in adult spleen and bladder tissues as we expected by the results of Western-blots. As inappropriate sampling can be excluded, possible reasons for the discrepancy may be: (1) the difference of PP13 expression in different individuals or (2) the cross-reactivity of anti-PP13 sera of any of the closest homologue proteins.

Computer assisted analyses of the deduced PP13 amino acid sequence revealed some interesting data: (1) PP13 can be regulated with phosphorylation by casein kinase II or tyrosine kinases. (2) PP13 turned out to be member of the beta-galactoside binding S-type animal lectin (galectin) superfamily and also to have homology to the carboxyl-terminal domains of some IgE binding proteins, as its similarity to the homologues is over 50% and 8 of the 16 invariant residues comprising the carbohydrate recognition/binding domains in galectins are conserved also in PP13. (3) The closest homologue of PP13 is the 16,5 kDa human eosinophil Charcot-Leyden Crystal (CLC) protein, a unique dual-function lysophospholipase found mainly in eosinophils.

Charcot-Leyden crystals (Charcot and Robin, 1853) - formed spontaneously in blood samples from patients with eosinophilia or present in tissues and biological fluids in cases of eosinophilic leukocyte infiltration - were shown to contain a human lysophospholipase as a sole component (Weller et al., 1980, 1982). Molecular cloning, crystal structure analysis and sugar-binding functional tests of CLC protein revealed that it is also the member of the galectin family and have IgE binding properties, as well (Ackerman, et al., 1993; Leonidas et al., 1995; Dyer and Rosenberg, 1996).

Beta-galactoside and IgE binding properties of PP13 have to be further examined, but PP13 - besides homology - possesses some of the characteristics of the galectin family. By Western-blot analysis PP13 was found to be composed of two

identical 16 kDa subunits held together by disulfide bonds. It has a carbohydrate content and also free/reactive thiol groups.

Lysophospholipase activity of PP13 was confirmed by ^{31}P NMR, a well-known method for these types of functional measurements (Plückthun and Dennis, 1982; Selle et al., 1993; Loo et al., 1997). Lysophospholipases are found in most cells, some cells may also have some different forms. Lysophospholipases are ranging in mass from 15 to 80 kDa and their function also vary from lysophosphatidic acid-mediated signal transduction in brain to intestinal digestion (Holtsberg et al., 1995). Lysophospholipases are esterolytic enzymes that catalyse the removal of a single fatty acid from the 1-position of lysophospholipids, which are generated by phospholipase A2 catalysing fatty acid hydrolysis from the 2-carbon position of phospholipds.

These data suggested that PP13 may also have multiple function. Hypothetically:

1) Cell migration is dependent on adhesion to extracellular matrix proteins for anchorage and traction. In addition to mediating adhesion, extracellular matrix binding to specific transmembrane receptors also effects cell growth and differentiation (Hay, 1991; Lin and Bisell, 1993). Also, trophoblast behavior during implantation is influenced by decidual extracellular matrix proteins (Aplin et al., 1988; Graham and Lala, 1991). Cells bind to extracellular matrix proteins by appropriate surface receptors called adhesion molecules, like integrins, cadherins, immunoglobulin superfamily and selectins (Coutifaris et al., 1991; Hynes, 1992, 1994).

Galectins are also involved in cell-cell and cell-matrix interactions, in cell-growth regulation and in apoptotical pathways (Kaltner and Stierstorfer, 1998; Perillo et al., 1998). For example Galectin-3 or Mac-2 murine macrophage cell surface protein (Cherayil et al., 1989) is a major non-integrin laminin-binding protein.

Galectins' appearance is developmentally regulated and temporally associated with the appearance of specific carbohydrate-containing structures in tissues (Barondes, 1984). As a member of the galectin superfamily, PP13 may also function in cell-growth regulation and the development and organization of microenvironment in the placenta.

2) Lysophospholipids have strong cytolitic and membrane-perturbing properties (Weltzien, 1979), so PP13 as a lysophospholipase may have a possible protective function during pregnancy for the implant and a certain role in the maintenance of pregnancy. For example we know that lysophospholipids have fusigenic properties and they are involved in the implantation mechanisms of rabbit embryos: cellular fusion of trophoblastic and uterine epithelial cells helps in embryo penetration into the uterine decidualized endometrium (Morin et al., 1992). PP13 therefore may function in the control mechanisms of human embryo implantation, as well. Another possibility, that PP13, as processing the lysophospholipid product of phospholipase A2, evaluates arachidonic acid production, particularly during the initiation of labor.

CHAPTER 5: GENERAL CONCLUSIONS

1., Four PP17 immunoreactive proteins were discovered in term placental tissue extract. We concluded by Western-blot analysis that these proteins are not placenta specific and expressed tissue dependently: PP17a (31.500 kDa) and PP17c (60.900 kDa) are found mainly in steroidogenic tissues, PP17b (48.000 kDa) is ubiquitous, PP17d (74.000 kDa) is expressed only in term placenta. In the following we confirmed the existence of a new protein family.

2., Two cDNAs have been isolated from a placental cDNA library encoding the 251 residue-long, 28.129 kDa PP17a variant. We determined the entire nucleotide and the deduced amino acid sequences, which were submitted to the GenBank database and are available under accession numbers AF051314 and AF051315. Computer assisted analyses of the sequences were performed.

3., Ten cDNAs encoding the 434 residue long 47.208 kDa PP17b variant were also isolated. After determination of the entire nucleotide and deduced amino acid sequences we submitted $PP17b_1$ sequences to the GenBank under accession number AF055574. Computer assisted analyses of the sequences were performed, as well.

4., PP17c immunoreactive protein turned to be dimer of PP17a variant, whose structural characteristics induce α-helical dimeric coiled coil formation.

5., PP17d is placenta specific and secreted into the circulation in elevating levels in the third trimester of pregnancy, therefore we suppose to develop a semiquantitative chemiluminescence Western-blot assay for seromonitorisation normal conditions in pregnancy. The cDNA of PP17d has not yet been isolated.

6., With Northern-blot analyses we demonstrated the expression of three different-size PP17 mRNAs in human healthy and tumorous tissues. This heterogeneity underlined our hypothesis about the alternative splicing of PP17 mRNAs as a background of the existence of PP17 protein family.

7., Overexpression of PP17b variant in cervical carcinoma patients were determined both in protein and mRNA levels. Serum levels of PP17b are increased at the diagnosis in cervical carcinoma patients and declines after curative operative treatment. This fact offers the possibility of seromonitoring, but only in operable cases.

8., Closest homologues of PP17 proteins (human adipophilin and mouse adipose differentiation-related protein) are adipose differentiation factors.

9., A newly discovered protein in 1998, TIP47 turned out to be entirely identical to the previously determined PP17b variant of the PP17 family. Since PP17b/TIP47 functions in mannose 6-phosphate receptor trafficking, theoretically it is possible that PP17b/TIP47 is involved in HSV-2 infections. As the connection of HSV-2 and HPV co-infection to cervical cancer is recently reinforced, the functional findings regarding TIP47 may explain our observations for PP17b in cervical cancer tissues and in sera of patients

10., Recently we have isolated a full-length cDNA encoding for the 139 residue-long, 16,118 kDa PP13. Complete nucleotide and amino acid sequence analysis (GenBank accession number: AF117383) and computer assisted structural search have been performed.

11., By Northern-blot analyses we found the 600 bp PP13 mRNA expressed in human placental tissue, but not in any of the other 15 tissues investigated.

12., By Western-blot analysis PP13 turned out not to be placenta specific as we found it in some human adult and fetal healthy tissues and in some tumors expressed. PP13 is not secreted into circulation neither in healthy controls, nor during pregnancies.

13., Alignment search showed that the human PP13 is a new member of the beta-galactoside binding S-type animal lectin (galectin) superfamily and it is a very close homologue of some IgE binding proteins. PP13 has the highest similarity to the 16,5 kDa human eosinophil Charcot-Leyden Crystal protein, a unique dual-function lysophospholipase.

14., Like Charcot-Leyden Crystal protein, PP13 also possesses lysophospholipase activity verified by ^{31}P NMR studies.

15., Hypothetically, as a member of the galectin family, PP13 may be involved in cell-cell and cell-matrix interactions, functioning in cell-growth regulation and the development and organization of microenvironment in the placenta. As a lysophospolipase, PP13 may have a possible protective function for the implant and a certain role in the maintenance of pregnancy. PP13 also can function in the control mechanisms of human embryo implantation, and probably in arachidonic acid production, particularly during the initiation of labor.

CHAPTER 6: REFERENCES

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CHAPTER 7: LIST OF PUBLICATIONS

7.1. Publications

Than, N.G., Sümegi, B., Than, G.N., Kispál, Gy., Bohn, H. (1998): Cloning and sequence analysis of cDNAs encoding human placental tissue protein 17 (PP17) variants. *Eur. J. Biochem.*, 258, 752-757.

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Than, N.G., Sümegi, B, Than, G.N., Berente, Z., Bohn, H. (1999) Isolation and sequence analysis of a cDNA encoding human placental tissue protein 13 (PP13), a new lysophospholipase, homologue of human eosinophil Charcot-Leyden Crystal protein. *Placenta*, submitted.

7.2. Abstracts

Than, N.G., Kispál, Gy., Sümegi, B., Than, G.N., Bohn, H. (1997) Amino acid sequence analysis of oncodevelopmental soluble placental tissue protein 17 (PP17) and measurements of the protein by RIA and by the newly developed highly sensitive chemiluminescence Western blot analysis. *Tumor Biol.*, 18, 110.

Than, N.G., Sümegi, B., Than, G.N., Bohn H. (1998) A new placental protein family with oncodevelopmental significance: Cloning and sequence analysis of the human placental protein 17 (PP17) family. *Tumor Biol.*, 19, (S2), 33, 1998.

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7.3. Presentations

7.3.1. Presentations in the topic

Than, G., Kispál, Gy., Szabó, D., Than, G.N., Bohn, H.: *"Measurements of soluble placental tissue protein 13 (PP_{13}) and 17(PP_{17}) by RIA and the highly sensitive chemiluminescence western blot analysis"*
3rd International Conference of the Hungarian Biochemical Society
Pécs, Hungary, 6-9th July 1997 (poster)

Than, N.G., Kispál, Gy., Sümegi, B., Than G.N., Bohn, H.: *"Amino acid sequence analysis of oncodevelopmental soluble placental tissue protein 17 (PP17) and measurements of the protein by RIA and by the newly developed highly sensitive chemiluminescence Western blot analysis"*
XXV. Meeting of the International Society for Oncodevelopmental Biology and Medicine
Montreux, Switzerland, 19-24th September, 1997 (poster)

Than, N.G., Kispál, Gy., Sümegi, B., Than, G., Bohn, H.: *"Humán placenta protein 17 (PP_{17}) génjének izolálása, aminosavszekvenciájának megállapítása; a fehérje expressziójának vizsgálata terhesek szérumában, lepényi- és cervix carcinoma extraktumban és gesztációs trophoblaszt tumor sejtvonalon"*
XXVI. Meeting of the Hungarian Society of Gynecologists and Obstetricians
Pécs, Hungary, 15-18th April, 1998 (poster)

Than, N.G., Sümegi, B., Bohn, H., Than, G., Kispál, Gy.: *"Egy humán onkodevelopmentális placenta protein: a PP17 klónozása és szekvencia analízise"*
3rd Workshop of Hungarian Biochemical Society
Sárospatak, Hungary, 11-14th May 1998

Than, N.G., Sümegi, B., Kispál. Gy., Than, G., Bohn, H.: *"A humán placenta protein 17 (PP17) család klónozása és szekvencia analízise"*
XXVIII. Membrane-Transport Conference, ***I. prize - Varga Vince Award***
Sümeg, Hungary, 26-29th May, 1998 (poster)

Than, N.G., Sümegi, B., Kispál, Gy., Than, G., Bohn, H.: *"Cloning and sequencing of human placental protein 17 (PP17) family"*
XXVIII. Membrane-Transport Conference
Sümeg, Hungary, 26-29th May 1998

Than, N.G., Sümegi, B., Than, G.N., Bohn, H.: *"A new placental protein family with oncodevelopmental significance: Cloning and sequence analysis of the human placental protein 17 (PP17) family"*
XXVI. Meeting of the International Society for Oncodevelopmental Biology and Medicine
Umeå, Sweden, 1-4th September, 1998 (invited speaker)

Than, N.G., Sümegi, B., Than, N.G., Bohn, H.: *"Cloning and sequencing of members of the human placental protein 17(PP17) family"*
4th Conference of the International Federation of Placental Associations
New Investigator's Award
Tokyo, Japan, 1-3rd October, 1998 (poster)

Than N.G.: *"Placenta protein 17 (PP17) variánsok klónozása és szekvencia analízise"*
Scientific Meeting of the University Medical School of Pécs
Pécs, Hungary, 19th April, 1999 (invited speaker)

Than N.G., Sümegi B., Than G., Bohn H.: *"A humán placenta protein 13 (Charcot-Leyden Crystal Protein homológ) klónozása és szekvencia analízise"*
4th Workshop of Hungarian Biochemical Society
Eger, Hungary, 10-13th May, 1999 (poster)

7.3.2. Other presentations

Than, G., Szánya, J.: *"A rapid and sensitive assay for large scale analysis of enzyme-immunoglobulin interaction"*
Annual Congress of the Student Researchworkers at the Medical School of Pécs
Pécs, Hungary, 3-5th March 1994

Szánya, J., Than, G.: *"Immunoglobulin-enzyme interaction analyzed in a citrate-synthase - anti-citrate synthase model"*
Annual Congress of the Student Researchworkers at the Medical School of Pécs
Pécs, Hungary, 3-5th March 1994

Than G., Szánya, J.: *"A rapid and sensitive assay for large scale analysis of enzyme-immunoglobulin interaction"*
5th European Medical Students' Conference
Humboldt University, Berlin, Germany, 20-22nd October 1994 (poster)

Than, G., Szánya, J.: *"Nagyszámu párhuzamos minta vizsgálatára alkalmas módszer kidolgozása enzim-immunglobulin kölcsönhatások tanulmányozásához"*
Annual Congress of the Student Researchworkers at the Medical School of Pécs
Pécs, Hungary, 23-25th February 1995

Szánya J., Than, G.: *"Enzim-immunglobulin kölcsönhatások modellvizsgálata citrát szintáz - anti-citrát szintáz ellenanyagok segítségével"*
Annual Congress of the Student Researchworkers at the Medical School of Pécs
Pécs, Hungary, 23-25th February 1995

Than, G.: *"Developement of education of oncology at Medical Universities respect with molecular epidemiological aspect"*
1st European Medical Students' Symposium
Athens, Greece, 15-16th April 1995

Szánya, J., Than, G.: *"Immunoglobulin-enzyme interaction analyzed in pig heart synthase - anti-citrate synthase model"*
1st European Medical Students' Symposium
Athens, Greece, 15-16th April 1995

Than, G., Szánya, J.: *"Analysis of immunoglobulin - enzyme interaction in citrate synthase - anti-citrate synthase model"*
VI. Health Sciences Students' Conference
La Laguna, Tenerife, Spain, 27-28th April 1995 (poster)

Németh, P., Szánya, J., Than, G.: *"Are antibodies able to modify enzyme functions?"*
9th International Congress of Immunology
San Francisco, USA, 23-29th July 1995 (poster)

Than, G.: *"Experiences in a new medical educational system: Clinical Training from a student's point of view"*
Skills training and OSCE in medical education - Educational Workshop
Pécsvárad, Hungary, 14-16th September 1995

Than, G.: *"Experiences in the Dutch Credit System and ECTS"*
TEMPUS SMART Evaluation Meeting
Budapest, Hungary, 22nd June 1997

Kispál, Gy., Csere, P., Than, G., Lill, R.: *"Mitochondrial iron metabolism"*
3rd International Conference of the Hungarian Biochemical Society
Pécs, Hungary, 6-9th July 1997

Németh, P., Tus, K., Than, N.G., Nagy, G.: *"Enzim-immunglobulin kölcsönhatások vizsgálata mono- és poliklonális ellenanyagokkal"*
3rd Workshop of Hungarian Biochemical Society
Sárospatak, Hungary, 11-14th May 1998

Nagy, G., Than, N.G., Tus, K.: *"Mitokondriális enzimek és autoantitestek kölcsönhatásának funkcionális vizsgálata"*
II. National Conference of PhD Students
Debrecen, Hungary, 30-31st August, 1998

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