

**Functional analysis of PP17b and PP20 soluble
placental proteins**

DOCTORAL DISSERTATION

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PhD THESIS

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2005.

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Abbreviations:

ADRP, adipose differentiation related protein; BSA, bovine serum albumin; CIM, confocal immunofluorescence microscopy; CIN, cervical intraepithelial neoplasia; dbcAMP, dibutyryl cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; ESI: Electrospray ionisation; EST, expressed sequence tag; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; ICC, immunocytochemistry; IHC, immunohistochemistry; IPTG, isopropyl- β -D-thiogalactoside; ISC, in situ carcinoma; MS, mass spectrometry; MLGM, milk lipid globule membrane; MPR, mannose 6-phosphate receptor; PBS, phosphate-buffered saline; PI, propidium iodide; PFA, paraformaldehyde; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonyl-fluoride; PP17, Placental Protein 17; PP20-B, PP20 purified from placenta by Dr. Bohn; PP20-R, recombinant PP20; PSD, post source decay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, tris-buffered saline; TIP47, tail interacting protein of 47 kDa, PP20, placental protein 20; TPK, thiamin pyrophosphokinase

INTRODUCTION

Pregnancy-related protein research

As their name suggests, pregnancy-related proteins were discovered through comparative examinations of pregnant and nonpregnant samples. As early as 1958 Thornes (using antiplacenta immunoserum) observed four components in the serum of pregnant women, which were not present in the blood of healthy nonpregnant 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 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) 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 *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**).

FETAL PROTEINS

Table 1.

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 (PSbetaG)
2	SP2	Sex-Hormone-Binding Globulin (SHBG)
3	SP3	Pregnancy-Associated α ₂ -Glycoprotein (α ₂ PAG) 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	Insulin-Like Growth Factor Binding Protein 1 (IGFBP-1)
13	PP13	galectin-13
14	PP14	(31-Lactoglobulin Homologue / Glycodelin-A
15	PP15	
16	PP16	
17	PP17	TIP47/Sandrin
18	PP18	Branched-chain aminotransferase

Table 1.

19	PP19	
20	PP20	human thiamin pyrophosphokinase
21	PP21	Membrane Cofactor Protein (MCP)
22	PP22	Cellular Thyroid Hormone Binding Protein
23	PP23	SOUL/Heme binding protein 2
24	PP24	Human Sphingolipid Activator Protein 1 (SAP-1)
25	PP25	unknown
26	PP26	

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	

Aims of the study

Women's Clinic and jointly Institute of Biochemistry and Medical Chemistry of the University of Pécs has been involved in the collaboration research of the pregnancy-related oncofetal proteins for thirty years. During the years of collaboration the team has performed the isolation, and the basic and applied research study in normal and pathologic circumstances of 9 novel proteins, and developed new measuring procedures (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). Since 1997 the team have been dealing with the sequential, structural, functional and expressional analyses of 8 placental proteins (PP13, PP17a, PP17b, PP18a, PP18b, PP20, PP23 and PP25) with genomical and proteomical methods (Than et al., 1998; 1999; 1999; 2001;2001; Bellyei et al., 2004).

The research group discovered the PP17 protein family at the millennium. Two members of the protein family could be cloned and sequenced (PP17a: GenBank Accession No. AF051314, AF051315; PP17b: GenBank Accession No. AF055574; PP17c and PP17d). The team have started the functional analysis of the PP17 protein family. The PP20 protein was not identified before, we knew only slightly more than a decade. PP20 is composed of two identical 27 kDa subunits. The carbohydrate content of PP20 was found to be relatively low (3.0% by weight) and the average amount of PP20 present in one human term placenta was found to be 0.5 mg.

The present study is aimed at a more in-depth study of these two placental proteins, including:

1. Databank search to reveal the expression, structure and regulation of the PP17 gene. Multiple sequence alignment, to prove to be a member of the growing lipid storage droplet protein family.
2. Immunofluorescence microscopy and protein sequencing to present the evidence for the association of PP17b to lipid droplets and milk lipid globule membranes.
3. Using a HeLa cell model to show the importance of protein kinase A and protein kinase C dependent pathways for the regulation of PP17 gene expression, and the effect of the phase of cell cycle, differentiation and apoptosis on expression of this gene.
4. Isolation of the cDNA of PP20 and analysis of the nucleotide and deduced amino acid sequences.
5. Databank search to demonstrate the genomic localization, structure and regulation of PP20 gene
6. Identification of PP20 by mass spectrometry
7. Analysis of PP20 / hTPK1 enzymatic activity by using HPLC-MS with electrospray (ESI) ionisation
8. Expression and localization of PP20
9. Construction of the comparative 3D modelling model of PP20

Investigated soluble placental tissue proteins

Placental protein 17 (PP17)

The isolation and characterization of PP17 was first reported in 1983 (Bohn et al., 1983). 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. The carbohydrate content was found to be 2.1% by weight. Localisation of PP17 was investigated by Inaba (Inaba et al., 1983). In human early placentas PP17 was localized mainly in the cytoplasm of villous syncytiotrophoblast, in human term placentas positive staining for PP17 was obtained in the cytoplasm of both basal and reflected chorionic trophoblastic cells. With the use of an electroimmunoassay (detection limit 1 mg/ml), 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. Since 1997 our laboratories have been performing detailed studies about PP17 soluble placental protein with genetical and proteomic methods. Our research group showed that the PP17 protein family consists of four PP17 variants (PP17a, PP17b, PP17c and PP17d of 30, 48, 60 and 74 kDa) (Than et al., 1997). The entire nucleotide and amino acid sequences of C-terminus sharing 30 kDa PP17a (AF051314, AF051315) and 48 kDa PP17b (AF055574) were determined and deposited to GenBank. Furthermore, their expression patterns in various human tissues and serum levels in different conditions were also studied and published in this Journal for the first time (Than et al., 1997, 1998). The closest homologues of PP17 variants were found to be human *adipophilin* (Heid et al., 1998) and mouse *adipose differentiation-related protein* (Jiang et al., 1992) involved in early

adipocyte differentiation; and human (Nishiu et al., 1998) and rat (Greenberg et al., 1993) *perilipins*, major hormonally regulated adipocyte-specific phosphoproteins. The subsequently GenBank deposited *TIP47* (AF057140) proved to be identical to PP17b. It was shown that TIP47-glutathione S-transferase fusion proteins bind to both the cation-dependent and independent mannose 6-phosphate receptors (MPRs) *in vitro*, and thus the protein was named TIP47 (tail-interacting protein of 47 kDa). It was proposed that TIP47 directs the retrieval of MPRs from the pre-lysosomal compartment with delivery back to the *trans*-Golgi network through the interaction with the cytoplasmic tails of MPRs (Diaz et al., 1998).

Parallel, a debate started on the possible function of TIP47, as a recent paper stated that TIP47 plays a role rather in intracellular lipid metabolism than in secretory protein sorting, taking into account that there is a high-level amino acid sequence similarity in the N-terminal region of TIP47 and other lipid droplet-associated proteins, which localize on the surface of lipid droplets in a lipid synthesis/storage status-responsive manner (Wolins et al., 2001). A reply paper reinforcing the protein's MPR transport function emphasized that TIP47 is not a lipid droplet component, and accused probable cross reactivity of TIP47 antibody with N-terminus of adipophilin of leading to that finding (Barbero et al., 2001). Most recently, evidence was presented using GFP-tagged TIP47, that it co-localizes with intracellular lipid droplets, showing that there is a disagreement between the cellular function of TIP47 (Miura et al., 2002).

In the past four years, the oncological significance and overexpression of PP17b in human uterine squamous cervical carcinoma tissues and HeLa (squamous cervical cancer) cells were established. Serum PP17b levels were found to be elevated in cervical carcinoma patients, which declined after radical surgery (Than et al., 1998, 1999, 1999). Normal cervical epithelia were negative for PP17b, while cytoplasm of the dysplastic cells were positive in low-grade dysplasias, and strongly positive in high-grade dysplasias. In invasive squamous

cervical carcinomas, cytoplasms of basal-type tumor cells were negative, while squamous-type dysplastic cells were strongly positive (Than et al., 2001). Now, by extensive databank search, structural similarities between human PP17 (TIP47), adipophilin and perilipin genes were revealed, and the analysis of the 5' flanking region of PP17 gene showed a number of potential transcription factor binding sites indicating its complex transcriptional regulation

Placental protein 20(PP20)

Isolation and physicochemical characterization of soluble placental tissue protein 20 (PP20) was described in 1985 (Bohn and Winckler). PP20 was found to be composed of two identical 27 kDa subunits which were held together by non-covalent bonds. The carbohydrate content of PP20 was found to be relatively low (3.0% by weight) and the average amount of PP20 present in one human term placenta was found to be 0.5 mg (Bohn et al., 1985). By immunohistochemical studies, in human early placenta, PP20 was mainly located in the cytoplasm of the syncytiotrophoblast, cytotrophoblast, and chorionic trophoblast. In term placentas the protein was clearly localized mainly in the cytoplasm of Hofbauer-like cells in the villous stroma, as well as in the reflected and basal chorionic trophoblast, but not localized in term villi (Inaba et al., 1987). With an electroimmunoassay, PP20 could not be detected in normal sera, pregnancy sera, cord blood sera or amniotic fluid, and the protein appeared not to be placenta specific (Bohn et al., 1985).

MATERIALS AND METHODS

(PP17B)

Materials

PP17 antigen (Op. 169/195) and anti-PP17 rabbit antibody (54ZB) were prepared by Dr. Hans Bohn (Behringwerke AG, Marburg/Lahn, Germany). We obtained FITC labeled goat anti-rabbit IgG (BD Biosciences, Heidelberg, Germany); anti-bax (4F11) and anti-bcl-2 (124) monoclonal antibodies, and Universal Kit (Immunotech, Marseille, France); HeLa S3 cell line (ATCC, Manassas, VI, USA). We purchased 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, antibiotic-antimycotic solution, benzamidine, bovine serum albumin, cyanogen-bromide activated agarose beads, dibutyryl cyclic AMP, DNase-free RNase, Dulbecco's modified Eagle's medium, fetal calf serum, horseradish peroxidase labeled goat anti-rabbit IgG, leupeptin, oleic acid, and phorbol myristate acetate (Sigma, St. Louis, MO, USA); Nile red (Molecular Probes Inc., Eugene, OR, USA); protein kinase A and C inhibitors (Calbiochem, Darmstadt, Germany); trypsin (Promega GmbH, Mannheim, Germany); ZipTipC18 pipette tips (Millipore, Bedford, MA, USA); ECL chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK); carboplatin/Paraplatin[®] (Bristol-Myers-Squibb, Sermonetta, Italy); 5-fluorouracil/5-FU "Lederelle"[®] (Wyeth-Whitehall, Wolf Rats Hausen, Germany); irinotecan/Campto[®] (Rhone-Poulenc Rorer, West Malling, UK); mitomycin/Mitomycin C[®] (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan); and paclitaxel/Taxol[®] (Bristol Arzneimittel GmbH, München, Germany).

Databank search

PP17b cDNA was compared to different EST and genomic databases by BLAST (Altschul et al., 1997) and UCSC Genome Browser, alignments of PP17b cDNA and related ESTs to genomic sequences were performed with LocusLink (Pruitt

et al., 2001), all provided by NCBI (Bethesda, MD, USA). Transfac Database was searched (Heinemyer et al., 1998) for putative transcription binding sites at the 5' flanking region of PP17/TIP47 gene using PatSearch (GBF-Braunschweig, Germany). Multiple amino acid sequence alignment of PP17b to its homologues was carried out with CLUSTALW at EMB-net (Lausanne, CH) (Thompson et al., 1994).

Cell culture and drug treatments

Confluent monolayers of synchronized HeLa cells were grown on 100-mm dishes in standard DMEM containing 1% antibiotic-antimycotic solution, supplemented with 10% FCS under 5% CO₂ condition and 95% humidified air at 37°C. For immunocytochemistry and confocal immunofluorescence microscopy, cells were cultured on poly-2-lysine coated glass coverslips, dried overnight and stored at -80°C. To increase triacylglycerol storage, cells were incubated in culture media supplemented with 600 µM oleic acid complexed to fatty acid free BSA (molar ratio of 6:1) for 20h. For apoptosis induction, cytostatic drugs (carboplatin 0.75 µg/ml, 5-fluorouracil 25 µg/ml, irinotecan 5 µg/ml, mitomycin 10 µg/ml, and paclitaxel 10 nM) were diluted in culture medium and applied for 24h. To induce differentiation, cells were treated with 0.5 mM dbcAMP for 72h or 80 nM PMA for 48h. There were cells incubated with 0.1 µM PKC inhibitor or 0.36 µM PKA inhibitor (10x Ki in each cases) parallel to treatments with paclitaxel, dbcAMP or PMA.

Subcellular fractionation

Lipid loaded HeLa cells were harvested and low-speed centrifuged. Pellets were dispersed by vortexing in hypotonic lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM benzamidine, 100 µM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride and 10 µg/ml leupeptin) for 10min at 4 °C. After further cell disruption in a Teflon/glass homogenizer, homogenates were centrifuged for

10min at 1000 x g at 4 °C, the supernatants were mixed with 70% sucrose (w/w) in a ratio of 1:1.5, and layered under a linear 0–40% sucrose (w/w) gradient. 6-ml tubes were centrifuged for 4h at 154,000 x g in a Beckman SW41Ti rotor at 4 °C. Lipid droplet fractions were collected in 1 ml by slicing off the tops of the tubes with a Beckman tube slicer, and then five additional 1-ml fractions were collected. Equal portions of the fractions were either separated by SDS-PAGE for Western blot or solvent extracted for lipid analysis.

Milk lipid globule membrane fractionation and monolayer preparation

Total protein of fresh milk obtained from human volunteers was extracted for 5-8 times with mixtures of chloroform and methanol (1:1 and 2:1, v/v) at ratios not exceeding 5 mg protein/ml. For the isolation of MLGM associated proteins, MLGM fractions were separated from milk by sucrose gradient centrifugation, and then proteins were further separated from lipids by chloroform / methanol extraction. Both total and MLGM associated proteins were then subjected to SDS-PAGE / Western blot. For immunofluorescence imaging, we developed MLMG monolayers mixing milk with 0.5% agar (w/v) in 1% concentration at 60 °C, then fixing the mixture on glass coverslips.

SDS-PAGE / Western blot

1 ng PP17 antigen and 10-10 µg protein from term placental total protein extract, HeLa total protein extracts and subcellular fractions, milk total protein extracts and MLMG fractions were subjected to 12% SDS-PAGE (w/v). Immunoblots were carried out with anti-PP17 antibody and horseradish peroxidase labeled secondary IgG as described earlier (Sambrook et al. 1989). Protein bands were revealed by ECL chemiluminescence system followed by quantitative densitometry using Scion Image for Windows.

Lipid analysis

Solvent extraction and thin layer chromatographic separation of neutral lipids were done as described in (Braesemle et al., 1997); densitometric quantification was similar as for proteins.

Immunoaffinity purification and protein identification by mass spectrometry

Anti-PP17 antibody was coupled to cyanogen-bromide activated agarose beads and incubated with lipid loaded HeLa cell or milk total protein extracts at room temperature for 30min. The gels were washed three times with 20 mM Tris-HCl buffer (pH 7.4, containing 150 mM NaCl) to remove unbound proteins. The immunoreactive proteins were removed with an equal volume of 2x Laemmli sample buffer, then proteins were separated by gradient (6-18 %) one-dimensional PAGE and visualized by Coomassie staining. Bands of interest were excised from the gel, reduced, alkylated and in-gel digested with trypsin as described previously (Shevchenko et al., 1996). Proteins were identified by a combination of MALDI-TOF MS peptide mapping and MALDI-PSD MS sequencing. The digests were purified with ZipTipC18 pipette tips with a saturated aqueous solution of 2,5-dihydroxybenzoic acid matrix (ratio of 1:1). A Bruker Reflex IV MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) was employed for peptide mass mapping in positive ion reflector mode with delayed extraction. The monoisotopic masses for all peptide ion signals in the acquired spectra were determined and used for database searching against a non-redundant database (NCBI, Bethesda, MD, USA) using MS Fit program (UCSF, San Francisco, CA, USA) (Clauser et al., 1999). Primary structures of tryptic peptide ions were confirmed by PSD MS sequencing.

Immunolocalization of PP17b in squamous cervical carcinoma tissue sections and in HeLa cells

Tissue sections were prepared from routine formalin-fixed, paraffin-embedded samples of invasive uterine squamous cervical carcinoma (n=20). Four μm sections were cut, mounted on slides, dried at 37 °C overnight, dewaxed and rehydrated. Both tissue sections and the cell culture samples described above were incubated with anti-PP17 antibody, and with monoclonal anti-bax and anti-bcl2 antibodies for the parallel assessment of apoptosis (Harima et al., 2000). Immunostaining was carried out according to the streptavidin-biotin-peroxidase technique, with hydrogen peroxide / 3-amino-9-ethylcarbazole development using Universal Kit (Bratthauer et al., 1994). Visual evaluation of hematoxylin-counterstained slides was performed by Olympus BX50 light microscope with incorporated photography system (Olympus Optical Co., Hamburg, Germany).

Confocal immunofluorescence microscopy

Fixed cells and MLMG monolayers were consecutively treated with anti-PP17 antibody followed by FITC labeled secondary IgG in PBS containing 0.1% saponin and 0.1% BSA. For neutral lipid staining, 0.01% Nile red dissolved in DMSO was added parallel to the secondary antibody solutions. Cell fluorescence was monitored with a Bio-Rad MRC-1024ES laser scanning confocal attachment (Herefordshire, UK) mounted on a Nikon Eclipse TE-300 inverted microscope (Kingstone, UK).

Flow cytometry and cell cycle analysis

Synchronized cultured cells were harvested, washed in PBS and fixed with 4% PFA for 20min at 4 °C. Immunofluorescent intracellular PP17 staining was performed in permeabilization buffer (0.1% saponin, 0.1% NaN_3 and 0.1% BSA in PBS) with a two-step labeling technique (Berki et al., 1998), using anti-PP17 antibody and FITC labeled secondary IgG for 30min each at 4 °C. For cellular

DNA content analysis, after intracellular staining, samples were incubated with 100 µg/ml RNase followed with 5 µg/ml PI for 30-30min at 24 °C. 10-10,000 events were measured in each sample on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), and statistically analyzed using CellQuest software. PP17 quantities were measured in FL-1, while cellular DNA contents in FL-2 channel. To determine PP17 gene expression in cell cycle phases, gates were set on different peaks of the FL-2 histograms.

Statistical evaluation

Values in the figures, tables and text are expressed as mean \pm SEM of n observations. Statistical analysis was performed by analysis of variance followed by Turkey's and chi-square tests. Statistical significance was set at $p < 0.05$.

(PP20)

Materials

PP20 antigen (Lot No. 41/60) and anti-PP20 polyclonal rabbit serum (461ZA) were prepared by Dr. Hans Bohn, Behringwerke AG, Germany (Bohn and Winckler, 1991). *E. coli* XL1-Blue MRF strain, human placental Uni-ZAP™ XR expression cDNA library and an *in vitro* packaging system were purchased from Stratagene (La Jolla, CA, USA). We obtained HeLa (human cervical epithelial adenocarcinoma), HepG2 (human liver hepatocellular carcinoma), Panc-1 (human pancreas ductal epitheloid carcinoma) and WRL-68 (human fetal hepatic cells) cell lines (ATCC, Manassas, VA, USA); BCA reagent (Pierce Biotechnology Inc., Rockford, IL, USA); ECL chemiluminescence system (Amersham Pharmacia Biotech Buckinghamshire, UK); FITC labelled goat anti-rabbit IgG (BD Biosciences, Heidelberg, Germany); DRAQ5 dye (Biostatus Ltd., Shepshed, UK); trypsin (Promega, Mannheim, Germany); ZipTipC18 pipette tips (Millipore, Bedford, MA, USA); HPLC quality methanol (Scharlau,

Barcelona, Spain), Glutathione Sepharose 4B column (Amersham Biosciences, Uppsala, Sweden); ammonium-acetate, antibiotic-antimycotic solution, bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), glutathione, isopropyl- β -D-thiogalactoside (IPTG), horseradish peroxidase labelled goat anti-rabbit IgG, phenylmethylsulfonyl-fluoride (PMSF), thiamin-hydrochloride, thiamin-pyrophosphate-hydrochloride, T7 and T3 and sequence specific oligonucleotide primers (Sigma-Aldrich Co., St. Louis, MO, USA).

Blood and tissue samples

Specimens (n=39) were collected from different types of human tissues. Normal fetal (n=12) and adult (n=25) tissues including first trimester (10-12th gestational week) and term placenta, or tumorous tissues (n=6) were investigated. After homogenization by ultraturrax in standard Laemmli sample solution containing 1 mM PMSF, all the samples were ultracentrifuged (10,000g for 10 min), the supernatants were then measured by bicinchoninic acid (BCA) reagent assay and equalized for 1 mg/ml protein content. Blood was collected from patients with carcinomas (ovarian-, chorio-, cervix-, corpus-) and from healthy pregnant women at the 8th, 14th, 20th, 24th, 28th and 32nd gestational weeks. Sera were separated by ultracentrifugation and diluted equally in standard Laemmli sample solution. All samples were stored at -20 °C until assay. Placentas were formalin-fixed, paraffin-embedded, cut for 4 μ m sections, mounted on slides, dried at 37 °C overnight, dewaxed and rehydrated for further immunohistochemistry or immunofluorescent confocal imaging.

Cell cultures

HeLa, HepG2, Panc1 and WRL-68 cells were grown on 100-mm dishes in standard DMEM containing 1% antibiotic-antimycotic solution, supplemented with 10% FCS under 5% CO₂ condition and 95% humidified air at 37 °C. Cells

were harvested and low-speed centrifuged, then the pellet was dispersed by vortexing in lysis buffer (50 mM Tris pH 7.4, 1 mM PMSF) for 10 min at 4 °C. After further cell disruption in a Teflon/glass homogenizer, the homogenate was pelleted, and the supernatant was measured by BCA reagent and equalized for 1 mg/ml protein content in 2x Laemmli solution for Western blotting.

Cloning and sequence analyses of PP20 cDNAs

A placental expression cDNA library was plated on 150-mm Petri dishes at 100,000 plaques/plate for the identification of cDNAs encoding PP20. λ phage protein expression was induced with 10 mM IPTG. From all plates, replicas were loaded onto nitrocellulose membranes and anti-PP20 serum was used to screen the filters. Primary immunoreaction was detected by alkaline phosphatase-conjugated anti-rabbit IgG and developed by the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tertazolium phosphatase reaction system. Positive plaques were isolated and plaque-purified by rescreening five more times by the same procedure. λ phages were converted into pBluescript SK- phagemids with R408 helper phage by the method of (Short et al., 1992). Miscellaneous procedures were performed as published in Sambrook et al. (1989). Plasmid preparation was carried out with QIAprep Spin Miniprep Kit. Both sense and antisense strands of the cDNAs were sequenced by primer walking. Sequencing by the dideoxynucleotide chain termination chemistry was performed by *Taq* BigDye terminator cycle sequencing using an automated ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, USA). Sequences were analyzed using the NCBI database.

Databank search

PP20 cDNAs were compared to different ESTs and genomic databases by BLAST algorithm (Altschul et al., 1997). Alignment of the longest PP20 cDNA and related ESTs to genomic sequences were performed with LocusLink (Pruitt

et al., 2001), all provided by NCBI (Bethesda, MD, USA). Transfac Database (Heinemeyer et al., 1998) was searched for putative transcription binding sites at 1kb of the 5' flanking region of PP20 / hTPK gene using the Patch algorithm (GBF-Braunschweig, Germany).

Protein identification by mass spectrometry

The highly purified PP20-B antigen was diluted in 2x Laemmli sample buffer, separated by 12 % (w/v) SDS-PAGE and visualized by Coomassie staining. Bands of interest were excised from the gel, reduced, alkylated and in-gel digested with trypsin as described previously (Shevchenko et al., 1996). Proteins were identified by a combination of MALDI-TOF mass spectrometry MS peptide mapping and MALDI post source decay (PSD) MS sequencing. The digests were purified with ZipTipC18 pipette tips with a saturated aqueous solution of 2,5-dihydroxybenzoic acid matrix (ratio of 1:1). A Bruker Reflex IV MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) was employed for peptide mass mapping in positive ion reflector mode with delayed extraction. The monoisotopic masses for all peptide ion signals in the acquired spectra were determined and used for database searching against a non-redundant database (NCBI, Bethesda, MD, USA) using MS Fit program (UCSF, San Francisco, CA, USA) (Clauser et al., 1999). Primary structures of tryptic peptide ions were confirmed by PSD sequencing.

Construction of bacterial PP20 / hTPK1 expression plasmid

From the pBlueScript plasmid containing the full length PP20 cDNA, the whole open reading frame was PCR amplified with (5'-AGGATCCATGGAGCATGCCTTACCCCGTTGG-3') and (5'-AAACTCGAGCACACTTATGGATGCCAG-3') primers. The resultant PCR product was cloned into the *Bam*HI and *Xho*I sites of the pGEX-4T-1 expression vector.

Expression and purification of PP20 / hTPK1

The PP20-R/pGEX-4T-1 expression vector was transformed into *E. coli* DH5 α competent host strain. After isolation of the plasmid, it was transformed into *E. coli* BL21 host strain. Bacteria were induced with IPTG, and the expressed protein was subsequently purified with Glutathione Sepharose 4B column in the presence of glutathione, after which thrombin cleavage was carried out. The purity of PP20-R was verified by immunoblotting with anti-PP20 serum.

Analysis of PP20 / hTPK1 enzymatic activity by using HPLC-MS with electrospray (ESI) ionization

Five portions of thiamin were dissolved in 1.5 ml of 100 mM Hepes (pH =7.4, 1 mM MgCl₂, 1 mM ATP) to a final concentration of 1 mM. Twenty μ g of PP20-B and PP20-R antigens were added to one of the samples; in control cases PP20 was excluded from the mixture or 20 μ g of heat denaturated PP20-B or PP20-R was added. All the samples were then incubated for 10 hours at 37 °C, following which the analysis of thiamin and thiamin-pyrophosphate was carried out using HPLC-MS with electrospray ionization (ESI). The HPLC-MS instrument consisted of a Dionex P 580 low-pressure gradient pump, Rheodyne 8125 injector with 20 μ l loop a Dionex UVD 340S diode array detector a Dionex AQA mass spectrometer. Instrument control and data acquisition were performed using Chromeleon data management software. The column (150 \times 4.6 mm) was packed with 5 μ m particle size endcapped C₁₈ reversed phase material. The mobile phase was 0.1 M ammonium-acetate (pH = 7.0) / methanol = 95/5. The flow rate was 1.2 ml/min. ESI ionization was performed in positive mode enabling to obtain positively charged molecular ions ([thiamin + H]⁺, [thiamin-pyrophosphate + H]⁺).

SDS-PAGE / Coomassie staining or Western blot

20 µg of PP20-B and PP20-R proteins were subjected to 12% (w/v) SDS-PAGE and visualized by Coomassie staining for subsequent MS protein identification. 10 ng PP20-B, PP20-R and 20 µg protein of serum, tissue and cell culture samples were subjected to 12 % (w/v) SDS/PAGE (BioRad, Hercules, Ca, USA). Immunoblotting was carried out as described in Sambrook et al. (1989). Protein bands were revealed by the ECL chemiluminescence analysis system.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were incubated with polyclonal anti-PP20 serum. Immunostaining was carried out according to the streptavidin-biotin-peroxidase technique, with hydrogen peroxide / 3-amino-9-ethylcarbazole development using the Universal Kit (Bratthauer et al., 1994). Control sections were incubated only with secondary IgG. Visual evaluation of hematoxylin-counterstained slides was performed using an Olympus BX50 light microscope with incorporated photography system (Olympus Optical Co., Hamburg, Germany).

Immunofluorescence confocal microscopy

After deparaffination, placental tissue sections were washed with distilled water and TBS (pH 7.4, 10 mM Tris, 148 mM NaCl) and digested with trypsin for 30 minutes. The slides were rinsed with TBS and PBS (pH 7.3, 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) followed by treatment with polyclonal anti-PP20 serum. Subsequently parallel labelling with FITC labelled secondary IgG, and 20 µM DRAQ5 dye nucleus labeling in PBS and 0.1% (v/v) BSA were carried out. Control sections were incubated only with secondary IgGs. Cell fluorescence was monitored by a Bio-Rad MRC-1024ES laser scanning confocal attachment (Herefordshire, UK) mounted on a Nikon Eclipse TE-300 inverted microscope (Kingstone, UK).

Construction of the 3D model of PP20 / hTPK

Multiple amino acid sequence alignment of PP20 / hTPK to its homologues was carried out with CLUSTALW at EMB-net (Lausanne, CH) (Thompson et al., 1994). The PROSITE (Falquet et al., 2002) and NetPhos (Blom et al., 1999) databases were searched for biologically significant patterns and putative phosphorylation sites on PP20 / hTPK by Protein Explorer (Sayle et al., 1995). A standard comparative modelling with MOLSCRIPT on a Linux PC workstation was performed as described by Kraulis (1991). Because of the high degree of homology of PP20 / hTPK to mouse TPK (89% sequence identity), it was satisfactory to use the mouse TPK as a single reference protein. The atomic coordinates of the reference protein were obtained from the Protein Data Bank (Berman et al., 2000). The resolution of the X-ray structure used as reference was 1.9 Å (Timm et al., 2001)] and the structure was obtained for the mTPK complexed with thiamin (PDB Acc. No.: 1IG3). Further refinement was not performed because of the high degree of identity between the target and reference structures.

RESULTS AND DISCUSSION

PP17b studies

Lipid droplet and milk lipid globule membrane associated Placental Protein 17b (PP17b) is involved in apoptotic and differentiation processes of human epithelial cervical carcinoma cells

PP17 gene: expression, structure and regulation

GenBank search revealed a high variety of alternatively spliced human ESTs – related to PP17a and PP17b cDNAs by length and sequence – in almost all types of healthy tissue. ESTs were highly expressed in placenta and epithelial origin tumors. These underlined our previous Northern- and Western blot results, showing that PP17a is mostly a steroidogenic tissue protein, while PP17b is an ubiquitously synthesized oncodevelopmental protein, both members of an alternatively spliced protein family, homologous to the perilipins. Genomic alignment of PP17b cDNA and longest EST (BI561840) sequences mapped PP17 gene (Locus ID: 10226) to 19p13.3 (genomic contig: NT_011255), containing 8 exons sized from 82 to 943 bp, spanning about 29.0 kb, with all exon-intron boundaries conforming to consensus sequences (Mount et al., 1982). This gene lacks a canonical TATA box, but a putative initiator element (Inr) was found in it, contained by genes with TATA-less promoter (Smale et al., 1989). The 5' end of the longest EST started at the consensus start site (A) of the Inr, confirming it to be the first nucleotide of the first noncoding exon. A downstream promoter element (Burke et al., 1997), a pyrimidine-rich element (Johnston et al., 1998) and several GC-rich consensus GCF (Bina et al. 2001) and SP-1 (Emami et al., 1998) transcription factor binding sites clustered in the vicinity of the Inr might serve in transcription initiation (**Fig. 1**).

*ccggacttcatgacttggc***gagggg***cc***tag****ggaggg***cc***agggaggagaggg**
*gccccgtggcggggaatccctcaacctgat***tggtcccctcgccgctcactcc****AGTGC**
GCCCCAACCTACCACGCAGTAAAAGCCACCCCGCTCGGCCCGACGGTTTTCCAAGCT
GTTTTGAAGTCGCGCAGCTGTTCTGGGACGTCCGTTGACCGCG**tgagtgccggg**
*gc...6.2kb Intron 1...ttcctctctctc***agCGTCTGCTGCAGAGACC****ATG****TCT**
GCCGACGGGCGAGAGCTGATGGCAGCACCCAGGTGACAGTGAAGAACCGGTACAGCAG
tgaggaagggcgg*...1.3kb Intron 2...acttctctcctgt***agCCCAGTGTGG**
TGGACCGTGTGGCCAGCATGCCTCTGATCAGCTCCACCTGCGACATGGTGTCCGACGCT
ATGCCTCCACCAAGGAGAGCTACCCGCACATCAAGACTGTCTGCGACGCAGCAGAGAAGG
GAGTGAAGACCCTCACGGCGGTGCTGTGACGGGGCTCAGCCGATCCTCTCCAAGCTGG
AGCCCCAGA**tggggtgaagtcca***...0.1kb Intron 3...aattggtgtggc***gT**
TGCATCAGCCAGCGAATACGCCACAGGGGGCTGGACAAGTTGGAGGAGAACCCTCCCAT
CCTGCAGCAGCCACGGAGA**gtaacgatgtccgtc***...7.3kb Intron 4...tgt*
*tgcattctct***agTCTTGGCGGACACCAAGGAGCTTGTGTGCTAAGGTGTCGGGGCC**
CAAGAGATGGTGTCTAGCGCCAAGGACACGGTGGCCACCCAATGTGCGAGGCGGTGGAC
GCGACCCGCGGTGCTGTGCAGAGCGGCTGGACAAGACAAAGTCCGTAGTGACCGCGGC
GTCCAATCGGTTCATGGCTCCCGCTTGGCCAGATGGTGTGAGTGGGTTCGACACGGT
CTGGGGAAGTCGGAGGAGTGGGCGGACAACCACCTGCCCTTACGGATGCCGAATGG
aagtgtggccagc*...4.1kb Intron 5...tcctttctctg***agCCCAGTCCGC**
ACATCCCTGGATGGCTTTGACGTCCGTCCGTGCAGCAGCAGCGGCAGGAACAGAGCTAC
TTCGTACGTCTGGCTCCCTGTGCGAGAGGCTCGGCCAGCACGCCATATGAGCACTCGCTG
GGCAAGCTTCGAGCCACCAAGCAGAGGGCACAGGAGGCTCTGCTGCAGCTGTCCAGGTC
CTAAGCTG**tgaggttcaggg***...2.9kb Intron 6...tctcccctgccc***agA**
TGAAACTGTCAAGCAAGCGTTGATCAGAAGCTGGTGAAGGCCAGGAGAAGCTGCACC
AGATGTGGCTCAGCTGGAACCAGAAGCAGCTCCAGGGCCCCGAGAAGGAGCCGCCAAGC
CAGAG**gtaccatgagggga***...5.1kb Intron 7...catctcccttctc***agCAGGT**
CGATCCCGGGCGCTCACCATGTTCCGGGACATTCGCCAGCAACTGCAGGCCACCTGTAC
CTCCCTGGGTCCAGCATTCAGGGCTCCCCACCAATGTGAAGGACCAGGTGCAGCAGGC
CGCCCGCAGGTGGAGGACCTCCAGGCCACGTTTTCCAGCATCCACTCCTTCCAGGACCT
GTCCAGCAGCATTCGGCCAGAGCCGTGAGCGTGTGCCAGCGCCCGGAGGCCCTGGA
CCACATGGTGAATATGTGGCCAGAACACACCTGTACCGTGGCTCGTGGGACCCCTTTC
CCCTGGAATCACTGAGAAAGCCCGGAGGAGAAGAAG**TAC****GGGGGAGAGGAGGACTCAG**
CGGGCCCGCTCTATAATGACAGCTGTGCTCTGGAGTCTCAACCCGGGGCTCATTTCAA
ACTTATTTCTAGCCACTCCTCCAGCTCTTCTGTGCTGTCCACTTGGGAAGCTAAGGCT
CTCAAACCGGCATCACCCATTTGACCCATCTCTCAGCCTCTCTGAGCTTGGAAAGAAGCC
TGTTCTGAGCCTCACCTATCAGTCAGTAGAGAGAGATGTCCAGAAAAATATCTTTCAG
GAAAGTCTCCCTGCAGAAATTTTTTCTTTGTTAAATATCAGGAATATAGGCCGGGTG
CGGTGGCTCACACCTGTAATCCAGCACTTTGGGAGGCTGAGGCGGGCGGAACACCTGAG
GTCAGGTGTTGAGACCAGCCAGGCCAACATGGTGAACCCCGTCTCTACTAAAAATACA
AAAAAAATGAGCCGGGCATGGTAGCAGGTGTCTGTTATCCAGTTAGGAGGCTGAGGCA
AGAGAACTCTTGAACCTGAGAGGCGGAGGTTGCAGTGAAGCAAGATCGGCCATTGCAC
TCCAGCTGGGGGACAAGAGTGAAGTGTCTCA*aaaaaaaaaaaaaaaaa***g***aaaaaaaaaat*
cagggatagttcatatcccacttcttggttacaccgatgtccctgaatatcagcctg

Fig. 1. Nucleotide sequence and possible transcriptional regulation of the human PP17 gene. The figure displays eight exons in boldface capitals, seven introns as well as the 5' and 3' flanking regions in lowercase italics, and the consensus GT/AG splice junction sites underlined. Start (ATG) and stop (TAG) codons in exons 2 and 8 are inverse typed. In the absence of a canonical TATA box, double underlined pyrimidine-rich element (-23), initiator element (Inr; putative initiation site boxed) and downstream promoter element (DPE; +50) may serve an identical function in PP17 gene. GC-rich consensus binding sequences transcription initiation factors (GCF highlighted, SP-1 boxed) are also indicated.

Analysis of the 1.5 kb 5' flanking region, attempting to get further insight into the possible regulation of PP17 gene, showed numerous different consensus transcription factor binding sequences clustered preceding the 5' end of the first exon (Table 2).

Transcription Factor	Binding position	Transcription Factor	Binding position	Transcription Factor	Binding position
AML-1	-704	AHR	-790	MYO-D	-172
AP-1	-920	ARNT	-175	NF- κ B	-169
	-892		-569	PAX-2	-642
	-870	c-MYC	-589	PAX-5	-209
	-817	CREB	-646	PEA-3	-928
	-539	GATA-1	-990	PPAR γ	-644
	-496	GCF	-147	P-300	-639
	-384		-144	SP-1	-961
	-27		-90		-542
	AP-2		-876	-74	-93
		-665	-54	-77	
-225		+4	USF	-572	
-217		LYF	YY1	-1022	
+35		-976	-845		
AP-4	-201	MZF-1	-292	-221	

Table 2. Possible transcriptional regulation of the human PP17 gene. Computed positions of binding sites for consensus transcription factors in PP17 gene promoter are indicated relative to the putative Inr.

Factors potentially involved in the transcription of PP17 gene include (a) *general activators or repressors*: GCF, SP-1, YY1 (Lee et al., 1993) and USF (Roy et al., 1991); (b) *co-activators*: AP-4 (Mermod et al., 1998) and P-300 (Lundblad et al., 1995); (c) *cAMP/PKA, PKC or phorbol ester responsible elements*: AP-1 (Bohman et al., 1987), AP-2 (Imagawa et al., 1987), CREB Fink et al., 1991), GCF and NF- κ B (Shirakawa et al., 1991); (d) *hematopoietic regulators*: AML (Miyoshi et al., 1995), GATA-1 (Trainor et al., 1990), LYF (Lo et al., 1991), MZF-1 (Kaushansky et al., 1991) and PAX-5 (Adam et al., 1992); (e) *adipose differentiation regulator*: PPAR γ (Tontonoz et al., 1994); (f) *myogenic factor*: MYO-D (Davis et al., 1990); (g) *keratinocyte specific factors*: AP-2, GCF and PAX-2 (Dressler et al., 1992); (h) *factors abundant in placenta*: AHR (Reyes et al., 1992), AP-2 and PPAR γ ; (i) *proliferation and/or apoptosis regulators*: AP-2, c-MYC (Gaubatz et al., 1995) and NF- κ B; (j) *embryo- and organogenic factors*: PAX-2 and PAX-5; (k) *proto-oncogenes or their targets*:

AML, AP-1, AP-2, PAX-2, PAX-5, PEA-3 (Wasylyk et al., 1989) and PPAR γ ; (l) *aryl hydrocarbon regulators*: AHR and ARNT (Reyes et al., 1992). From these, it may be concluded that (a) ubiquitous PP17b synthesis could be derived from possible gene regulation by factors involved in development of different cells; (b) oncodevelopmental significance of PP17b must be re-emphasized by locating potential binding sites for factors engaged in proliferation, oncogenesis or development; (c) PP17b could be involved in lipid metabolism and droplet formation regulated by PPAR γ ; (d) apoptotic and (e) differentiation pathways could utilize the as yet unestablished function of PP17b.

PP17b is a member of the growing lipid storage droplet protein family

By multiple sequence alignment, PP17b proved to have a close structural relationship to human adipophilin and perilipin, members of the newly discovered lipid droplet associated protein family, sharing a common N-terminal motif (Lu et al., 2001). Alignment of their cDNAs to genomic sequences, and superimposition of exon-intron boundaries to the aligned proteins revealed some common characteristics of their genes (**Fig. 2**).

PP17b	MSADGAEADGSTQVTVEEPVQQPSVVDVRSAMPLISSTCDMVSAAYASTK	1- 50
hADFP	MAS-----VAVD-P--QPSVVTRVNLPLVSSTYDLMSAYLSTK	1- 37
hPLIN	MAVN---KGLTLLDGDLP-EQENVLQVQLPVVSGTCECFQKTYTSTK	1- 45
PP17b	ESYPHVKTVCDAAEKGVRTLTAAVSWAQPILSKLEPQIASASEYHRGL	51-100
hADFP	DQYPYLKSVCEMAENGVKTIITSVAMTSALPIIQKLEPQIAVANTYACKGL	38- 87
hPLIN	EAHPLVASVCNAYEKGVQSASSLAAWSMEPVVRRRLSTQFTAANELACRGL	46- 95
PP17b	DKLEENLPMLRQPTEKVLADTKELVSSKVSQAQEMVSSAKDTVATQLSEA	101-150
hADFP	DRIEERLPILNQPSTQIVANAKGAVTGAKDAVTTTGTGAKDSVASTITGV	88-137
hPLIN	DHLEEKIPALQYPPEKIASSELKDTIS-----TRLRSARNSISVPIAST	96-138
PP17b	VDATRGAVQSGVDKTKSVVTGGVQSVMGSRLLGQMVLSGVDTVLGKSEEWA	151-200
hADFP	MDKTKGAVTGSVEKTKSVVSGSINTVLGSRMMQLVSSGVENALTKSELLV	138-187
hPLIN	SDKVLGAALAGCELAWGVARDTAEFAANTRAGRLASGGADLALGSIKVV	139-188
PP17b	DNHLPLTDAELARIATSLDGFVDVASVQQQRQEQSYFVRLGSL-----	201-243
hADFP	EQYLPLTEELEKEAKKVEGFDLV-----QKPSYYVRLGSL-----	188-224
hPLIN	EYLLPADKEESAPAPGHQQAQKSP-----KAKPSLLSRVGALTNTLSRYT	189-233
PP17b	ERLRQHAYEHSGLKLRATKQRAQE-ALLQLSQALSMLMETVKQGV-----	244-286
hADFP	TKLHSRAYQQALSrvKEAKQKSQQ-TISQLHSTVHLIEFARKNVYS---A	225-270
hPLIN	VQTMARALEQGHTVAMWIPGVVPLSSLAQWVGASVAMQAVSRRRSEVRVPW	234-283

PP17b	DQKLVEGQEK LHQMWLSWN-QKQLOGPEKEPPK PEQVESR ALTMFR----	287-331
hADFP	NQKIQDAQDK LYLSWVEWK-RSIGYDDT DESHCAEQFESR TLAIAR----	271-315
hPLIN	LHSLAA AQEEDHED QTDTEGEDTEEEEE ELETEENK FSEVAALPGPRGLLG	284-333
PP17b	DIAQQLQATCT SLGSS IQGLPTNVKDQV QARRQVEDLQATFSS-----	332-375
hADFP	NLT QQLQTTCH TLLS NIQGVQNIQDQ AKHMGVMAGDIYSVFRN-----	316-349
hPLIN	GVAHTLQKTLQ TTISAVTWAPAAVLGM AGRVLHLTP APAVSSTKGRAMSL	334-383
PP17b	IHSFQDLSS SILAQSRER VASAREALDH MVE--YVAQ NTPV T WLVGPF FAP	376-423
hADFP	AASFKEVSD SLLTSSKGQLQ KMESLDD VMD--YLVN NTPLN WLV GPF FYP	350-397
hPLIN	SDALKGVT DNVVDTVVHY VPLPRLSL MEPESEFRDID NPPAE VERREAER	384-433
PP17b	GITEKAPEEK K-----	424-434
hADFP	QLTESQNAQD QGAEMDKSS QETQRSE HKTH-----	398-437
hPLIN	RASGAP SAGPEP APRLAQ PRRSLR SAQSPGAPP GP GLEDEVATPAAP RP G	434-483
hPLIN	FPAVPREKPK RRVSDSFFRPSVMEPIVGR THYSQLR KKS	483-522

Fig. 2. Structural relation of human PP17, adipophilin and perilipin genes and proteins. After multiple sequence alignment of PP17b, adipophilin (hADFP) and perilipin (hPLIN), and alignment of their cDNAs to genomic sequences, aligned proteins were superimposed with corresponding exon boundaries. Identical amino acids are shown in boldface letters, subsequent exons are indicated by alternate highlighting.

Although genomic sizes and locations (PP17: 29.0 kb, 19p13.3; hADFP: 12.2 kb, 9p21.3; hPLIN: 15.6 kb, 15q26) and intron sizes were divergent, homology was proven by the similar number and length of exons, the corresponding analogous peptide lengths, and the high number of identical and conserved residues. The most conserved regions in all three proteins were encoded by exons 3 and 4, where PP17b had 38-56% identity and 68-82% similarity to its closest homologues. On its C-terminus PP17b had a lower level of sequence similarity to perilipin (29-42%) than to adipophilin (50-70%), and the number of identical residues with the latter was also significantly reduced (26-43%) (Table 3).

Exon	Exon length (bp)			Peptide length (aa)			Identity/similarity (%)		Following intron (kb)		
	PP17b	ADFP	PLIN	PP17b	ADFP	PLIN	ADFP	PLIN	PP17b	ADFP	PLIN
1.	111	54	102	-	-	-	-	-	6.2	1.0	2.0
2.	82	52	58	22	10	15	18 / 27	21 / 42	1.3	0.1	4.0
3.	198	195	204	66	65	68	56 / 82	38 / 68	0.1	2.5	1.6
4.	84	84	84	28	28	28	54 / 75	54 / 79	7.3	2.4	1.2
5.	285	285	264	95	95	88	43 / 70	20 / 42	4.1	1.0	0.3
6.	201	183	174	67	61	58	36 / 70	13 / 29	2.9	1.2	1.7
7.	126	135	192	42	45	64	26 / 50	14 / 40	5.1	1.7	0.4
8.	943	879	246	114	133	82	40 / 70	15 / 38	-	-	1.0
9.	-	-	1577	-	-	129	-	-	-	-	-

Table 3. Conserved regions in human PP17, adipophilin and perilipin genes and proteins. Exon lengths, corresponding peptide lengths, identities / similarities to PP17b, and following intron sizes were compared in each genes. Exons were highlighted analogous to the alignment. Though intron sizes are divergent, structure of PP17 and hADFP genes are highly **conserved**, with their close relation to hPLIN gene. The most conserved regions in all three proteins (boldface) are encoded by exons 3 and 4.

This is the first comparison of PP17b with two human members of the newly discovered “PAT domain gene family” (Lu et al., 2001), suggesting their common genetic origin. Shared characteristics in the regulation of PP17 gene with other family members were also found: (a) the promoter region of the murine perilipin gene is similar to the human PP17 gene, lacking also TATA box (Lu et al., 2001); (b) the mouse ADRP gene contains several transcription factor binding sites (AP2, PAX-2, C-MYC, SP1) (Eisinger et al., 1993), as does PP17 gene, (c) the expression of human adipophilin is highly inducible by PPAR γ , which plays a fundamental role in lipid catabolism and adipocyte differentiation, as well as in epithelial differentiation (Gupta et al., 2001). In light of these findings collectively, the concept of PP17b being a member of the lipid storage droplet protein family was to be analyzed further.

PP17b is localized on lipid droplets and milk lipid globule membranes

As previous findings by other groups were contradictory on the function of TIP47 – recently detecting lipid droplet association of the previously believed mannose 6-phosphate receptor transporter with a polyclonal antibody [13] – this question was now examined on invasive squamous cervical carcinomas and HeLa cells using our highly specific anti-PP17 antibody. In fixed-embedded tissue sections of squamous cervical carcinoma, mainly tumor cells with squamous differentiation were stained in a punctuated pattern. In higher magnification, positive granules showed an unstained core, mimicking lipid droplets (**Fig. 3A**). Similarly, lipid loaded HeLa cells had a characteristically

granular cytoplasmic PP17 localization (**Fig. 3B**). By confocal imaging, there was a large difference between cells cultured under low or high lipid concentrations. Compared to control cells (**Fig. 3C**), in lipid-loaded cells, spherical structures stained with anti-PP17 antibody in the cytoplasm (**Fig. 3D**). Large clusters of these globules strongly double-stained with anti-PP17 antibody and Nile Red, appearing to be neutral lipid droplets. In higher magnification, even distinct PP17 positive rings surrounding the droplet surfaces could be detected (**Fig. 3E**). Confocal images supported our computational finding that PP17b belongs genetically and structurally to a new protein family, and also reinforced the postulation that PP17b is a constituent of lipid droplets. Moreover, the same PP17 positive ring could be detected on the surface of double-labeled milk lipid globule membranes, with weaker reticular PP17 staining inside of MLMGs, which was probably the result of the surface protein internalization as small lipid droplets developed into large MLMGs (**Fig. 3F**).

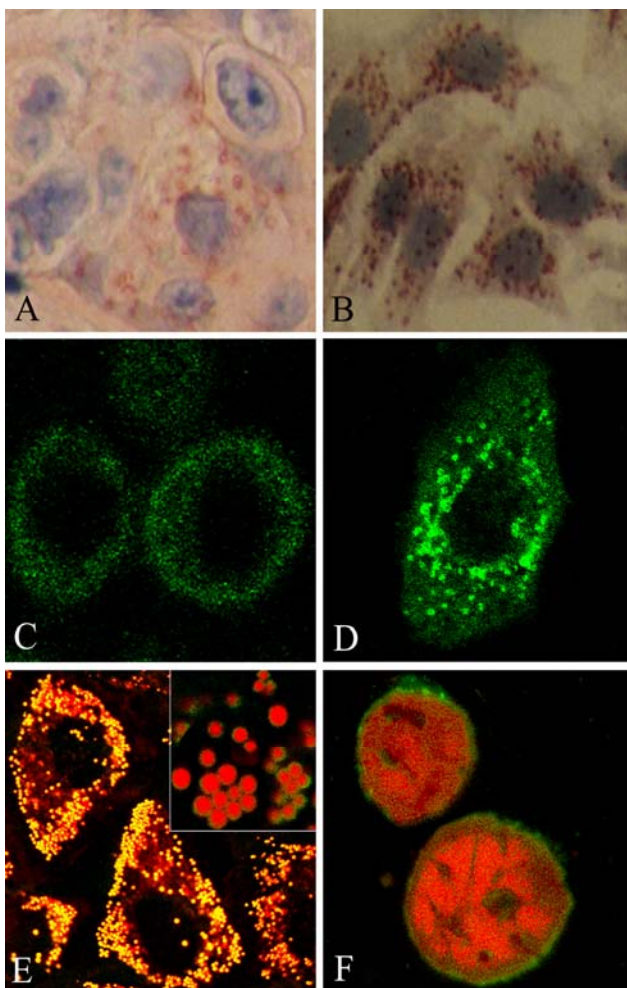


Fig. 3. Lipid droplets in invasive squamous cervical carcinomas and HeLa cells are stained with anti-PP17 antibody. (A) In invasive squamous cervical carcinoma, tumor cells have punctuated, ring-like cytoplasmic PP17 staining (IHC, haematoxylin counterstain). (B) Lipid loaded HeLa cells have dominantly granular PP17 staining (ICC, haematoxylin counterstain). (C) Compared to controls, (D) in lipid-loaded cells, spherical structures stained with anti-PP17 antibody (CIM). (E) In lipid-loaded cells, clusters of small lipid-droplets are double-labeled with anti-PP17 antibody (green) and Nile Red (red), co-localization is represented in yellow. Inset magnifies lipid droplets surrounded by distinct PP17 positive ring (CIM). (F) A strong PP17 staining around the surface, and weaker signs inside of double-labeled MLMGs is present.

All of these allow some parallels to be indicated: (a) it is thought that perilipins may bring small lipid droplets together, probably by protein-protein interactions (Brasaemle et al., 2000), while PP17a and PP17b have coiled-coil structures, and were detected to dimerize or oligomerize in natural or even denatured conditions (Than et al., 1998; Diaz et al., 1998), which might enable them to play a role in lipid droplet aggregation and formation; (b) alternatively spliced perilipin isoforms have different distribution in steroidogenic cells or adipocytes (Brasaemle et al., 1997), while a tissue specific distribution of PP17 variants was also discovered, as PP17b was ubiquitously expressed, while PP17a expression was restricted to steroidogenic tissues only (Than et al., 1998); (c) adipophilin was purified from milk and its cDNA was isolated from a mammary gland clone collection (Nielsen et al., 1999), while human mammary gland and mammary adenocarcinoma ESTs similar to PP17b cDNA was found by BLAST, and subsequently PP17b cDNA was also found to be differentially expressed in breast cancer cell lines (Mellick et al., 2002), indicating that the staining of MLMGs was probably not due to a simple cross-reaction.

To disclose cross-reaction with adipophilin at all and to assess the exact subcellular distribution of PP17 variants detected by our highly specific antibody, fractionation and Western blotting of HeLa cells were subsequently performed. In cells cultured under low lipid concentrations, small amounts of PP17a, PP17b and PP17c were found in the buoyant lipid droplet fraction, while almost all the staining for these proteins could be detected in the cytosol (**Fig. 4A**). In lipid-loaded cells, amounts of PP17a, PP17b and PP17c were increased in the cytosol fraction, and in parallel an intense elevation of PP17b in the lipid-droplet fraction was detected, as evidence for droplet-association of PP17b (**Fig. 4B**). In total milk, high amounts of PP17b and PP17c were identified, whereas mainly PP17b was associated to MLMG fractions (**Fig. 4C**).

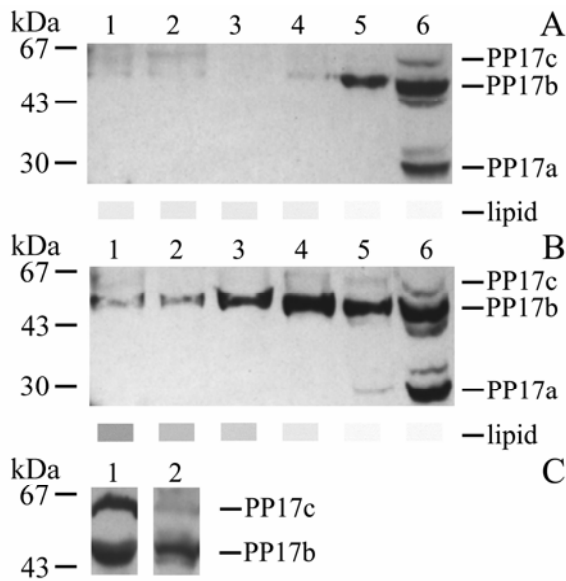


Fig. 4. Western blot of PP17 variant

distribution in HeLa cells and human milk. (A)

In control cells, small amounts of PP17a, PP17b and PP17c (PP17a dimer) was present in the buoyant fraction and moderate amounts in cytosol.

(B) In lipid-loaded cells, amounts of PP17a, PP17b and PP17c were slightly increased in cytosol, while the quantity of PP17b was significantly elevated in lipid-droplet fraction. Lane 1 represents floating lipid-droplet fraction, **Lane 2-5 are intermediate fractions**, while lane 6 is cytosol fraction.

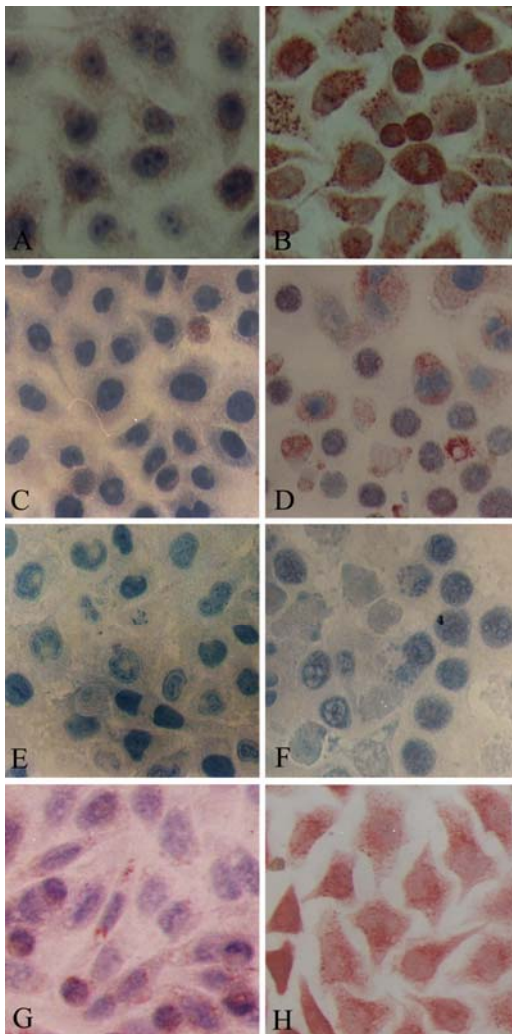
Amounts of neutral lipids in each fraction were

quantified with densitometric scanning and shown semiquantitatively. (C) In total milk (lane 1), high amounts of PP17b and PP17c were found, while in MLMG fraction (lane 2), mainly PP17b was detected. Markers indicate molecular masses in kDa.

Following this, the PP17 immunoreactive 30, 48 and 60 kDa proteins were purified from lipid loaded HeLa cell extracts and human milk, then MALDI-TOF MS peptide mapping and MALDI-PSD MS sequencing were performed. Each protein band yielded a good quality peptide map, and most of the input masses were matched to the candidate protein sequences. The majority of the tryptic peptides matched with the theoretical masses within 62 ppm. MALDI-TOF MS data of the 48 kDa protein permitted the identification of PP17b, and mass maps of the 30 and 60 kDa proteins matched PP17a with 46% coverage of the protein sequence. PSD data obtained for precursors also confirmed the identity of these proteins. These data show the specificity of our original antibody, excludes cross-reactivity with its human homologues, reinforces dimerization of PP17a to PP17c, and also confirms the lipid-droplet association of PP17b.

PP17b is involved in apoptosis and differentiation of epithelial cells

Several putative transcription factor binding sites involved in apoptosis and differentiation were localized in PP17 gene promoter. Using well-characterized apoptosis and differentiation models, induction of PP17 gene expression through the supposed pathways were detected, parallel to the morphological changes. PP17 quantities were measured in apoptotic conditions, treating cells with carboplatin, 5-fluorouracil, irinotecan, mitomycin or paclitaxel in clinically achievable concentrations, in various dose-time combinations. Apoptosis was assessed by typical cytomorphological alterations in the nucleus and cytoplasm, and by the elevated bax/bcl2 oncoprotein ratio, widely used for squamous epithelial cells and tissues (Harima et al., 2000). The effect and time course of different apoptosis inducing agents on PP17 gene expression was varied. Paclitaxel had the highest apoptotic effect, which appeared after 12h and peaked



at 24h, correlating well with increased PP17 protein synthesis, specifically in small round cells exhibiting clearly apoptotic morphology, with picnotic nuclei and narrow cytoplasm (**Fig. 5B**).

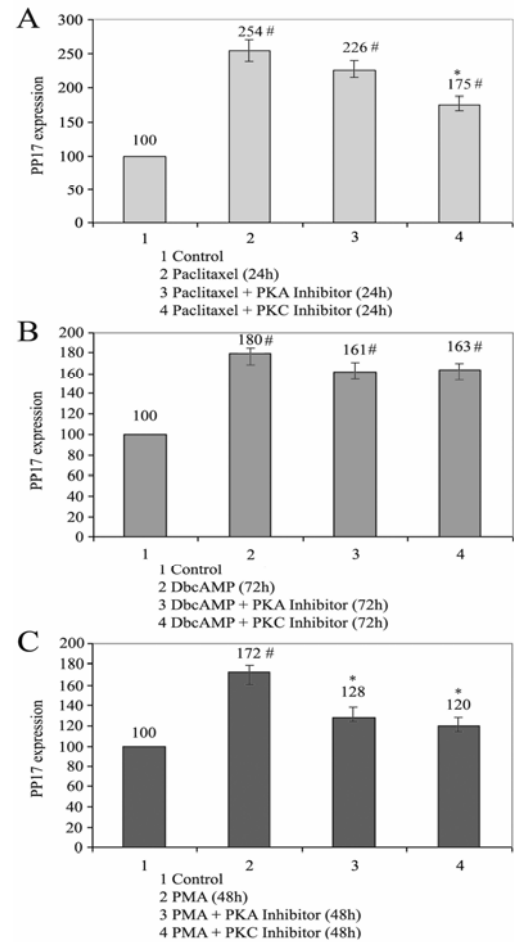
Fig. 5. PP17 immunostaining of apoptotic and differentiated HeLa cells. *A, C, E and G* show control cells, *B, D, and F* paclitaxel (10 nM for 24h) treated cells, while *H* dbcAMP (0.5 mM for 72h) treated cells. In *A, B, G and H*, cells were PP17 stained, in *C and D* with anti-bax, in *E and F* with anti-bcl-2 antibody. Compared to controls, after paclitaxel treatment, **synthesis** of PP17 variants (*B*) and bax (*D*) proteins was strongly increased, while bcl-2 (*F*) was unaltered. During differentiation, PP17 variant **synthesis** was highly elevated. Punctuated PP17 immunostaining was detected either in apoptotic or in differentiated cells.

By flow cytometry, a strict dose and time dependency of its PP17 inducing effect (+49% after 18h, +154% after 24h) were observed (**Fig. 6A**). Parallel treatment with PKC inhibitor caused significant reduction in PP17 protein synthesis after 24h (+75%), while PKA inhibitor had less influence on this effect of paclitaxel (+126%) (**Fig. 6B**).

Fig. 6. Flow cytometric measurements on PP17 induction during apoptosis or differentiation. (A) Parallel treatment of PKC inhibitor with paclitaxel caused significant ($*p<0.05$) reduction in PP17 synthesis compared to paclitaxel alone, while PKA inhibitor did not have so strong effect. (B and C) During cell differentiation, PP17 synthesis was notably elevated, which could be significantly ($*p<0.05$) reduced by PKA and PKC inhibitors only in case of PMA. Values indicated above the bars are the averages of three separate flow cytometric measurements. (# $p<0.05$: significant to controls.)

Cells were treated with DbcAMP or PMA to obtain data on PP17 gene involvement in cell differentiation pathways, and both notably induced differentiation and PP17 protein synthesis (**Fig. 5H and Table 4**).

Compared to controls, 72h treatment with dbcAMP caused the highest PP17 increase (+80%), which did not elevate further in even higher concentration ranges, and could only be moderately reduced by PKA (+61%) or PKC (+63%) inhibitors (**Fig. 6C**). There was an evident degree of cell differentiation after PMA treatment, although it was less effective in the induction of PP17 protein synthesis (+72%), but parallel PKC or PKA inhibitor treatment decreased PP17 induction significantly (+20 / +28%) (**Fig. 6D**).



Drug	Concentration	Time	Synthesized proteins		
			PP17a	PP17b	PP17c
Control cells	-	-	+/-	+/-	+/-
Apoptosis induced cells					
Carboplatin / Paraplatin [®]	0.75 µg/ml	24h	+/-	+	+/-
Irinotecan / Campto [®]	5.00 µg/ml	24h	+/-	+	+/-
5-fluorouracil / 5-FU Lederelle [®]	25.00 µg/ml	24h	+/-	+	+/-
Mitomycin / Mitomycin C [®]	10.00 µg/ml	24h	+	++	+
Paclitaxel / Taxol [®]	10.00 nM	24h	+	+++	+
Differentiation induced cells					
Phorbol myristate acetate	80.00 nM	48h	+/-	++	+/-
Dibutyryl cyclic AMP	0.50 mM	72h	+/-	++	+/-

Table 4. Synthesis of PP17 variants in induced and control HeLa cells. Exposition of HeLa cells to cytostatic and cell differentiation inducing drugs were carried out as described in Materials and Methods. Whole protein extracts were Western blotted, then the revealed bands were densitometrically analyzed. Amounts of PP17 variants are shown semi-quantitatively.

In case of paclitaxel, a time dependent shift in cell cycle was detected. On average, 65-75% of the control cells were in G_0/G_1 and 25-35% in M phases. Paclitaxel stopped the cells in M phase after 18h parallel to increasing (+49%) PP17 protein synthesis, which peaked after 24h (+154%) (**Fig. 7A**). It was remarkable that PP17 protein synthesis was ~40% higher in M than in other phases of the cell cycle either in control, apoptotic (**Fig. 7B-C**) or differentiated cells, which may also show PP17 gene involvement in differentiation.

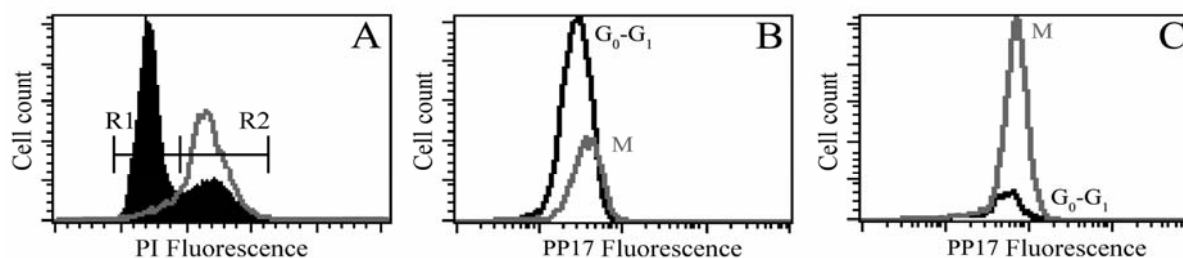


Fig. 7. Cell cycle and cycle dependent PP17 fluorescence in control and paclitaxel induced cells.

(A) Cells in G_0-G_1 phases were gated under R1 and cells blocked in M phase under R2. PI fluorescence of control cells was shown in black filled histogram, paclitaxel induced cells in gray open histogram. (B and C) Cell cycle dependent PP17 fluorescence of control and paclitaxel induced cells. Time dependent shift in cell cycle was caused by paclitaxel, stopping cells in M phase, with increased PP17 **protein synthesis** reaching its peak (+154%) after 24h. PP17 fluorescence was 40% higher in cells in M than in G_0-G_1 phases in both cases. Figures are representatives of three separate experiments.

It is known that paclitaxel markedly increases the binding of NF- κ B and AP-1 transcription factors to their binding sites (Lee et al., 1997). PP17 gene promoter has been shown to contain several NF- κ B and AP-1 binding sites, therefore it is likely that paclitaxel might induce PP17 gene expression by the activation of NF- κ B and AP-1 transcription factors. Furthermore, it is known that PKC inhibitors abolish paclitaxel induced NF- κ B activation (Lee et al., 1997), which is in concordance to our observation that a PKC inhibitor suppressed paclitaxel induced PP17 synthesis. Paclitaxel induced gene expression, cell death and differentiation are regulated by complex protein kinase networks including ERK1,2, c-Jun NH₂-terminal kinase and p38-MAP kinase pathways (Lee et al., 1998), which may explain the complex regulatory effects that have been seen under different conditions.

It was published that a gene involved in squamous cell differentiation can be effectively induced by PMA utilizing AP-1 binding sites, and its expression is inhibited by PKC inhibitors (An et al., 1993). This is also consistent with our observations that PMA activated PP17 gene expression, which was decreased by a PKC inhibitor. The PKC / Ras / MEKK1 / MKK1-dependent/AP-1 kinase cascade involved in the regulation of PMA induced gene expressions (Vuong et al., 2000) may be another possible means of PP17 gene regulation.

PP20 studies

Cloning, sequencing, structural and molecular biological characterization of placental protein 20 (PP20) / human thiamin pyrophosphokinase (hTPK)

Cloning and sequence analyses of PP20 cDNAs

Immunoscreening of 2×10^6 recombinant plaques of the human placental cDNA library with anti-PP20 serum yielded 4 positive recombinant clones. Through the determination of the entire nucleotide sequences of all cDNAs, the clones turned out to contain full-length inserts encoding the same protein. The largest, 2431 bp cDNA (GenBank Acc. No.: AY206415), contained an 85 bp 5' untranslated region followed by a coding region of 731 bp and a 3' untranslated region of 1614 bp including two proposed (5'-AATAAA-3') polyadenylation signals. The first ATG was likely to be the translation initiator codon located in an optimal context (5'-TCCGTTATGG-3') typical for vertebrate mRNAs recognized by the 40S ribosome subunit (Kozak, 1987) (Fig. 1). The mature protein consisted of 243 amino acids with a predicted molecular mass of 27.265 kDa and a theoretical pI of 5.03. The entire nucleotide sequences of PP20 cDNAs proved to be identical to human thiamin pyrophosphokinase (GenBank Acc. No.: AAK01351), except that the longest PP20 cDNA contained 3 additional nucleotides at its 5' end compared to hTPK1 cDNA. By alignment search, PP20 had high homology (89% identity, 95% homology) to mouse thiamin pyrophosphokinase (mTPK).

Genomic localization, structure and regulation of PP20 / hTPK gene

GenBank searches revealed human ESTs in various adult and fetal human tissues with a predominant expression in placenta. Genomic alignment of the longest PP20 cDNA and ESTs mapped PP20 / hTPK gene (Locus ID: 27010) to chromosome 7q34-q36 (genomic contig: NT_007914), containing 9 exons, not 8

as published previously (Nosaka et al., 2001; Zhao et al., 2001), sized from 59 to 1721 bp. The gene coding sequence was extremely long (384 kb) with all exon-intron boundaries conforming to consensus sequences (Mount, 1982). The sequence contained a TATA-box-like sequence (-38) and an initiator element (Inr) as do other genes with a consensus TATA-box less promoter (Smale et al., 1989). The 5' end of the longest PP20 cDNA started at position +4 compared to the putative start site (A) in the Inr, which consisted of a consensus sequence. A downstream promoter element (DPE; +38) and consensus GC-rich sequences, binding sites for SP-1 and GCF transcription factors might also act in the transcription initiation (Fig. 8).

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cggacgcagagcacagggcgcggtcccacttgctccggctcccggaagcgggccccggc
caccctcgctccccggggcctaaaacctgcgagcagaggaccgcccgccaaggctcctcAGC
CGAGCGCCGAGCGGTGATCGCCGTAGCTCCCGCAGCCTGCGATCTCCAGTCTGGCTC
CTACCAGCCATTGTgtaagtg...0.3kb Intron 1...aatgcagAGGCCAATAATCCGTT
ATCGAGCATGCCTTTACCCCGTTGGAGCCCCTGCTTTCCACTGgtactgtattt...5.9kb
M E H A F T P L E P L L S T
Intron 2...ttttctctagGGAATTTGAAGTACTGCCTTGAATCTTAATCAGCCTTTG
G N L K Y C L V I L N Q P L
GACAACTATTTTCGTCATCTTTGGAACAAAGgtaacc...8.2kb Intron 3...tgaagCT
D N Y F R H L W N K
CTTTAAGAGCCTGTGCCGATGGAGGTGCCAACCGCTTATATGATATCACCGAAGGAGAG
L L R A C A D G G A N R L Y D I T E G E
AGAGAAAGgtatgc...3.4kb Intron 4...tctttcagCTTTTTCCTGAATTCATCAAT
R E S F L P E F I N
GGAGACTTTGATTCTATTAGGCCTGAAGTCAGAGAATACTATGCTACTAAGgttaattat
G D F D S I R P E V R E Y Y A T K
aa...2.5kb Intron 5...aatacatagGGATGTGAGCTCATTCAACTCCTGATCAAGAC
G C E L I S T P D Q D
CACACTGACTTTACTAAGTGCCTTAAATGCTCCAAAAGAAGATAGAAGAAAAAGACTTA
H T D F T K C L K M L Q K K I E E K D L
AAGgtaatat...2.5kb Intron 6...tgcttagGTTGATGTGATCGTGACACTGGGAGGC
K V D V I V T L G G
CTTGCTGGGCGTTTTGACCAGATTATGGCATCTGTGAATACCTTGTTCGAAGCGACTCAC
L A G R F D Q I M A S V N T L F G A T H
ATCACTCCTTTTCCAATTATAATAATCCAAGAGGAATCGTGATCTACCTGCTCCAACCA
I T P F P I I I I Q E E S L I Y L L Q P
gtgagtc...4.3kb Intron 7...tctccttcagGGAAAGCACAGGTTGCATGTAGACT
G K H R L H V D T
GGAATGGAGGGTATTGGTGTGGCCTTATTCTGTTGGACAGCCTTGTATGCAGGTTACA
G M E G D W C G L I P V G Q P C M Q V T
ACCACAGGCCTCAAGTGGAACTCAgtaagta...9.5kb Intron 8...atcttcttacAG
T T G L K W N L
CAAATGATGTGCTTGCTTTTGGAACTTGGTCAGTACTTCCAATACCTACGACGGGTCTG
T N D V L A F G T L V S T S N T Y D G S
GTGTTGTGACTGTGAAACTGACCACCCACTCCTCTGGACCATGGCCATCAAAAAGCTTAA
G V V T V E T D H P L L W T M A I K S *
CTGTTGACTGGCATCCATAAGTGTGCCTCTGCCTTATCTCATTCTCAACAGTTCATTGC
TCAACAAGAACGATTACCTGGGTTTGCAAGAATCTAACCTCTCTAGGGGAAGCCCACT
GGGTTTAAAGATGTAGTGTGATAGATAACAGGTAACATTATAAATGACAGATCTCAAT
TTTATAGTAGTGGAAAGATACATGCTAAGAAAGCAAATAAGCTCTATTATATTGCGTTG
GAACCTAATGGGAATCATTCCACTATACAATTGACTACTGATTATTCTTTACATTATT
AATCATTCCATTATCTAGAAAATGTTTTAATTTGAATCAGAGAAAAGTGTGAGGT
TCCTCTTGAGTCTAGAATCCTTAAATGTCTAACACAAGGCTACCTCTGAGTACCT
TTTAGTATTGTTTCTGTATGATATATATATCTTATACTGAAAAAAATTCCTTTC
AGATTGGGGTGTAGAAAGTGCACCAGGTCCTGACCTTATTACTGTCTTTGGTATTGT
CTTAATAAATCAAGAATCATTGACCTAATTGTTAAATTTAAAAATAGGTAGTTAGCAAT
AGGTGGAAAGAGAAATGATGTGAAAGATAAATGATGATTCGTGGAGCCCTACTCACACAT
TAACCCCAAATTCAAAAGTAAGAATGCAAAAGTCTAGAGGGGGTAACAGTCTGCATCAT
CATCAACACTAAATGGAGAAAGCTGTGCAGAGGAAACTTAAGCATAAAAAATTAATTCG
TTTCTGACATACCTTAGACTGAAAAACTGTTGGTTCATCCAGAAGTGTATTATATTACC

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Fig. 8. Nucleotide / deduced amino acid sequences and putative transcriptional regulation of the human PP20 / hTPK gene. The figure displays nine exons in boldface capitals, eight introns as well as the 5' and 3' untranslated regions in lowercase italics, and the consensus GT/AG splice junction sites underlined. Start (ATG) and stop (TAA) codons in exons 2 and 9 are inverse typed. The double underlined TATA-box like sequence (-38), an initiator element (Inr; putative initiation site shaded) and downstream promoter element (DPE; +38) might act in the transcription initiation. Polyadenylation signal is boxed. The consensus GC-rich sequences, binding sites for SP-1 (shadowed) and GCF (boxed) transcription factors were indicated. The deduced amino acid sequence of PP20 / hTPK protein is shown highlighted under the nucleotide sequence.

AGAAAATGAGTTTGTCTATGGGGATACATGAACCTTCATATACTAAGGAGCCTAACTCCAA
 AGCCTGCGTTCTCATCCAGCTGTGATATTCACCTAAGTTTCCGGACCCCTTTTCCTTAGCT
 GTAAAAATGGAAGCGGTTGACTGATGGTGTCTGAGGTTCTTTCCACACTGAAATTCTAA
 ATATTGACACTTAGCAGTCATAGGGCTGATAATACACACAGTTACTGACTTAGCCTAAAC
 AACCTGGTGCATCGAAAATGTATTACCTTTCTTTTGTAAAGAGACCATCTTCTATCTTCT
 TTCCACCTTTCTCTGTTTATGAAACCAACTGTTGACATACAAACCATGATTGAAGGAGA
 ACCTGTCCAACATGTTTTATGTACACAAATCCCTATGTGTCTATAAGAAAAGTGAAAGTA
 ACTGTTTTCTTCTGGTGTATGACAGTGTGAGACTCAGGTTGTCTGTAGAGAATGAAAG
 GAGCAGTGGCCCGCGTGATTGTGGCATTAAAGGAGCAGTGGCCCATGTGACTGTGGCATT
 TTCGGCACTTTTCATTACTTTCTGCTTGACCGGAAGTTGAGGCTTAGCTATGTTCCATC
 TTCAGTTTCTGAAGACTAGTTATATATATTCCTTACTAGAAATATATTATAATATATAAAA
 GAAATATATCTGTGATTTAAAAATTTGCTACCAAAGAAATGCATGTTCTGTGTGCCCTGA
 AAATGTTACCAGTGTAAATAAATGGATACTTATCAAAAAGaatatagtttctctga

Analysis of 1 kb of the promoter region showed numerous putative consensus transcription factor binding sequences clustered preceding the 5' end of the first exon (Table 5).

Transcription Factor	Binding position	Transcription Factor	Binding position	Transcription Factor	Binding position	Transcription Factor	Binding position	Transcription Factor	Binding position		
AP-1	-12	CBAF	-807	GATA-1	-252	MZF-1	-254	Pax-2,	-783		
	-377		CP-1		-143		-765		NF-1	-807	Pax-5,
	-483		-807		-827		NF-E		-883	Pax-8	-783
AP-2	-60	CREB	-782	HINF-C	-67	NF-GMa	-751	STAT-1 &	-76		
	-106	CTCF	-770	HNF-3 α	-493	PITX2	-393	STAT-3	-76		
	-219	E2F	-16		-727	PXR	-266	SXR	-1016		
	-362	EZF-2	-329	ISGF-3	-291	SP-1	-19	T3R	-329		
	-431	FXR	-255		-710		-65		-829		
	-461		-867		-804		-82	T3R β 1	-288		
-532	GCF	-66		-848	-102			-716			
AP-4	-772	GR	-290	LUN-1	-92		-189	TFIID	-968		
c-Ets-1	-360		-354		-391		-367	VDR	-255		
	-370		-387		-412	-419	-329				
c-Ets-2	-355		-431		-920	-425	-583				
c-Myb	-446		-711	Lyf-1	-414	-510	-829				
CAR	-439	-736		-548	-563	YY1	-960				

Table 5. Putative transcriptional regulation of the human PP20 / hTPK gene. Putative binding sites for transcription factors in 1kb of the PP20 / hTPK gene promoter were searched in Transfac Database by the Patch algorithm. Their binding positions were indicated relative to the putative Inr.

For the ubiquitous expression of the protein, the numerous AP-1 (Bohmann et al., 1987), AP-2 (Imagawa et al., 1987) and SP-1 (Lee et al., 1993) sites might be responsible. Four potential c-Myb sites may be responsible for the regulation of the exquisite balance between cell division, differentiation and survival (Ramsay et al., 2003) and may account for the expression of the gene in the gastrointestinal tract and haematopoietic system. The presence of various ISGF-3, STAT-1 and STAT-3 (Kraus et al., 2003) binding sites suggested that PP20 / hTPK expression could be regulated by interferon. To verify the exact functional

role of these putative transcriptional factor binding sites, further in vitro experiments should be performed.

Identification of PP20-B as human thiamin pyrophosphokinase and determination of the enzymatic activity

After SDS-PAGE, in the case of placental derived PP20-B major bands at 27 and 54 kDa were detected by Coomassie staining. No additional bands in lower or higher molecular weight regions could be identified, indicating high purity of the protein. The two bands were cut from the gel, then MALDI-TOF MS peptide mapping with MALDI-PSD MS sequencing was performed. Both protein bands yielded good quality peptide maps, and most of the input masses matched the candidate protein sequences. The majority of the tryptic peptides matched with the theoretical masses within 97.4 ppm. MALDI-TOF MS data of the 27 and 54 kDa proteins in both cases permitted the identification of human thiamin pyrophosphokinase (Acc. No.: AAK01351), and suggested that the 54 kDa band was a PP20 / hTPK dimer. PSD data obtained for precursors also confirmed the identity of these proteins.

Mass spectrometry with ESI proved to be a useful method. Using the soft ionization technique, only the protonated sample molecules and the molecular ions could be generated without fragmentation, therefore identification became easy. By our experimental conditions, overfragmentation could be neglected and the molecular peaks were observed with high intensity. The thiamin pyrophosphate eluted at 2.91 min, indicating evidence of thiamin pyrophosphokinase activity for both PP20-B and PP20-R. Thiamin was eluted at 7.34 min (**Fig. 9A**). (The retention time of ATP was 2.02 min, and for AMP 2.38 min.) Our compounds of interest were identified by their retention times, UV-VIS and mass spectra. The molecular thiamin-pyrophosphate peak (scan filter: 50 V) could be observed at 424.7 amu, with high intensity (**Fig. 9B**). The other mass peaks were from the earlier eluted polar compounds contaminating

the thiamin pyrophosphate peak. The molecular thiamin peak (scan filter: 100 V) could be observed at 264.8 amu (**Fig. 9C**). No fragmentation of thiamin occurred (Yamanaka et al. 1994; Carerei et al., 1996). The peak purity test of thiamin-pyrophosphate showed that the UV spectra did not match each other particularly well. This was indicated by the degree of match, which was 964, with a standard deviation of 6.15%. By thiamin, the two curves covered each other perfectly. This was expressed by a match factor of 999, and the relative standard deviation of the match was 0.21%. (Match factor 1000 means the peak is completely pure. If the match factor is over 950, peak purity is completely acceptable.)

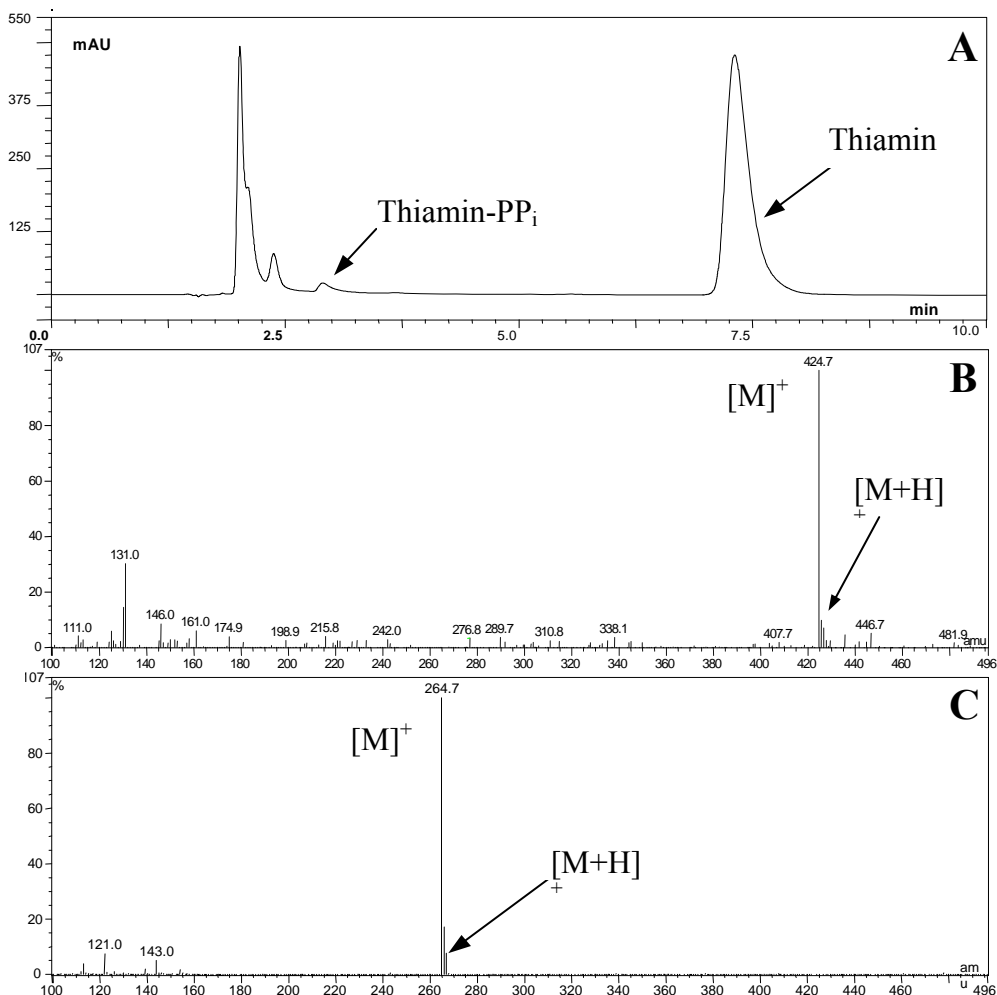


Fig. 9. Enzymatic analysis of the thiamin pyrophosphokinase activity of PP20-B by mass spectroscopy. (A) Chromatogram of the mixture containing PP20-B / hTPK and thiamin after being incubated overnight. **(B)** Mass spectrum of thiamin-PP_i ionized at 3.5 kV the scan filter was 50 V and **(C)** the mass spectrum of thiamin ionized at 3.5 kV, the scan filter was 100V.

Thiamin pyrophosphokinase is present in all types of human cells. Its function is the catalyzation of pyrophosphorilation of thiamin (vitamin B1) with ATP and magnesium ion. In humans, thiamin is absorbed from the gut, transported into cells, and converted into an active coenzyme form, thiamin pyrophosphate (TDP) (Grassl, 1998; Zempleni et al., 1992). TDP serves as a cofactor for 2-oxoacid dehydrogenase like pyruvate, 2-oxoglutarate, branching chain alpha ketoacid dehydrogenase or transketolase in pentose phosphate cycle. TDP-dependent enzymes are essential in carbohydrate metabolism and mitochondrial energy metabolism. Thiamin is an essential vitamin for vertebrates (Fridrich, 1998), therefore its utilization and pyrophosphorilation have high significance to cell viability and function in vertebrates. TDP synthesis can be important in the placenta because of its high energy requirement. Thiamine / TDP deficiencies have been seen in placental infarcts, indicating that TDP function and TDP expression can have a role in placental diseases (Grassl, 1998; Zempleni et al., 1992). Reports of several diseases and syndromes can be found in the literature in connection with thiamin deficiency or decreased thiamin pyrophosphokinase activity. According to two studies examining thiamin metabolism, thiamin and TPK enzyme deficiency are associated with diabetes mellitus, sensorineural deafness and thiamin-responsive megaloblastic anemia (Akinci et al., 1993; Rindi et al., 1992). Another publication referred to DIDMOAD syndrome (diabetes insipidus, diabetes mellitus, optic atrophy, deafness) associated with megaloblastic and sideroblastic anemia, neutropenia, and borderline thrombocytopenia. The authors proposed that an inherited abnormality of thiamin metabolism was responsible for the multisystem degenerative disorder (Borgana-Pignatti et al., 1989). Another study supposed that adverse effects of the antibiotic metronidazole might be due to its conversion to a thiamin analogue and consequent vitamin B1 antagonism. A metabolic product was found to be a close structural analogue of thiamin and an effective inhibitor of TPK *in vitro*

(Altson et al., 1987). A recent prospective controlled cohort study confirmed that metronidazole did not represent a major teratogenic risk in humans when used in the recommended doses, but a reduced neonatal birth weight was found in the metronidazole group compared with controls (Diav-Citrin et al., 2001). This could be explained by metronidazole's metabolic conversion and possible serious effect on placental metabolism through PP20 / hTPK pathway.

Expression and localization of PP20 / hTPK

In Western blot analysis, PP20-B and PP20-R proteins migrated in 27 and 54 kDa bands as described before (Bohn et al., 1985). Immunologically identical bands were detected in human term placental tissue. From 39 other types of human tissue, the highly specific anti-PP20 serum reacted with both the 27 kDa protein and with the dimer in all fetal and adult tissues, as they were expressed in equal amounts and ubiquitously (**Fig. 10A**). The expression of PP20 decreased from the first trimester to the third, and there was no remarkable difference between the amounts of the protein in normal placentas and those from pregnancies affected by preeclampsia or IUGR (**Fig. 10B**).

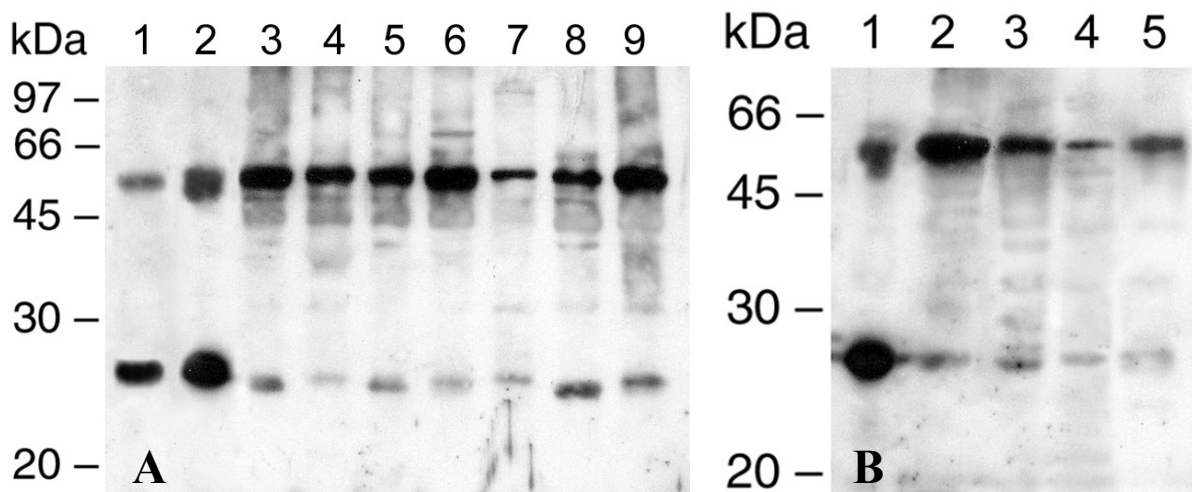


Fig. 10. Identification of the purified (PP20-B), recombinant (PP20-R), and placenta expressed PP20 / hTPK by Western blotting with polyclonal anti-PP20 serum. (A) 10 ng PP20-R (lane 1), 10 ng PP20-B (lane 2), 20-20 µg first trimester placenta (lane 3), rectum (lane 4), thyroid gland (lane 5), lung (lane 6), malignant melanoma (lane 7), renal tumour metastasis (lane 8), liver adenocarcinoma

(lane 9); **(B)** 10 ng PP20-R (lane 1), 20-20 µg first trimester placenta (lane 2), term placenta (lane 3), preeclamptic placenta (lane 4) and IUGR placenta (lane 5) tissues extracts were electrophoresed on 12% (w/v) SDS/PAGE. After Western blotting using polyclonal anti-PP20 serum and horseradish peroxidase labelled secondary IgG, protein bands were revealed by ECL chemiluminescence system. The positions of molecular mass markers are displayed on the left.

The immunoreactive protein bands could also be detected universally in the examined tumorous tissues as well as in various cell line extracts. PP20 / hTPK was not detectable in the circulation of pregnant woman, or in the sera of healthy and tumorous patients (**Table 6**).

Healthy adult tissues		Healthy fetal tissues	
Muscle	+	Muscle	+
Heart	+	Heart	+
Adrenal gland	+	Adrenal gland	+
Brain	+	Brain	+
Lung	+	Lung	+
Stomach	+	Stomach	+
Pancreas	+	Pancreas	+
Liver	++	Liver	+
Spleen	+	Spleen	+
Skin	+	Skin	+
Kidney	+	Kidney	+
Bladder	+	Bladder	+
Placenta (in term)	+		
Ovarium	+	Tumorous adult tissues	
Corpus uteri	+	Malignant melanoma	+
Cervix uteri	+	Kidney tumor	+
Mammary gland	+	Liver adenocarcinoma	+
Thyroid gland	+	Colon adenocarcinoma	+
Rectum	+	Cervical carcinoma	+
Adipose tissue	+	Brain tumor	+
Gall bladder	+		
		Serum samples	
Cell line extracts		Ovarium carcinoma	+
HepG2	+	Chorio carcinoma	+
HeLa	+	Cervical carcinoma	+
Panc-1	+	Corpus carinoma	+
WRL-68	+	Pregnant women (7 th -41 st week)	+

Table 6. Quantity of PP20 / hTPK in human normal/adult foetal and tumorous tissue extracts and adult serum samples. 10-10 µg protein from tissue extracts were subjected to 12 % (w/v) SDS/PAGE. After Western-blotting, protein bands were revealed with ECL chemiluminescence analysis, quantitative densitometric measurements were done by Scion Image for Windows.

We were unable to confirm the results of other research groups who found differences in the expression levels of hTPK, especially higher amounts in testis, small intestine and kidney by Northern blot (Zhao et al., 2001). Others stated that the mouse TPK mRNA levels were expressed in a tissue specific manner and found in high levels in kidney and liver (Nosaka et al., 1999). Our findings confirm the data that PP20 / hTPK is ubiquitously expressed in a wide variety of human tissues and appears to be an important housekeeping metabolic gene in humans.

With immunohistochemistry, diffuse and stronger labelling in the cytoplasm of the syncytiotrophoblasts and very weak staining of the trophoblasts and Hofbauer cells could be seen in first trimester placentas (**Fig. 11A**). A moderate labelling in the cytoplasm of the syncytiotrophoblasts and very weak staining of the trophoblasts and Hofbauer cells in the third trimester (**Fig. 11B**) and preeclamptic placenta (**Fig. 11C**) were found. By the more sensitive immunofluorescent confocal imaging, we detected the subcellular localization of PP20, which appeared to be diffuse in the cytoplasm (**Fig. 11D**).

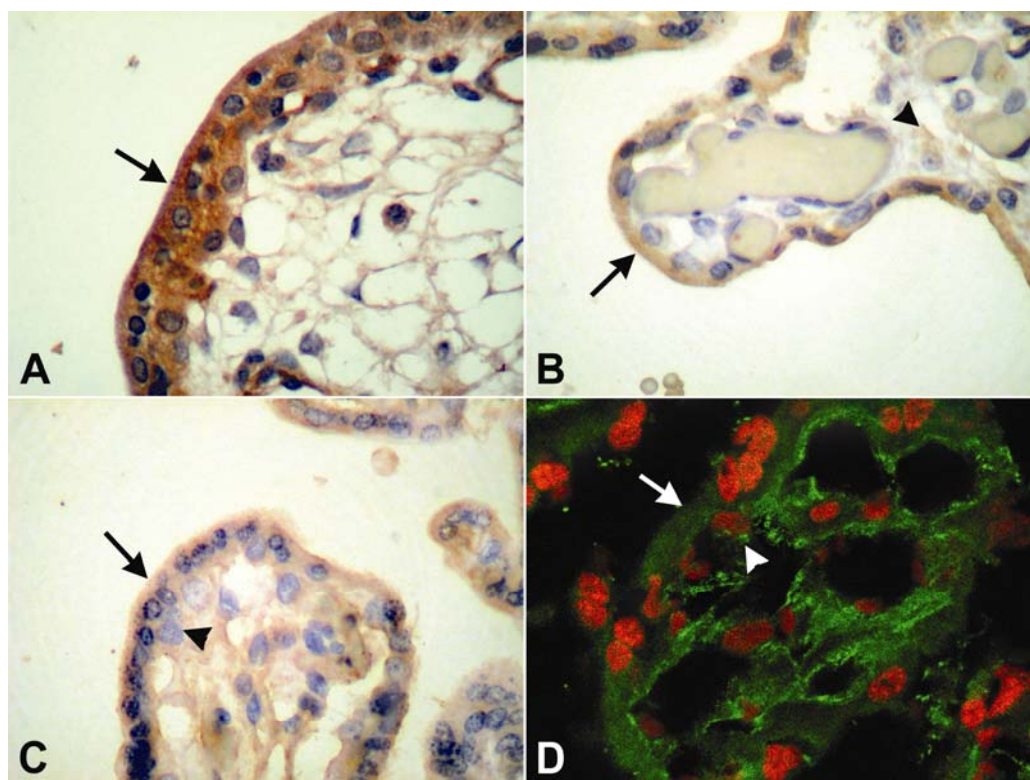


Fig. 11. Localization of PP20 / hTPK in human placental tissues. Formalin-fixed, paraffin-embedded tissue sections of human first trimester (**A**), term (**B, D**) and preeclamptic placentas (**C**) were stained for immunohistochemistry or immunofluorescent confocal microscopy, respectively, using polyclonal anti-PP20 serum. The immunohistochemical sections were counterstained with haematoxylin (400x magnification). A stronger PP20 expression was seen in the syncytiotrophoblasts (arrow) of the first trimester placenta (**A**), and moderate expression could be detected in the syncytiotrophoblasts (arrow) with a weak staining of the cytotrophoblasts (arrowhead) and Hofbauer cells in term and preeclamptic placentas (**B, C**). The confocal image was counterstained by DRAQ5 nucleus dye (red) (1000x magnification). Diffuse PP20 staining (green) could be recognized in the cytoplasm of syncytiotrophoblasts (arrow), and its discrete labelling in the cytotrophoblasts (arrowhead) (**D**).

Immunohistochemical studies have previously shown that in human early placenta PP20 was mainly located in the cytoplasm of the syncytiotrophoblast, cytotrophoblast, and in term placentas, and the protein was clearly localized mainly in the cytoplasm of Hofbauer-like cells in the villous stroma as well as reflected and basal chorionic trophoblast (Inaba et al., 1987). These findings suggested that the villous syncytiotrophoblast might lose the ability to produce this protein by placental aging. Our investigations supplemented this data as we studied first and third trimester normal placentas as well as placentas from pregnancies affected by preeclampsia or IUGR.

Structural model of PP20 / hTPK

Comparative structural modelling satisfying all spatial restraints revealed that the fold and backbone structure of PP20 / hTPK corresponded to the structure of mouse TPK. The root mean square deviation (RMSD) between the mouse and PP20 / hTPK was 0.17 Å, which indicated minor structural differences only. PP20 / hTPK also consisted of an N-terminal alpha/beta-domain and the C-terminal beta-sandwich domain. The overall dimensions of the subunits were about 56 Å in length and 31 Å in width. The thiamin binding-site was formed by a beta-bulge between Ser216B-Asn219B, with the loops between Gly199B-

Asn203B, and between Asp95A-Asp100A, as in the mouse enzyme (**Fig. 12B**). The vicinity of the suspected ATP binding-site was also identical to that of the mouse protein, except for the 129 alanine-glycine change, as shown in the sequence alignment (**Fig. 12A**), where the binding-sites were shaded. Sequential alignment was performed at the subunit level.

Fig. 12A.

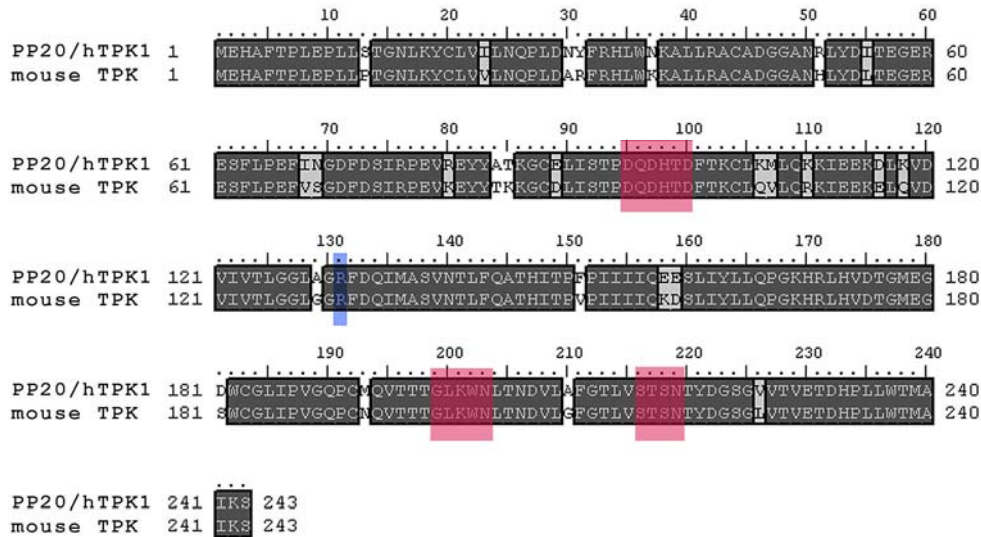


Fig. 12B.



Fig. 12. 3D homology modelling and sequence alignment of PP20 / hTPK to mouse TPK. (A) Identical residues in PP20 / hTPK and mouse TPK were shaded dark grey, conservative changes in light grey, the thiamin binding site in red and the suspected ATP binding site in blue. **(B)** 3D model of the PP20 / hTPK homodimer enzyme was constructed by homology modelling with MOLSCRIPT [20] based on the mTPK crystal structure. The 3D model was rendered by Raster3D. The peptide backbones were represented as ribbons, the thiamin binding-sites as balls and sticks, and thiamins as spacefills.

Both polypeptide chains consisted of 243 amino acids, and no deletions or insertions were present. The best alignment was obtained without gap insertion. Analysis of various TPK amino acid sequences in their catalytic centre indicated a high level of conservation in mammals, compared to bacteria, but in fungi there were greater differences. In the first cluster, out of 6 amino acids 3 were different in prokaryotes and in lower eukaryotes. In the second cluster, out of 5 amino acids, 3 or 4 were different. In the third cluster, 50 % of the amino acids were different in bacteria and fungi from the human sequence. This data showed that TPK is an enzyme with an active center very significantly different in human enzymes compared to bacterial and fungal, raising the possibility of designing specific drugs which could inhibit bacterial and/or fungal TPKs (**Fig. 13**). The PP20 model has been deposited at the EBI Macromolecular Structure Database with the accession code PDB: 1OLY.

PP20/hTPK1	95	D	D	H	T	D	100	...	199	G	L	K	W	N	203	...	216	S	T	S	N	219	
Mouse TPK1	95	D	D	H	T	D	100	...	199	G	L	K	W	N	203	...	216	S	T	S	N	219	
Schizosaccharomyces pombe	427	C	O	N	T	T	D	432	...	526	G	L	E	W	N	530	...	543	S	S	C	N	546
Giardia lamblia	137	E	Q	D	S	T	D	142	...	260	G	L	K	W	D	264	...	277	S	G	C	N	280
Bacillus cereus	50	E	K	D	Q	T	D	55	...	145	G	F	K	Y	P	149	...	164	C	I	S	N	168
Bacillus anthracis Ames	95	E	K	D	Q	T	D	100	...	170	G	F	K	Y	P	174	...	187	C	I	S	N	190
Fusobacterium vincentii	78	E	K	D	Y	T	D	83	...	166	G	F	K	Y	N	170	...	185	C	I	S	N	189
Enterococcus faecalis	74	E	K	D	T	T	D	79	...	171	R	S	K	Y	L	175	...	190	Y	A	S	N	194
Clostridium tetani	75	E	K	D	F	T	D	80	...	168	G	A	K	Y	P	172	...	189	G	V	S	N	193
Brucella melitensis	73	A	K	D	M	T	D	78	...	167	N	A	K	W	P	171	...	186	T	V	S	N	190

Fig. 13. Multiple sequence analysis of TPKs at their catalytic centre. At the first cluster, aspartates and threonine were highly conserved from bacteria to mammals, but more changes occurred in fungi. At the second cluster, only lysine was conserved from bacteria to mammals in contrast to fungi, whilst the other four amino acids were conserved in mammals. In the third cluster, serine and asparagine were highly conserved.

We concluded that the thiamin binding site of the human TPK enzyme was highly similar to that of the mouse enzyme. However, sequences of human and fungal TPKs were only 30% identical (Nosaka et al., 1999). In addition, bacterial TPKs were also highly different from the human enzymes, having only about 25% identity (Nosaka et al., 1993). In the case of fungal TPKs, in the first region of the active site two negatively charged aspartates substituted to neutral

cysteine and asparagines. In the second region, a positively charged lysine was exchanged to negatively charged glutamate, showing the important structural and charge differences in the active site. In the case of bacterial enzymes, in the first region histidine, which could be positively charged at neutral pH, was substituted by neutral or negatively charged amino acids. In the second region GL and WN pairs were changed to different space-filling groups, or in some cases to partially negatively charged tyrosine groups. This data showed a significant difference in the active sites of TPKs in different species. Even higher differences could be seen in the charge and size of space filling, raising the possibility for designing drugs which specifically inhibit bacterial and fungi enzymes without affecting human TPK. Therefore, this study indicates the possibility that special TPK inhibitors could be used as potential antimicrobial agents (Timm et al., 2001), which not only have no effect on PP20 / hTPK generally, but their development and administration may also enable safe antimicrobial therapy during pregnancy.

General Conclusions (PP17b, PP20)

1. GenBank analysis of EST clones underlines that alternatively spliced PP17a occurs mainly in steroidogenic tissues, while PP17b is synthesized in almost all types of tissue, especially in placenta and epithelial origin tumors.
2. Sequence data show high level sequence similarity at their N-termini between PP17b and neutral lipid droplet associated proteins including perilipins and adipophilin, which latter was also involved in adipose cell differentiation. Taken altogether, a comparison of PP17b and its gene to perilipins and adipophilin, members of the “PAT domain gene family,” similar exon structures, sequence homology and many common transcription factor regulatory sequences in the promoter regions were found, suggesting their common genetic origin and functional similarities.
3. With different techniques based on immunological reactions, considerable evidence was obtained to the effect that PP17b/TIP47 was a neutral lipid droplet associated protein, which also occurs in significant quantities in milk lipid globule membranes. Because of the controversy in the literature on its function, to avoid possible immunological cross-reactivity, a very specific independent technique, MALDI-TOF MS analysis was used, and both PP17 variants – PP17b most markedly – were proved to bind to the surface of neutral lipid droplets. Furthermore, our previous data showed that both PP17a and PP17b could aggregate even in the presence of low concentrations of SDS, raising the possibility that these proteins could be involved in the formation of different-size lipid droplets. By binding to lipid micelles and having self-aggregating properties, PP17 variants could

facilitate lipid droplet aggregation, which is clearly detectable in the case of milk lipid globule membranes. This property of PP17b indicates its function as a neutral lipid droplet associated protein and its involvement in lipid droplet formation/mobilization, in accordance with its possible function in cell and tissue differentiation.

4. With computer analysis of its 5' up-stream sequence, several transcription factor binding sites were identified, including mostly proliferation and / or apoptosis regulators, embryo- and organogenic factors, proto-oncogenes or their targets, which also points to the possible complex PP17 gene regulation.

5. Induction of apoptosis and differentiation indeed up-regulated PP17 expression, while kinase cascade inhibition led to a transcription factor activation block on the induction of PP17 expression, providing evidence for the importance of those transcription factors in PP17 gene regulation. These data also indicate that PP17b could play an important role in tumor cell development and differentiation. Since providing rich lipid supply to cells induced lipid droplet formation and PP17b overexpression, this indicates that PPAR γ could have a role in the regulation of PP17 expression. Furthermore, these data suggest that the main function of PP17a and PP17b is involvement in lipid droplet formation and in rearrangement of lipid membranes, which processes could also be important in cell differentiation and division. The high concentration of PP17b in milk lipid globule membranes indicates its potential role in exporting lipid droplets and membranes.

6. In the case of several previously known “placental proteins,” which turned out to have a general function in different human tissues, more specific structural or functional names were given, such as galectin-13 (PP13), glycodelin (PP14) or branched-chain aminotransferase (PP18). As (1) PP17b is synthesized ubiquitously, while PP17a is found mainly in steroidogenic tissues; (2) both PP17 variants are generally involved in lipid droplet formation, like alternatively spliced perilipins, which were shown to bind either to steroid or neutral lipid droplets; (3) neither the name “*placental protein 17b (PP17b)*” nor “*tail-interacting protein of 47 kDa (TIP47)*” gives the appropriate information on the structure, function, regulation, or the origin of this protein; (4) there is still a lack of an official name for the “PP17/TIP47” gene; and (5) there is a common need to elucidate this controversial situation, it is therefore now proposed that the PP17 variants be renamed to *sandrin A (PP17a)* and *sandrin B (PP17b)* (Steroid And Neutral lipid DRoplet-associated proteIN), and their gene to SNDR.
7. Four cDNAs have been isolated from placental library encoding the 243 residue-long protein, having two variants (27 kDa and 54 kDa). The 54 kDa variant was verified to be a dimer. By its primary nucleotide sequence, PP20 proved to be identical to human thiamin pyrophosphokinase (hTPK), as confirmed by protein sequence analysis.
8. GenBank search information revealed PP20 / hTPK gene was located on chromosome 7q34-q36 and was predominantly expressed in the placenta. The genomic sequence contained 9 exons, not 8 as published previously when the exon division and 5'-upstream region of the gene were not yet resolved

9. Analysis of the 1kb promoter region showed numerous putative transcription factor binding sites, which might be responsible for the ubiquitous PP20 / hTPK expression. This may also be in accordance with the presence of the protein in tissues responsible for the regulation of the exquisite balance between cell division, differentiation and survival.
10. TPK activity of the purified and recombinant protein was proved by mass spectrometry.
11. PP20 / hTPK was found in all human normal and tumorous adult and fetal tissues in nearly equal amounts, but not in sera.
12. By immunohistochemical and immunofluorescent confocal imaging methods, diffuse labelling in the cytoplasm of the syncytiotrophoblasts and weak staining of the trophoblasts were observed, and the amount of PP20 / hTPK decreased from the first trimester to the end of gestation.
13. A 3D model of PP20 / hTPK was computed (PDB No: 1OLY) by homology modelling. A high degree of structural homology showed that thiamin binding-site was highly similar to that of mouse enzyme, but highly different from the bacterial ones. Comparison of the catalytic centre sequences revealed differences, raising the possibility of designing new drugs which specifically inhibit bacterial and fungal enzymes without affecting PP20 / hTPK and offering the possibility for safe antimicrobial therapy during pregnancy.

ACKNOWLEDGEMENTS

This work was performed under the inspiring guidance and careful supervision of the late Professor Gábor Nándor Than, MD, PhD, DSci, who passed away in March 31, 2002. All the authors and his colleagues dedicate this work to his memory. I am also very thankful for my tutors , Professor Balazs Sümegi, PhD, DAc and Nandor G. Than, MD, PhD for their tutorial work, for Tímea Berki MD, PhD, Flow cytometric measurements, for György Szekeres MD, PhD, histological examinations, for Andras Szigeti, Arpad Boronkai and all the doctors and assistants of Institute Biochemistry and Medical Chemistry participating in the project for their theoretical and technical help, all of them working at Medical University of Pécs. This work was supported by Hungarian Grants ETT T-09 163/01; FKFP 0010/1999, 0166/2001; OMFB-BIO 00041/2001; and OTKA T/020622, T/023076, T/029824, T/046473, M/36996.

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