Eur. J. Biochem. **271**, 1065–1078 (2004) © FEBS 2004 doi:10.1111/j.1432-1033.2004.04004.x

Functional analyses of placental protein 13/galectin-13

Nandor G. Than[1,2], Elah Pick[4], Szabolcs Bellyei[2], Andras Szigeti[2], Ora Burger[4], Zoltan Berente[2], Tamas Janaky[5], Arpad Boronkai[2], Harvey Kliman[6], Hamutal Meiri[4], Hans Bohn[7], Gabor N. Than[3] and Balazs Sumegi[1,8]

[1]*First Department of Obstetrics and Gynaecology, Semmelweis University, Budapest, Hungary;* [2]*Department of Biochemistry and Medical Chemistry and* [3]*Department of Obstetrics and Gynaecology, University of Pecs, Hungary;* [4]*Diagnostic Technologies Ltd, Haifa, Israel;* [5]*Department of Medical Chemistry, University of Szeged, Hungary;* [6]*Department of Obstetrics and Gynaecology, Yale University, New Haven, CT, USA;* [7]*Behringwerke AG, Marburg/Lahn, Germany;* [8]*Research Group for Mitochondrial Function and Diseases, Hungarian Academy of Sciences, Pecs, Hungary*

Placental protein 13 (PP13) was cloned from human term placenta. As sequence analyses, alignments and computational modelling showed its conserved structural and functional homology to members of the galectin family, the protein was designated galectin-13. Similar to human eosinophil Charcot–Leyden crystal protein/galectin-10 but not other galectins, its weak lysophospholipase activity was confirmed by ^{31}P-NMR. In this study, recombinant PP13/galectin-13 was expressed and specific monoclonal antibody to PP13 was developed. Endogenous lysophospholipase activity of both the purified and also the recombinant protein was verified. Sugar binding assays revealed that *N*-acetyl-lactosamine, mannose and *N*-acetyl-glucosamine residues widely expressed in human placenta had the strongest binding affinity to both the purified and recombinant PP13/galectin-13, which also effectively agglutinated erythrocytes. The protein was found to be a homodimer of 16 kDa subunits linked together by disulphide bonds, a phenomenon differing from the noncovalent dimerization of previously known prototype galectins. Furthermore, reducing agents were shown to decrease its sugar binding activity and abolish its haemagglutination. Phosphorylation sites were computed on PP13/galectin-13, and phosphorylation of the purified protein was confirmed. Using affinity chromatography, PAGE, MALDI-TOF MS and post source decay, annexin II and beta/gamma actin were identified as proteins specifically bound to PP13/galectin-13 in placenta and fetal hepatic cells. Perinuclear staining of the syncytiotrophoblasts showed its expression in these cells, while strong labelling of the syncytiotrophoblasts' brush border membrane confirmed its galectin-like externalization to the cell surface. Knowing its colocalization and specific binding to annexin II, PP13/galectin-13 was assumed to be secreted to the outer cell surface by ectocytosis, in microvesicles containing actin and annexin II. With regard to our functional and immunomorphological results, PP13/galectin-13 may have special haemostatic and immunobiological functions at the lining of the common feto-maternal blood-spaces or developmental role in the placenta.

Keywords: brush border membrane; carbohydrate binding; galectin; lysophospholipase; placental protein.

Placental protein 13 (PP13) is a member of the group of the so-called 'pregnancy-related proteins' [1] that might be highly expressed in placenta and some maternal/fetal tissues during pregnancy. The structural and functional characteristics of these proteins and their possible role in placental development and regulation pathways are receiving increased interest at present. PP13 was first isolated from human placenta and characterized by Bohn *et al.* in 1983. It was found to be comprised of two identical 16 kDa subunits held together by disulfide bonds, and to have the lowest carbohydrate content (0.6%) of any known placental proteins [2]. Later, cloning of PP13 was performed in parallel by two research groups [3,4], and its sequence was deposited separately at the GenBank database (AF117383, AY055826). At that time, sequence analysis and alignment showed that PP13 shared the highest homology to human eosinophil Charcot–Leyden crystal (CLC) protein/galectin-10 [5], and similarly to CLC, PP13 purified from human placenta (PP13-B) showed weak lysophospholipase (LPLA) activity [3]. However, conserved structural identity of PP13 to the members of the galectin family was also found [3]. Subsequently, computational 3D modelling based on its primary structure and homology to prototype galectins [6] revealed a characteristic 'jellyroll' fold (deposited to

Correspondence to N. G. Than, Department of Biochemistry and Medical Chemistry, University of Pecs, 12 Szigeti Street, Pecs H-7624, Hungary. Fax: + 36 72 536 277, Tel.: + 36 30 9512 026, E-mail: gabor.than@aok.pte.hu

Abbreviations: CLC, Charcot–Leyden crystal; CRD, carbohydrate recognition domain; FITC, fluorescein isothiocyanate; GPC, glycero-3-phosphorylcholine; IPTG, isopropyl thio-β-D-galactoside; iLPC, 2-acyl-glycero-3-phosphorylcholine; LPC, L-α-lysophosphatidyl-choline; LPLA, lysophospholipase; PLA, phospholipase; PP13, placental protein 13; PP13-B, PP13 purified from placenta; PP13-R, recombinant PP13; PSD, post source decay.

Dedication: This manuscript is dedicated to the memory of the late Professor Gabor N. Than, whose inspiring leadership of his research team will be remembered forever.

(Received 8 December 2003, revised 14 January 2004, accepted 20 January 2004)

Brookhaven Data Bank, Accession No. 1F87), a single conserved carbohydrate recognition domain (CRD) and predicted sugar binding capabilities of PP13, and it was therefore designated as galectin-13 [7].

As several galectins have recently proved to be very closely related to PP13/galectin-13 [8,9], and there were also some incongruities in its tissue expression in studies performed by polyclonal antibodies to PP13 and PP13 cDNA [3], more powerful, specific monoclonal antibodies to PP13 had to be developed. By the expression of recombinant PP13 protein (PP13-R), it became possible to perform more detailed functional studies on the protein. Because LPLA activity of CLC protein/galectin-10 has recently been assigned to its interaction with putative eosinophil LPLAs or their known inhibitors [10], elucidation of intrinsic LPLA, phospholipase (PLA) or sugar binding activities of PP13/galectin-13 had to be reconsidered. In this study, immunoaffinity purification and mass spectrometry (MS) studies indicated the binding of PP13/galectin-13 to proteins involved in phospholipid metabolism and cytoskeletal functions, but no intracellular LPLA was detectably bound to it. On the other hand, intrinsic LPLA activity for not only the purified PP13-B, but also the bacterially expressed PP13-R was confirmed. With sugar binding assays, the results of previous predictions on the sugar binding specificity of its CRD [7] were strongly underlined. In contrast to other known prototype galectins, PP13/galectin-13 was found to be a homodimer linked by disulphide bonds. Unlike most thiol-dependent galectins, reducing agents were shown to decrease its sugar binding activity and abolish its haemagglutination. In addition, putative phosphorylation sites were computed, and phosphorylation of the purified protein was empirically proved.

As not only the information on their structural and carbohydrate binding characteristics of galectins, but also their exact morphological localizations in cells and tissues are essential for the understanding of their interaction with glycoconjugates and diverse biological functions, to obtain better insight into the physiological role and involvement in placental development and functions of PP13/galectin-13, as well as its predicted role in different pregnancy complications [11], a detailed immunolocalizational study was also performed. Its *in vitro* characterization in a collaborative study between the leading groups of PP13/galectin-13 research adequately revealed the putative physiological functions of the protein, and gave a possible hypothesis for its importance in placental developmental processes and its conjunction with fetal haemopoetic tissues.

Experimental procedures

Materials

PP13 antigen denoted here as PP13-B (Op. 234/266) and rabbit polyclonal antibody to PP13 (160 ZB) was prepared by H. Bohn (Behringwerke AG, Marburg/Lahn, Germany). NSO/1 myeloma cell line was kindly provided by C. Milstein (MRC, Cambridge, UK). We used anti-annexin II rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), fluorescein isothiocyanate (FITC) labelled anti-mouse IgG (Molecular Probes, Eugene, OR, USA) and FITC-labelled anti-rabbit IgG (BD Pharmingen, San Diego, CA, USA). We obtained WRL-68 human fetal hepatic cells (ATCC, Manassas, VA, USA); D_2O (Isotec Inc., Miamisburg, OH, USA); pUC57-T vector (MBI Fermentas, St. Leon-Rot, Germany); pQE30 vector, M15 (pREP4) *Escherichia coli* and Ni-nitrilotriacetic acid column (Qiagen Inc., Valencia, CA, USA); Protein A column (Affiland, Ans-Liege, Belgium); bicinchoninic acid reagent (Pierce Biotechnology Inc., Rockford, IL, USA); ECL chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK); DRAQ5 dye (Biostatus Ltd, Shepshed, UK); Universal Kit (Immunotech, Marseille, France); Pro-Q Diamond phosphoprotein gel staining kit (Molecular Probes, Eugene, OR, USA); trypsin (Promega GmbH, Mannheim, Germany); ZipTipC18 pipette tips (Millipore, Bedford, MA, USA). *N*-acetyl-D-lactosamine, L-fucose, galactose, glucose, lactose, maltose, mannose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine; cyanogen-bromide activated sepharose 4B, L-fucose-agarose, glucose-agarose, lactose-agarose, maltose-agarose, mannose-agarose, *N*-acetyl-D-galactosamine-agarose, *N*-acetyl-D-glucosamine-agarose; 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phospho-L-serin, L-phosphatidyl-inositol, L-phosphatidyl-ethanolamine; L-α-1-lysophosphatidylcholine, lysophosphatidylethanolamine, L-α-1-lysophosphatidylinositol, L-α-1-lysophosphatidyl-L-serin; isopropyl thio-β-D-galactoside (IPTG); antibiotic-antimycotic solution, bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid) (Hepes), phenylmethylsulfonyl fluoride and horseradish peroxidase labeled anti-rabbit and anti-mouse IgGs were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Databank search

PP13/galectin-13 cDNA and amino acid sequences were compared to various EST, genomic and protein databases by BLAST at NCBI (Bethesda, MD, USA) [12]. Multiple sequence alignments were carried out with CLUSTALW at EMBnet (Lausanne, Switzerland) [13]. The PROSITE [14] and NetPhos [15] databases were searched for biologically significant patterns and putative phosphorylation sites. The carbohydrate binding moiety and cysteine residues potentially involved in intermolecular cross-linking were localized on the 3D model of PP13/galectin-13 (PDB 1F87) with RASMOL [16].

Construction of bacterial PP13/galectin-13 expression plasmids

Full length PP13/galectin-13 cDNA was isolated by the standard RACE method [17,18] using 4 μg of total placental RNA and specific primers. The resulting PCR fragments were inserted into pUC57-T cloning vector. Insert-containing clones were selected and sequenced by automated DNA sequencing at the Biological Services of the Weizmann Institute (Rehovot, Israel). Subsequently, the whole open reading frame of the cDNA containing the consensus Kozak sequence at its 5′ end [19] was PCR amplified with (5′-CGATACGGATCCATGTCTTCTTTACCCGTGC-3′) and (5′-TAAGTCGAGCTCATTGCAGACACACACT

GAGG-3′) primers. The resultant PCR product was cloned into the *Bam*HI and *Sac*1 sites of the pQE30 expression vector.

Expression and purification of recombinant PP13/galectin-13

The PP13-R/pQE30 expression vector was transformed into M15 (pREP4) *Escherichia coli* host strain and the bacteria were induced with IPTG. The expressed protein was subsequently purified with Ni-nitrilotriacetic acid column in the presence of the His_6-tag. The primary structure and purity of PP13-R was verified by sequence analysis [20] and by immunoblotting with both polyclonal and monoclonal antibodies to PP13. The specific antibody recognition of both PP13-R and PP13-B were investigated by sandwich ELISA performed with two different monoclonal anti-PP13 IgGs described below.

Preparation of monoclonal antibodies to PP13/galectin-13

Monoclonal antibodies to PP13 were produced at the Hybridoma Center of the Weizmann Institute. Female Balb/c mice (Jackson Laboratory, Bar Harbor, ME, USA) were immunized with 0.05 mg PP13-B. Hybridomas were prepared from mice spleen cells by hybridizing with NSO/1 myeloma cells as described previously [21]. Cells were screened by direct ELISA using PP13-B as antigen. Anti-PP13 Ig producing clones were subsequently injected intraperitoneally into mice. Antibodies were isolated from the ascitic fluid, purified on Protein A column and checked for subclass and protein content by immunoblots and sandwich ELISA.

PP13/galectin-13 lysophospholipase and phospholipase activity detection by NMR

PP13-B purified from placenta and bacterially expressed PP13-R (20 μg each) were dissolved in 500 μL aqueous solutions (200 mM Hepes, 5.0 mM $CaCl_2$ and 130 mM NaCl, pH 7.4) of 5.0 $mg{\cdot}mL^{-1}$ of the different lysophospholipids listed in Materials. Aliquots without PP13 proteins were used as controls. The solutions were prepared and stored at 37 °C in 5 mm (outside diameter) NMR tubes and their ^{31}P-NMR spectra were recorded at various time intervals. During NMR measurements a 2 mm (outside diameter) insert tube filled with D_2O was placed in the NMR tubes. To detect phospholipase activity of PP13-B and PP13-R, 7.2 $mg{\cdot}mL^{-1}$ of the phospholipids listed in Materials were used, and 25 μL Triton X-100 was added to the aliquots to enable dissolution of the substrate. ^{31}P-NMR spectra were obtained on a Varian ^{UNITY}INOVA 400 WB spectrometer at 161.90 MHz, 37 °C. Proton decoupling provided 128 transients, using 30 °C flip angle pulses with 3.4 s delays and a 0.6 s acquisition time, in order for the peak integrals to represent the relative concentrations of the phosphorous containing species. The chemical shifts were referred to the deuterium resonance frequency of the D_2O in the insert tube. The relative concentrations (in molar fractions) of the species observed during the whole course of the study were determined by deconvolution of the spectra, using the routine built into the NMR software (VNMR 6.1 B; Varian Inc., Palo Alto, CA, USA).

PP13/galectin-13 sugar binding assays

Binding of PP13-R to different sugars was studied essentially as described in [22], but protein binding was followed by the endogenous fluorescence of PP13/galectin-13 (excitation at 280 nm, emission at 360 nm). PP13-R (50 μg) was dissolved in 200 μL sodium phosphate buffer (50 mM, pH 7.3, containing 0.15 M NaCl, 20 mM EDTA) and added to 50 μL activated sugar-coupled agarose beads as listed in Materials. In parallel experiments, 1 mM dithiothreitol was also added to the mixture. The solutions were incubated in 0.5 mL microtubes at 37 °C for 1 h with vigorous shaking. Tubes were then centrifuged at 10 000 ***g*** for 20 s to sediment agarose beads. For quantification of unbound PP13-R, fluorescence of the supernatants was determined in a protein concentration range of 2–100 $\mu g{\cdot}mL^{-1}$, measured by an LS50B PerkinElmer Luminescence Spectrometer (Shelton, CT, USA). For controls, uncoupled agarose beads (Sepharose 2B) were used. After removing the unbound PP13-R, specifically bound PP13-R was eluted with different sugars in different concentrations (1 mM–1 M) and fluorescence of the supernatants was measured by the same method. For positive controls, PP13-R (50 μg) was dissolved in buffer, for negative controls only buffer was used.

PP13/galectin-13 haemagglutination assay

Lectin activity of both PP13-B and PP13-R was determined by measurement of their capabilities to agglutinate human erythrocytes. Agglutination assays were performed in a 96 well microtiter plate with serial twofold dilutions (0.21–200 $\mu g{\cdot}mL^{-1}$) of the proteins in $NaCl/P_i$. Assays were also carried out by the addition of dithiothreitol, mannose or *N*-acetyl-lactosamine (1 mM each) to the mixtures. Samples (50 μL) were gently mixed with 2% suspension of erythrocytes (50 μL) and incubated at room temperature for 1 h. Agglutination activity was determined on the basis of the sedimentary state of the erythrocytes.

PP13/galectin-13 dimerization assay

For the detection of dimerization, PP13-R was diluted (0.16–0.6 $mg{\cdot}mL^{-1}$) in Laemmli solution prepared with or without 10% (v/v) 2-mercaptoethanol and subjected to 12% (w/v) SDS/PAGE, then visualized by Coomassie staining. Protein bands were identified by subsequent MALDI-TOF mass spectrometry.

Pro-Q Diamond phosphoprotein gel staining

PP13-B, PP13-R, ovalbumin (positive control) and BSA (negative control) (20 μg each) were pretreated in reducing conditions, run on 15% SDS/PAGE and stained with Pro-Q Diamond phosphoprotein gel stain according to the manufacturer's protocol. For detecting phosphoproteins, the gel was visualized and photographed in UV light. For detecting its total protein content, Coomassie staining was applied.

Cell culture

WRL-68 cells were grown on 100 mm dishes in standard DMEM containing 1% (v/v) antibiotic-antimycotic solution, supplemented with 10% (v/v) fetal calf serum, under 5% CO_2 condition and 95% humidified air at 37 °C. Cells were harvested and low-speed centrifuged at 2000 ***g***, then the pellet was dispersed by vortexing in lysis buffer (50 mM Tris pH 7.4, 1 mM phenylmethylsulfonyl fluoride) for 10 min at 4 °C. After further cell disruption in a Teflon/glass homogenizer, the homogenate was pelleted, and the supernatant was coupled to cyanogen-bromide activated Sepharose 4B by the instructions of the manufacturer.

Tissue preparations

One hundred milligram tissue blocks from a term human placenta obtained from Histopathology Ltd. (Pecs, Hungary) were homogenized in lysis buffer (50 mM Tris pH 7.4, 1 mM phenylmethylsulfonyl fluoride) for 10 min at 4 °C in a Teflon/glass homogenizer. After pelletting the homogenates, supernatants were either coupled to cyanogen-bromide activated, PP13-bound Sepharose 4B for immunoaffinity purification, or measured by bicinchoninic acid reagent and equalized for 1 $mg{\cdot}mL^{-1}$ protein content in 2× Laemmli solution for Western blotting. Other parts of the placenta were formalin fixed, paraffin embedded, cut for 4 μm sections, mounted on slides, dried at 37 °C overnight, dewaxed and rehydrated for immunohistochemistry and immunofluorescence confocal microscopy.

Affinity purification of PP13/galectin-13 bound proteins

Both PP13-B and PP13-R were coupled to cyanogen-bromide activated Sepharose 4B and incubated with protein extracts from human placenta or WRL-68 fetal hepatic cells at 24 °C for 1 h. For controls, samples were incubated with uncoupled Sepharose 4B. Gels were washed three times with 20 mM Tris/HCl buffer (pH 7.4, 150 mM NaCl) followed by four rinses with 20 mM Tris/HCl buffer (pH 7.4) to remove unbound proteins. Specifically bound proteins were removed by an equal volume of 2× Laemmli buffer, separated by 15% SDS/PAGE and visualized by Coomassie staining.

Protein identification by mass spectrometry

Bands of interest either in Coomassie stained PP13-B or PP13-R, as well as PP13-B or PP13-R bound and eluted protein extracts were excised from the gels, reduced, alkylated and gel digested with trypsin as described in [23]. Proteins were identified by a combination of MALDI-TOF 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 nonredundant database (NCBI, Bethesda, MD, USA) using MS FIT program (UCSF, San Francisco, CA, USA) [24]. Primary structure of tryptic peptide ions was confirmed by PSD MS sequencing.

SDS/PAGE and Western blotting

Ten nanograms each of PP13-B and PP13-R (or 50 ng each in the case of monoclonal antibodies) and 10 μg of human placental protein extract was subjected to 15% (w/v) SDS/PAGE followed by immunoblotting with polyclonal or monoclonal antibodies to PP13 and horseradish peroxidase labeled secondary IgGs as described in [25]. Protein bands were revealed by ECL chemiluminescence system.

Immunohistochemistry

Formalin fixed, paraffin-embedded tissue sections were incubated either with monoclonal or polyclonal antibodies to PP13. Immunostaining was carried out according to the streptavidin/biotin/peroxidase technique using Universal Kit [26]. Control sections were incubated only with secondary IgGs. Visual evaluation of hematoxylin counterstained slides was performed with an Olympus BX50 light microscope with incorporated photography system (Hamburg, Germany).

Immunofluorescence confocal microscopy

Paraffin embedded tissue sections were deparaffinated and treated with either monoclonal or polyclonal antibodies to PP13 followed by FITC-labelled secondary anti-mouse or anti-rabbit IgGs and 20 μM DRAQ5 nucleus labelling dye in $NaCl/P_i$ containing 0.1/0.1% (v/v) saponin and BSA. To visualize the localization of annexin II, anti-annexin II primary and FITC-labelled secondary IgGs were used. Control sections were incubated with only secondary IgGs, and antigen depletion was carried out on distinct slides. Fluorescence was scanned with a Bio-Rad MRC-1024ES laser confocal attachment (Herefordshire, UK) mounted on a Nikon Eclipse TE-300 inverted microscope (Kingstone, UK).

Statistical evaluation

Values in the figures and text were expressed as mean ± SEM of n observations. Statistical analysis was performed by analysis of variance followed by Student's t-test and chi-square test. $P < 0.05$ was considered to be statistically significant.

Results

PP13/galectin-13 is a member of a new subfamily among prototype galectins

From the GenBank search of related EST sequences, it could be assumed that PP13/galectin-13 mRNA was expressed only in placenta, fetal liver and spleen [3]. *PP13/galectin-13* gene mapped to chromosome 19 (19q13.1) in the close vicinity of genes of four known (galectin-10 [27], galectin-7 [28], galectin-4 [29] and placental protein 13-like

protein [8]) and three putative ('similar to placental protein 13' at locus XP_086001/AC005515-I [9], 'unnamed protein' at locus BAC85631/AC005515-II [9] and 'Charcot–Leyden Crystal 2 protein' at locus AAP97241) galectins at 19q13.1–13.2 with similar exon structures, indicating their common genetic origin. PP13/galectin-13 was found to have a close relationship with the predominantly placental expressed 'similar to placental protein 13' (69% identity, 80% similarity) and placental protein 13-like protein (68% identity, 79% similarity) as well as CLC protein (56% identity, 69% similarity). The putative 'Charcot–Leyden Crystal 2 protein' and 'unnamed protein' also had a considerably high relationship to PP13/galectin-13. Putative serine and tyrosine kinase phosphorylation sites localized on the outer surface of PP13/galectin-13 were predicted at positions 44–52 (Ser48), 37–45 (Tyr41) and 76–84 (Tyr80) by computations (Fig. 1A). With RASMOL, four cysteine residues were revealed on the surface of PP13/galectin-13 (Fig. 1B). By CLUSTALW alignments, Cys136 and Cys138 on beta-sheet F1 were found to be missing from all homologues. Cys19 and Cys92 on beta-sheets F2 and F3 were missing from distant homologues, but some of the newly described closest homologues contained them (Fig. 1A).

PP13/galectin-13 possesses weak endogenous LPLA activity

For both PP13-B and PP13-R, the highest degree of transformation was found for L-α-lysophosphatidylcholine (1-acyl-glycero-3-phosphorylcholine, LPC); other lysophospholipids showed at most 5% (molar) transformation during the same period (data not shown). In the course of LPC transformation, four species could be distinguished and quantified in the ^{31}P-NMR spectra (Fig. 2A), and their relative concentrations showed similar time-dependence (Fig. 2B); however, the reaction rates varied in the three solutions (PP13-B, PP13-R and control) (see below). The peak at 0.72 p.p.m. could be assigned to the starting material, which was involved in an isomerization equilibrium with 2-acyl-glycero-3-phosphorylcholine (iLPC) ($\delta = 0.56$ p.p.m) [30], independent from the presence of PP13 proteins. In a slower reaction, LPC was transformed into two other species, one appearing at 1.00 p.p.m., and the other at 0.82 p.p.m. The former signal could be assigned to glycero-3-phosphorylcholine (GPC) based on its chemical shift [3,30–32]. The relative concentrations of the three major species ($\delta = 1.00$, 0.82 and 0.72, respectively), expressed in molar fractions, are shown in Fig. 2B. The relative concentration of iLPC fluctuated between 0.10 and the limit of quantitation over the whole course of the reactions, roughly following the change of LPC (data not shown). The kinetics of the transformation of LPC could not be exactly described by classical models. However, the reaction appeared to move toward equilibrium, as judged by the time-dependence of the relative concentrations of the major species. The species appearing at 0.82 p.p.m. might well be an intermediate in the transformation, as its molar fraction increased in the first period and decreased after reaching a maximum value. Attempts are underway to identify this presumed intermediate. Determination of the enzymatic activities was difficult because the concentrations of both the intermediates and the products remained under the limit of quantitation for several tens of hours. However, a rough estimate could be made by the first spectra showing GPC in quantifiable concentrations: PP13-B showed 4.8 mol% transformation in 306 h, PP13-R gave 4.5 mol% in 210 h whereas control samples showed 1.1 mol% in 272 h. In terms of specific activity, these data read as 0.69, 0.94 and 0.18 nmol·min^{-1}·mg^{-1}, respectively, whereas approximately 1300 μmol·min^{-1}·mg^{-1} was found for human brain LPLA [33] and 2.5 μmol·min^{-1}·mg^{-1} for an LPLA isolated from human amnionic membrane [34]. Phospholipase (PLA) activity of PP13-B and PP13-R was tested analogously, using phospholipids as substrates. No change could be observed in ^{31}P-NMR spectra for any phospholipids, thus neither PP13-B nor PP13-R appeared to possess detectable PLA activity under these circumstances.

PP13/galectin-13 has strong sugar binding capabilities

Nonmodified agarose beads (Sephadex 2B) did not bind PP13-R at all, while all types of sugar-coupled agarose beads bound more than 95% of PP13-R after 1 h incubation. Different sugars (1 mM–1 M) eluted the protein from various sugar-coupled agarose in different manners, with the following elution capacity: *N*-acetyl-lactosamine > mannose > *N*-acetyl-galactosamine > maltose > glucose > galactose > fucose > lactose (Fig. 3A). In 1 M concentration, *N*-acetyl-lactosamine had significantly the highest efficacy (95–100%) to elute PP13-R from all kinds of beads, while mannose was less effective, having an elution capacity between 15 and 30%. On average, *N*-acetyl-galactosamine was the third most effective to specifically compete with PP13-R binding (12–19%). The elution capacities for other sugars were determined to be below 8% in the following order: maltose (0–8%), glucose (0–4%), galactose (0–7%), fucose (0–4%), lactose (0–2%). These latter sugars had higher elution efficacy only in some special combinations: maltose/fucose-agarose (21%) and maltose-agarose (42%); glucose/maltose-agarose (23%); lactose/lactose-agarose (7%) (Fig. 3A). In the presence of 1 mM dithiothreitol during the 1 h binding period, approximately half of PP13-R was bound to different sugar-coupled agaroses (e.g. lactose-agarose: 60%, glucose- and mannose-agarose: 55% each), and the elution of specifically bound PP13-R with different sugars was four-times more effective compared to nonreducing conditions. 100 mM mannose eluted 31–100% of PP13-R from glucose-, mannose- or lactose-agarose, while without the presence of dithiothreitol the elution was only 8–16% (Fig. 3B). The order of the elution capacity of the different sugars for PP13-R from the various sugar-coupled agaroses was the same in reducing and nonreducing conditions, but in the presence of dithiothreitol, sugar elution of specifically bound PP13-R from sugar-coupled agaroses was significantly higher. Mannose (100 mM) eluted all bound PP13-R from lactose-agarose, 50% from mannose-agarose and 43% from glucose-agarose.

PP13/galectin-13 possesses lectin activity

Lectin activity of PP13-B and PP13-R was confirmed by measurements of their agglutination capabilities of human erythrocytes. In nonreducing conditions, very small

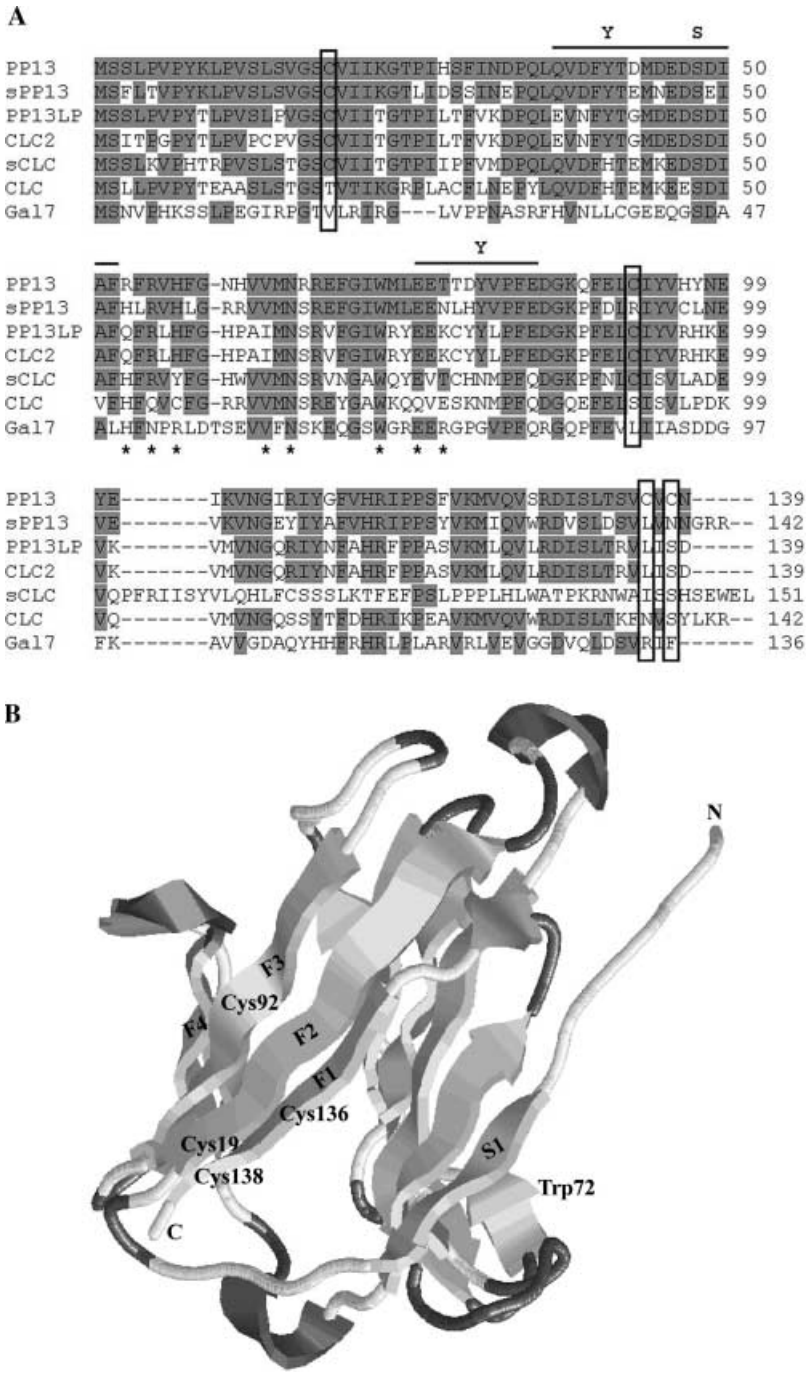

Fig. 1. Computational analyses of PP13/galectin-13. (A) Multiple sequence alignment between human PP13/galectin-13 and its homologues. Alignments were performed with CLUSTALW using amino acid sequences of the close homologues. The order of the protein list was based upon their homology to PP13/galectin-13. sPP13, similar to placental protein 13; PP13LP, placental protein 13-like protein; CLC2, Charcot–Leyden Crystal 2 protein; sCLC, unnamed protein, similar to CLC; CLC, Charcot–Leyden Crystal protein/galectin-10; Gal7, galectin-7. Identical residues to PP13/galectin-13 are shown with a grey background. Putative tyrosine and serine kinase phosphorylation sites on the surface of PP13/galectin-13 are shown above (Y, S), amino acid positions are shown next to the sequences. Cysteine positions in PP13/galectin-13 are boxed, and asterisks mark the highly conserved residues comprising the carbohydrate recognition domains in all galectins. Sequential differences at cysteine residues of PP13/galectin-13 compared to the homologues might explain its unique behaviour in dimerization. (B) Structural model of human PP13/galectin-13 visualized by RASMOL. The highly conserved Trp72 on beta-sheet S6a in the carbohydrate recognition moiety of PP13/galectin-13 was shown. The opposite surface of the monomer contains beta-sheets F1 (Cys136 and Cys138), F2 (Cys19) and F3 (Cys92) comprising the cysteines potentially involved in dimerization by cross-linking two subunits in a yet to be established manner. N- and C-termini of the molecule, as well as beta-sheets S1 and F1-F4 are indicated.

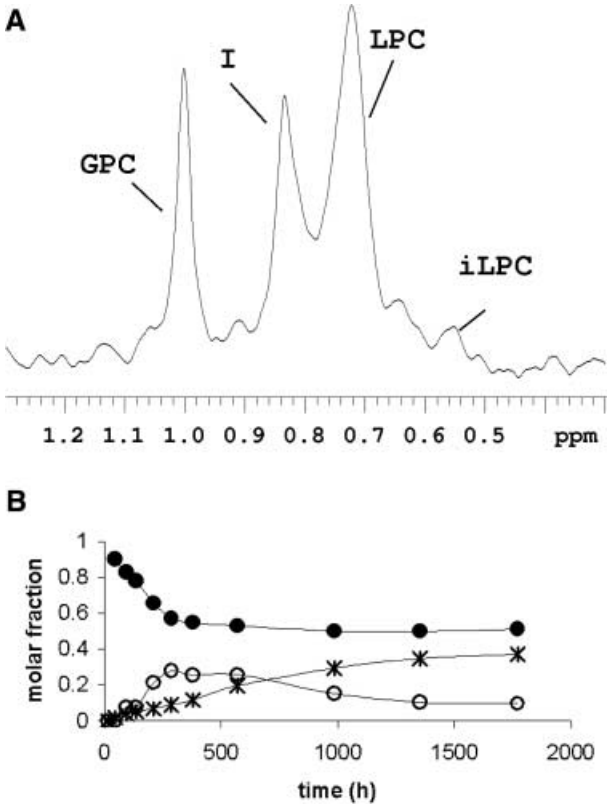

Fig. 2. Lysophospholipase activity of PP13-R determined by NMR spectroscopy. (A) A representative ^{31}P-NMR spectrum of the reaction mixture with starting composition of 40 μg·mL^{-1} PP13-R, 5 mg·mL^{-1} LPC, 200 mM Hepes, 5 mM $CaCl_2$ and 130 mM NaCl at pH 7.4. The peaks could be assigned as GPC (δ = 1.00 p.p.m.), the presumed intermediate (I) (δ = 0.82 p.p.m), LPC (δ = 0.72 p.p.m.) and iLPC (δ = 0.56 p.p.m.). (B) Time course of the relative concentrations of LPC (●), presumed intermediate (○) and GPC (*) in the presence of PP13-R.

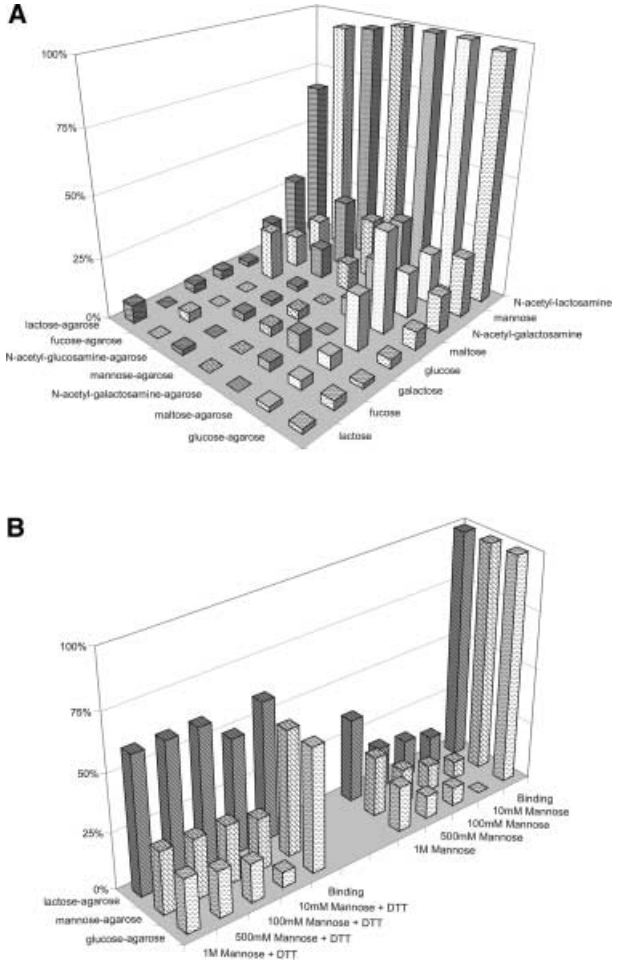

Fig. 3. Sugar binding experiments on PP13/galectin-13. (A) Elution of PP13-R from different sugar-coupled agarose beads by various sugars. Experiments were as detailed in Experimental procedures. The strength of PP13-R binding to different kinds of sugar-coupled agarose beads in the lack of reducing agent increased from lactose-agarose to glucose-agarose (left to right). Specifically bound PP13-R was competitively eluted by sugars (1 M) listed (back to front). The following elution capacity of various sugars was recognized: *N*-acetyl-lactosamine > mannose > *N*-acetyl-galactosamine > maltose > glucose > galactose > fucose > lactose. (B) Comparison of the elution of PP13-R from different sugar-coupled agarose beads by mannose in reducing and nonreducing conditions. Experiments were as detailed in Experimental procedures. In the presence of 1 mM dithiothreitol, approximately half of PP13-R bound to the different sugar-coupled agarose beads compared to the case without reducing agent (lactose-agarose: 60%, mannose-agarose: 55%, glucose-agarose: 55%). The elution of specifically bound PP13-R was four times as effective compared to cases without dithiothreitol. 100 mM mannose eluted 8, 11 and 16% of PP13-R from glucose-agarose, mannose-agarose and lactose-agarose, respectively, in a dithiothreitol (DTT)-free environment, while 31, 47 and 100% of PP13-R was eluted from the same sugar-coupled agarose beads in the presence of dithiothreitol. In reducing conditions, the difference between the affinities of all sugar-coupled agarose beads to PP13-R binding was not altered (data not shown).

amounts of both PP13-B and PP13-R induced haemagglutination, and strong agglutination was detected at and above 50 μg·mL^{-1} applied protein concentrations (Fig. 4), which was very similar to the phenomenon seen in cases of other galectins [35]. The pattern and effectiveness of both PP13-B and PP13-R were identical in agglutination of erythrocytes. However, no haemagglutination occurred in reducing conditions with the addition of 1 mM dithiothreitol to the mixture. Different sugars also had an inhibitory effect on haemagglutination capabilities of PP13-R. At and above concentrations of ≈1 mM *N*-acetyl-lactosamine and mannose, previously found to be the best ligands of PP13-R, abolished its haemagglutination activity (Fig. 4).

PP13/galectin-13 dimerizes via disulphide bonds

Galectins were known to be dimerized by noncovalent interactions [6,9]. From earlier data [2], as well as in our experiments, PP13/galectin-13 was found to be composed of two identical subunits held together by disulphide bonds. In nonreducing conditions, dimerization occurred at and above 0.21 mg·mL^{-1} PP13-R concentrations (Fig. 5A). When PP13-R was dissolved in Laemmli solution containing 10% (v/v) 2-mercaptoethanol, no dimerization of

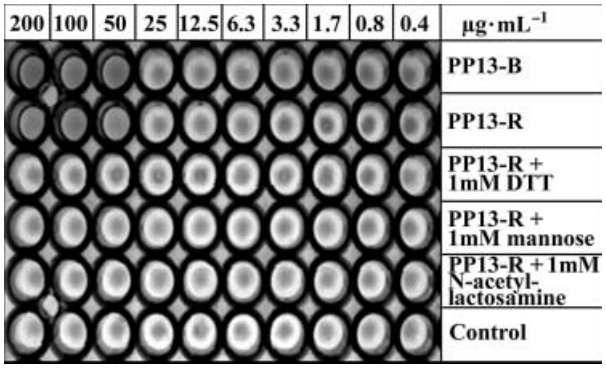

Fig. 4. Lectin activity of PP13/galectin-13 determined by haemagglutination assay. Agglutination assays were performed in a 96-well microtiter plate with serial twofold dilutions of PP13-B and PP13-R. PP13 proteins were diluted in 50 μL NaCl/P_i, then 50 μL of 2% (v/v) suspension of human erythrocytes was added to the samples and incubated at room temperature for 1 h. The top row contained PP13-B, while others contained PP13-R. Control wells contained no protein. In the case of both PP13-B and PP13-R in nonreducing conditions, strong haemagglutination could be seen at and above 50 $\mu g \cdot mL^{-1}$ final protein concentration. The agglutination capability of PP13-R was inhibited by dithiothreitol or different sugars at and above 1 mM concentrations.

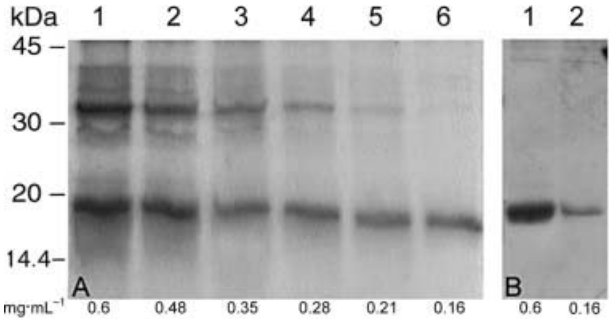

Fig. 5. Dimerization of PP13/galectin-13 in reducing and nonreducing conditions. Dimerization assays were performed by 12% (w/v) SDS/PAGE and Coomassie staining with different dilutions of PP13-R (0.16–0.6 $mg \cdot mL^{-1}$). (A) In nonreducing conditions, dimerization occurred at and above 0.21 $mg \cdot mL^{-1}$ PP13-R concentrations. At 18 kDa, PP13-R expressed with His_6-tag, while at 36 kDa, dimer of PP13-R could be seen. (B) In reducing conditions, where Laemmli solution contained 10% (v/v) 2-mercaptoethanol, no dimerization of PP13-R was visible at all, even at higher protein concentrations.

PP13-R was found at all, even at higher protein concentrations (Fig. 5B).

Placental expressed PP13/galectin-13 is phosphorylated

Pro-Q Diamond phosphoprotein gel stain specific for phosphorylated protein side chains was used to detect previously predicted putative phosphorylation of PP13/galectin-13. Both placental purified and bacterially expressed PP13 was examined along with ovalbumin (positive control) and BSA (negative control). A strong signal of phosphorylated groups in the lane of ovalbumin

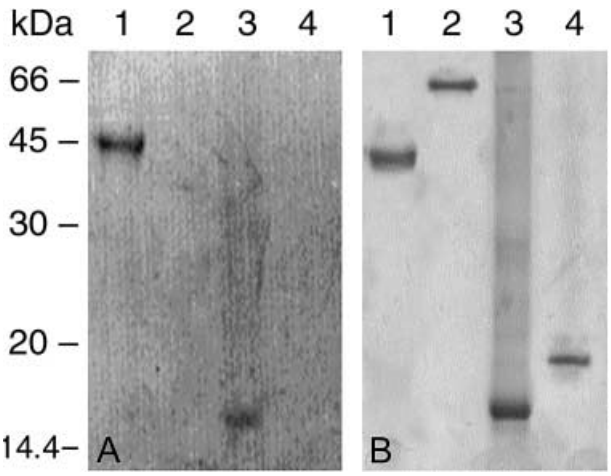

Fig. 6. Phosphorylation of PP13-B and PP13-R visualized by Pro-Q Diamond phosphoprotein and Coomassie gel stain. (A) Samples of ovalbumin (lane 1), albumin (lane 2), PP13-B (lane 3) and PP13-R (lane 4) were run on 12% (w/v) SDS/PAGE, then the gel was stained to visualize phosphoproteins and photographed. Signals of phosphorylated groups only in the lanes of the positive control ovalbumin and PP13-B purified from placenta could be specifically detected. No signal in the lanes of the negative control albumin and bacterially expressed PP13-R was found. (B) Subsequently, the same gel was stained by Coomassie staining to show total protein content.

and a weak signal in the lane of PP13-B purified from placenta could be specifically detected. No signal in the lanes of albumin and bacterially expressed PP13-R was found (Fig. 6A). An equal amount of protein content for each lane was verified by Coomassie staining (Fig. 6B).

PP13/galectin-13 binds annexin II and beta/gamma actin

By Coomassie staining after SDS/PAGE, in cases of PP13-B and PP13-R, major bands at 16 or 18 kDa were detected. No additional bands in lower or higher molecular mass regions could be identified, indicating high purity of both protein preparations. Bands were cut from the gels, then MALDI-TOF MS peptide mapping with MALDI-PSD MS sequencing was performed, recognizing both PP13-B and PP13-R as PP13/galectin-13. Next, human term placental tissue and fetal hepatic cell (WRL-68) extracts were bound either to PP13-B or PP13-R coupled to Sepharose 4B, or to Sepharose 4B alone. Again, using Coomassie staining, the same major protein bands at 16 kDa (in the case of PP13-B), at 18 kDa (in the case of PP13-R), or at 38 and 41 kDa (in cases of both PP13-B and PP13-R) could be detected either in placental or in fetal hepatic protein extracts bound to either PP13-B (data not shown) or to PP13-R (Fig. 7, lanes 1–2), while Sepharose 4B did not specifically bind any proteins at all (Fig. 7, lanes 3–4). By MALDI-TOF MS peptide mapping and MALDI-PSD MS sequencing, all protein bands yielded good quality peptide maps, and most of the input masses matched the candidate protein sequences. The eluted 16 or 18 kDa proteins were identified as PP13-B or PP13-R subunits dimerized with PP13-B or PP13-R subunits coupled to Sepharose 4B. MALDI-TOF MS data of the 38 kDa

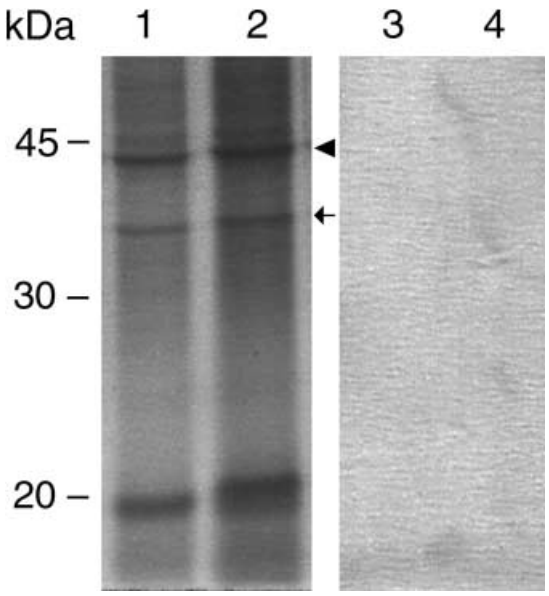

Fig. 7. Identification of PP13-R and its specific intracellular ligands separated by affinity purification, Coomassie staining and MS. Total protein extracts from placenta or human fetal hepatic cell line were incubated with either PP13-R coupled to Sepharose 4B or Sepharose 4B alone. Specifically bound proteins were eluted from columns with Laemmli solution containing 10% (v/v) 2-mercaptoethanol, then 12% (w/v) SDS/PAGE were performed. After excision from the gels, proteins were identified by MALDI-TOF MS peptide mapping and MALDI-PSD MS sequencing. Strongly bound proteins from placenta (lane 1) and human fetal hepatic cell line (lane 2) were detected at 38 and 41 kDa, while Sepharose 4B did not specifically bind any proteins from either placenta (lane 3) or fetal hepatic cells (lane 4). Annexin II (arrow) and beta/gamma actin (arrowhead) could be identified in both lanes 1 and 2. The 18 kDa band was identified as the eluted His-tag expressed PP13-R subunit dimerized with the PP13-R subunit coupled to Sepharose 4B.

protein in both cases permitted the identification of human annexin II (Accession No. NM_004039) (Table 1A), while the mass map of the 41 kDa protein matched beta/gamma actin (Table 1B) in both cases (Accession No. NM_001101 and NM_001614). PSD data obtained for precursors also confirmed the identity of these proteins.

Polyclonal and monoclonal antibodies to PP13 have specific recognition to PP13/galectin-13

To investigate and compare the specificity of polyclonal and newly developed monoclonal antibodies to PP13, Western blot testing was performed utilizing PP13-B, PP13-R proteins and human placental tissue extracts. As previously shown, polyclonal antibody to PP13 bound specifically to PP13-B extracted from human term placenta and also reacted with the same size protein in some fetal tissues such as liver and spleen [3]. Here it was observed that polyclonal antibody to PP13 could recognize PP13-R in a similar pattern as purified PP13-B and placental expressed PP13/galectin-13, with no other proteins recognized (Fig. 8A). From the newly developed monoclonal antibodies to PP13, clone 215 developed against a PP13/galectin-13 specific epitope had the strongest reaction with PP13-B and PP13-R, and also recognized the placental expressed PP13 with no cross-reaction to other proteins of the placenta (Fig. 8B).

PP13/galectin-13 is localized predominantly on the brush border membrane of placental syncytiotrophoblasts

In human term placental tissue, special localization of PP13/galectin-13 was found by different immunological techniques. Monoclonal antibody to PP13 gave a significantly weaker staining on immunohistochemical sections, while it had stronger staining with confocal imaging than polyclonal antibody to PP13. With both antibodies, labelling mainly on the brush border membrane of the syncytiotrophoblasts could be seen by immunohistochemistry, with a parallel weak staining of the cells (Fig. 9A,B). By the more sensitive immunofluorescence confocal imaging, a similar, but more intense PP13 staining of the brush border membrane was detected, also with a discrete perinuclear labelling of the syncytiotrophoblasts by both monoclonal and polyclonal antibodies (Fig. 9C,D). Parallel annexin II staining of the syncytiotrophoblasts as well as intense staining on the brush border membrane could be seen (Fig. 9E).

Discussion

Although PP13 was first isolated and cloned from human term placenta [2,3], its expression in human fetal liver and spleen tissues has also been detected [3]. As PP13 showed conserved sequential, structural and computed functional homology to members of the growing β-galactoside-binding galectin family [6], it was designated as galectin-13 [7]. In this study it was verified that PP13/galectin-13 mRNA and related ESTs were predominantly expressed in placenta, but also in fetal liver and spleen tissues. The PP13/galectin-13 gene mapped to the close vicinity of genes of four known and three putative galectins [8,9,27–29] with similar exon structures and surrounding untranslated regions in a tight cluster on chromosome 19. The encoded proteins also proved to share 80% of the highly conserved galectin residues, which suggested a gene multiplication event in this galectin subfamily. In contrast to the evolutionarily ancient galectins expressed in many tissues, this subfamily comprising PP13/galectin-13 appeared to have already developed in nonprimates but expanded in primates, as members are predominantly expressed in specific tissues, with many of them abundant only in placenta. Not only this fact but also their specific transcriptional regulation underlined the differential placental expression of these genes, as numerous placenta-specific transcriptional factor binding sites were found in the promoter regions [9]. An analogous gene duplication event on chromosome 11 occurred in the case of eosinophil major basic proteins, of which human major basic protein-2 is present only in eosinophils, while human major basic protein-1 is abundant in placenta, and both are involved in immune functions [9,36]. Similarly, genes of mannose-specific C-type lectins, *DC-SIGN* and *DC-SIGNR*, and their homologue CD23 (*FcERII*) were described to be evolutionary duplicated on chromosome 19p13.3. Their concomitant expression was shown in placenta and dendritic cells with specific immunobiological

Table 1. Assignments of proteolytic fragments from tryptic digests of PP13/galectin-13 affinity purified 38 and 41 kDa proteins. Protein identification and sequencing were described in Experimental procedures. Most of the input masses matched the candidate protein sequences. MALDI-TOF and MALDI-PSD MS data identified the 38 kDa protein as annexin II and the 41 kDa protein as beta/gamma actin.

Measured mass (MH^+)	Calculated mass (MH^+)	Delta (p.p.m.)	Modifications	Fragment	Missed cleavages	Database sequence
Annexin II						
1035.6177	1035.5297	85	–	213–220	0	(K) WISIMTER (S)
1086.5769	1086.4856	84	–	29–37	0	(K) AYTNFDAER (D)
1086.5769	1086.6821	−97	–	287–295	1	(K) VLIRIMVSR (S)
1094.5963	1094.5271	63	pyroGlu	69–77	0	(R) QDIAFAYQR (R)
1111.6201	1111.5536	60	–	69–77	0	(R) QDIAFAYQR (R)
1244.6868	1244.6235	51	–	136–145	0	(R) TNQELQEINR (V)
1439.8798	1439.7238	108	2Met-ox	291–302	1	(R) IMVSRSEVDMLK (I)
1460.7615	1460.6732	60	–	234–245	0	(K) SYSPYDMLESIR (K)
1542.9514	1542.8491	66	–	50–63	0	(K) GVDEVTIVNILTNR (S)
1588.8890	1588.7681	76	–	234–246	1	(K) SYSPYDMLESIRK (E)
1778.0156	1777.8642	85	–	120–135	0	(K) GLGTDEDSLIEIICSR (T)
1909.0682	1908.8827	97	–	180–196	0	(R) AEDGSVIDYELIDQDAR (D)
2065.2063	2064.9838	108	–	179–196	1	(R) RAEDGSVIDYELIDQDAR (D)
Beta/gamma actin						
1198.7517	1198.5228	191	–	44–54	0	(K) DSYVGDEAQSK (R)
1198.7517	1198.7061	38	–	22–32	0	(R) AVFPSIVGRPR (H)
1203.6632	1203.5614	85	2Met-ox	33–43	0	(R) HQGVMVGMGQK (D)
1499.7963	1499.6767	80	pyroGlu	353–365	0	(K) QEYDESGPSIVHR (K)
1515.8512	1515.7497	67	–	78–88	0	(K) IWHHTFYNELR (V)
1628.0516	1627.7716	172	pyroGlu	353–366	1	(K) QEYDESGPSIVHRK (C)
1791.0558	1790.8925	91	–	232–247	0	(K) SYELPDGQVITIGNER (F)
1954.2281	1954.0650	83	–	89–106	0	(R) VAPEEHPVLLTEAPLNPK (A)
2215.3066	2215.0705	107	–	285–305	0	(K) DLYANTVLSGGTTMYPGIADR (M)
2807.5914	2807.3119	100	–	207–231	1	(K) EKLCYVALDFEQEMATAASSSSLEK (S)

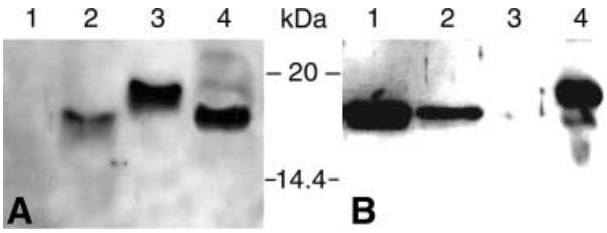

Fig. 8. Identification of purified, recombinant and placenta expressed PP13/galectin-13 by Western blotting with polyclonal and monoclonal antibodies to PP13. (A) Human term placental tissue extract (20 μg, lane 2), PP13-R (10 ng, lane 3), PP13-B (10 ng , lane 4), or (B) PP13-B (50 ng , lane 1), term placental tissue extract (30 μg, lane 3) and PP13-R (50 ng , lane 4) were run on 15% (w/v) SDS/PAGE. Lanes 1 (A) and 3 (B) represent empty lanes containing no proteins. After Western blotting using either polyclonal (A) or monoclonal (B) antibodies to PP13 and horseradish peroxidase labeled secondary IgGs, protein bands were revealed with ECL chemiluminescence system. The positions of molecular mass markers are displayed in the middle.

functions [37,38]. As several other galectins are also involved in inflammation and immune defences [9], our findings suggest that the newly evolved and differentially expressed PP13/galectin-13 with its homologues might have special immune functions at the fetomaternal interface. In the near future this phenomenon must be analyzed in the light of previous clinical data on PP13 serum levels in different disorders of pregnancy [11].

Because of the highly conserved homology with several other galectins, it was likely that PP13/galectin-13 exhibited sugar binding activity. Indeed, in our previous report based on homology modelling [7], the possible functional and structural characteristics of PP13/galectin-13 were predicted, including a CRD which resembled the β-galactoside-binding site of galectins. In this study, binding experiments showed that PP13/galectin-13 was effectively bound to different sugar containing agarose gels, and that various sugars could compete this effect with different affinities to the PP13/galectin-13 binding site. As in the case of most galectins when similar sugar concentrations were applied, *N*-acetyl-lactosamine had the highest affinity to its CRD. Similarly to CLC protein/galectin-10 but not other previously analyzed galectins, PP13/galectin-13 also had high affinity to mannose, which could be understood in terms of the similarities in their CRDs [7,9].

N-acetyl-galactosamine also had a certain affinity to PP13/galectin-13 CRD, in contrast to other sugar derivates, which only slightly displaced the protein from sugar-coupled agaroses. Interestingly, homology modelling data had also indicated that *N*-acetyl-lactosamine would bind the most effectively to the PP13/galectin-13 binding site, and in the case of other sugars, there were only minor discrepancies between the previously suggested and experimentally observed binding affinities [7]. Strong lectin activity of PP13-B and PP13-R was also proven by their haemagglutination activity and by haemagglutination inhibition assays, where excess sugar molecules competed with red

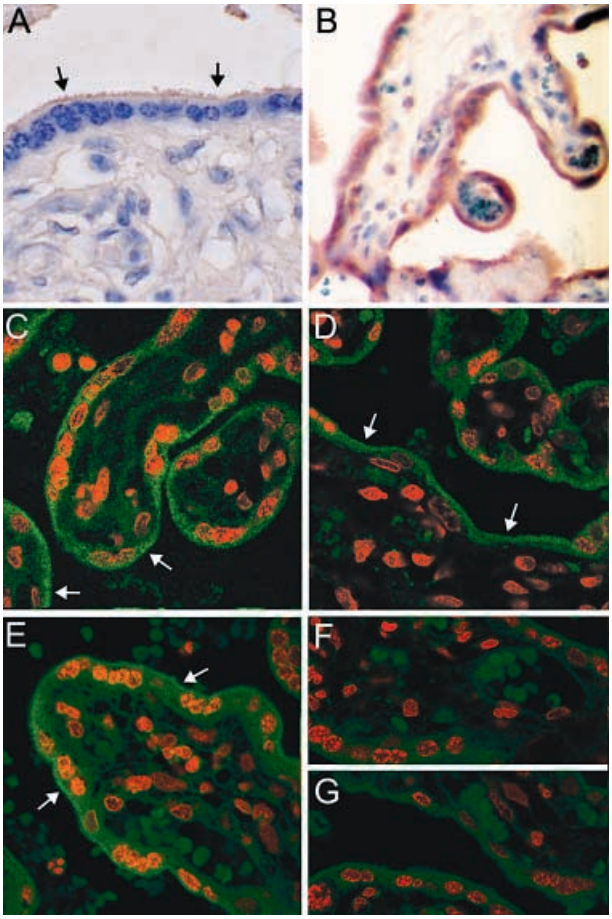

Fig. 9. Localization of PP13/galectin-13 in human normal term placental tissue. Formalin-fixed, paraffin embedded tissue sections of human term placenta were stained for immunohistochemistry or immunofluorescence confocal microscopy, respectively, using both (A,C) monoclonal (clone 215) or (B,D) polyclonal antibodies to PP13. (A,B) Immunohistochemical sections were counterstained with haematoxylin (300× magnification). A moderate PP13 expression could be seen in the brush border membrane of the syncytiotrophoblasts (arrows) by monoclonal (A) and polyclonal (B) antibodies to PP13 with an additional weak staining of the cells. (C–G) Confocal images were counterstained by DRAQ5 nucleus dye (red) (750× magnification). (C,D) Intense PP13 staining (green) could be recognized in the brush border membrane of the syncytiotrophoblasts (arrows) and also a discrete perinuclear labelling of the syncytiotrophoblasts with both the monoclonal (C) and polyclonal (D) antibodies to PP13. (E) Annexin II staining (green) could be seen in the syncytiotrophoblasts and more intense staining could be found in the brush border membrane (arrows). (F,G) In control sections stained only with anti-mouse (F) or anti-rabbit (G) secondary IgGs, no staining of the syncytiotrophoblasts or the brush border membrane could be seen.

blood cell sugar residues and proved to be more likely to bind to PP13 proteins leaving red blood cells to sediment. These experimental data and the specific and predominant localization of PP13/galectin-13 on the brush border membrane of syncytiotrophoblasts were in close agreement with a systematic study on the structure and distribution of specific glycans in human placenta, which showed that residues containing *N*-acetyl-lactosamine, mannose and *N*-acetyl-glucosamine were widely expressed on villous surfaces [39]. This may provide an explanation of the binding specificity of PP13/galectin-13, and suggests a similar binding pattern of its newly described, mainly placental-expressed homologues.

In vitro, PP13/galectin-13 dimerization occurred at and above 0.21 $mg{\cdot}mL^{-1}$ concentrations in nonreducing conditions, while in the presence of dithiothreitol, no dimerization was detected at all. Furthermore, in reducing conditions, approximately half of PP13-R was bound to sugar-coupled agaroses, and the protein's haemagglutination activity was also abolished, all of which could be explained by the loss of dimerization. Cystein residues in galectins (formerly known as 'S-type' or 'thiol-dependent' lectins) were considered to be important, because some but not all galectins, such as galectin-1 and galectin-2, might lose their sugar binding activity under nonreducing conditions [40,41]. Compared to the structure of CLC protein/galectin-10, which is known to be a monomer [42], four additional cysteine residues were found in PP13/galectin-13, which might localize on a putative dimerization surface. Interestingly, Cys136 and Cys138 on beta-sheet F1 in PP13/galectin-13 were found to be missing from all homologues, whereas Cys19 and Cys92 on beta-sheets F2-F3 were missing from homologues including CLC/galectin-10, only the newly described closest relatives contained them. This data may vindicate the surprising experimental findings on its dimerization via disulphide bonds, a phenomenon yet not described for other galectins. Superposing the 3D model of PP13/galectin-13 monomer on well known models of galectin dimers [43–45] with RASMOL revealed, that if PP13/galectin-13 dimerizes such as galectin-1 and galectin-2 in a two fold rotation perpendicular to the beta-sheets, then only beta-sheets S1 and F1 containing Cys136 might participate in the dimerization. If PP13/galectin-13 dimerizes more like to galectin-7, then beta-sheets F1-F5 might comprise the putative dimerization interface containing Cys19, Cys92 and Cys136, as well. As our data is still not enough to describe an exact dimerization interface, X-ray crystallographic experiments have been started to reveal the proper dimeric structure and carbohydrate binding of the protein.

Our data also showed that dithiothreitol equally decreased the binding strengths between PP13-R and different sugars, but no change occurred in the order of their binding capabilities. As the CRD of PP13/galectin-13 was situated just opposite the cysteine-rich region, it is understandable that the monomeric form could not cross-link red blood cells, but the mechanism of how the reducing agent could decrease the sugar binding potential of the protein remains to be elucidated. Our sequence alignment also suggested that the new subfamily members might also dimerize via disulphide bonds, which should be critically analyzed later. As these proteins were primarily expressed in the low blood flow organ placenta, special alterations in the oxygenation could easily affect their biological functions through their dimerization status and sugar binding affinities.

It is important to mention that computations localized putative serine and tyrosine kinase phosphorylation sites on the outer surface of PP13/galectin-13 at positions 44–52 (Ser48), 37–45 (Tyr41) and 76–84 (Tyr80), in close vicinity to its CRD, similarly to placental protein 13-like protein [8].

Experimental data showed that *in vivo* placental expressed and purified PP13-B was phosphorylated, while the *in vitro* bacterial expressed PP13-R was not. Knowing that galectin-3 was reported to be phosphorylated at Ser6, and phosphorylation modulated its carbohydrate affinity and biological functions as an 'on/off' switch [46,47], our preliminary data raised the possibility of phosphorylation having an influence on PP13/galectin-13 functional properties. In our conditions, carbohydrate binding affinities were similar both in cases of PP13-B and PP13-R; the only difference was detected in their lysopholypase activity. Further detailed experiments must be performed to establish the importance and the exact mechanism of this phenomenon.

Formerly it was found that PP13/galectin-13 had a weak lysophospholipase activity [3], which had previously been observed in the case of CLC protein/galectin-10 [5]. However, it was shown later that this enzymatic activity might not be derived from CLC protein/galectin-10, but from another protein associated with it [9]. Although PP13-B purified from human term placenta did not contain a level of impurity comparable to that which the CLC protein/galectin-10 preparation probably had, we overexpressed the cloned and His-tagged PP13-R in *E. coli*, and purified it on a Ni-NTA column. Next, the enzymatic activity of PP13-B and PP13-R were compared. ^{31}P-NMR analysis showed that both PP13-B and PP13-R had a very weak endogenous LPLA activity, even PP13-R showed higher catalytic activity than the purified one, indicating that PP13/galectin-13 itself possesses LPLA activity. PLA activity of neither the purified nor the recombinant PP13/galectin-13 could be confirmed under these circumstances. The exact LPLA catalytic centre of PP13/galectin-13 must to be further analyzed.

Although the possibility that the weak LPLA activity of PP13-B coming from an associated and copurified protein could be excluded, it still remained an interesting question as to which intracellular proteins were interacting PP13/galectin-13. By immobilizing PP13-B and PP13-R, the proteins extracted from term placental tissue and fetal hepatic cells and specifically bound to PP13 proteins were determined. By means of MALDI-TOF MS peptide mapping and sequencing, the 38 kDa and 41 kDa proteins, which bound both to PP13-B and PP13-R, were identified as human annexin II and beta/gamma actin, respectively. The 16 or 18 kDa proteins were identified as the eluted PP13-B or PP13-R subunits.

It is well known that cells differ widely in their capacity to produce and secrete galectins, and galectin secretion is also responsive to developmental events. During the complex mechanisms of human placentation, correlating with the differentiation pathways of the trophoblasts, changes in the distribution patterns of galectin-1 and galectin-3, homologues of PP13/galectin-13, were already seen [48]. Studies also revealed that actin filaments might play an important role in translocation of lectins during differentiation processes [49], and could also be involved in focal concentration of cytosolic galectin at specific cytoskeletal regions, for example at evaginating plasma membrane domains during ectocytosis [50]. The exact mechanisms of how PP13/galectin-13 is transported to the outer surface of the syntitiotrophoblasts' plasma membrane have not yet been studied, but it is assumed to be released through an alternative, nonclassical route, similarly to other galectins [50]. Fibroblasts were previously shown to secrete galectins by ectocytosis in microvesicles, which also contained actin (derived from disassembling microfilaments) and annexin II in high amounts [50]. As PP13/galectin-13 was bound to these molecules very specifically and colocalized with annexin II on the brush border membrane, PP13/galectin-13 potentially utilizes this ectocytotic pathway for externalization, in the same way as the secretion of galectin-3 [51]. *In vivo*, microvesicles were found to be labile and disrupted spontaneously, releasing their lectin cargo very rapidly. Although PLAs were considered to be candidates for catalyzing the hydrolysis of the sn-2 fatty acyl bond of phospholipids to liberate free fatty acids and lysophospholipids, and to release soluble extracellular galectins, to date no particular PLA has been found to carry out this exact function [52]. PP13/galectin-13 possesses a relatively weak but definitive LPLA activity, which is probably insufficient for catabolizing significant amounts of phospholipids, but which may contribute to its penetration through the vesicle membrane.

Annexin II, a member of a Ca^{2+} and phospholipid binding protein family, is present as a heterotetramer on the apical extracellular surface of the syncytiotrophoblasts [53], colocalized with PP13/galectin-13. It is thought to play a role in the differentiation of the placenta and in the functions of the mature microvilli [54]. It was recognized as a profibrinolytic coreceptor for tissue plasminogen activator and plasminogen on endothelial cells, and was shown to stimulate the tissue plasminogen activator-dependent conversion of plasminogen to plasmin [55,56]. In addition, annexin II was also found to promote plasmin inactivation, regulate ion channels, PLA2 and prothrombin activation [57]. Therefore, its interaction with PP13/galectin-13 may play an important role in placental haemostatic processes.

In conclusion, PP13/galectin-13 was localized mostly in the brush border membrane of the syncytiotrophoblasts' lining at the common feto-maternal blood spaces of the placenta. As a 'prototype' galectin, it has a single sugar binding domain, which emerges into the extracellular space. Sugar binding assays proved that PP13/galectin-13 had high affinity to sugar residues widely expressed in placenta, and bound to surface antigens of red blood cells. The protein dimerized via disulphide bonds, which unique feature might affect its biological activity upon the oxygenation changes in the low blood flow organ placenta. Phosphorylation of the protein could be another regulator for its special biological functions, the mechanism of which must also be elucidated. With regard to the fact that red blood cells do not show a remarkable rate of aggregation to syncytiotrophoblasts in the case of normal placental function, and plasma levels of PP13/galectin-13 did not correlate with the intensity of the placental synthesis of the protein, a transitory stay of PP13/galectin-13 in the cell membrane followed by the release from the brush border membrane of the syncytiotrophoblasts' is involved. It is not yet known whether the release is catalyzed by an autonomous PLA, or whether PP13/galectin-13 has its own ability to split the chemical bonds. As protein separation techniques did not identify any PLA bound to PP13/galectin-13, and measurements proved a weak LPLA activity of the protein itself, a hypothesis could

be set up that the low self-LPLA activity of the protein is enough to satisfy the demands on securing a slow, constant PP13/galectin-13 release, which is capable of preventing erythrocyte adhesion in the area with reduced blood flow. A considerable amount of detected PP13/galectin-13 also deserved attention in fetal liver and spleen, which are also known to be elemental haemopoietic organs with reduced blood flow. In contrast, as with other galectins involved in immunobiological functions, our data suggested that PP13/galectin-13 and its newly described, differentially expressed close homologues might have special immune functions at the feto-maternal interface. Involvement of PP13/galectin-13 and its homologues in developmental processes of the placenta is also hypothesized.

Acknowledgements

The authors are grateful to Dr Ilana Maor at Diagnostic Technologies Ltd. for her assistance in the collaboration. Special thanks to Dr Gyorgy Szekeres, Rita Keszthelyi and Zsuzsa Halas (Histopathology Ltd, Hungary) for their technical assistance in immunostaining, Erzsebet Osz (University of Pecs, Hungary) for her assistance in NMR measurements, Prof Nathan Sharon (The Weizmann Institute of Science, Israel) for his helpful discussions in methodology and Steve Starkey for critical reading of the manuscript. Zoltan Berente is grateful to the Hungarian Academy of Sciences for the Bolyai Janos Scholarship. Experiments were carried out at the facilities of the University of Pecs except PP13-R expression and the preparation of monoclonal antibody to PP13 (Diagnostic Technologies Ltd, Israel), immunhistology with monoclonal antibodies (Yale University, USA) and protein identification with MS (University of Szeged, Hungary). This work was supported by Hungarian Grants ETT T-09 163/01; FKFP 0166/2001; OMFB-BIO 00201/2002; and OTKA T/020622, T/023076, T/029824, M/36996. It was also funded in part by the Office of the Chief Scientist, Israel Ministry of Industry and Trade Grant #31851.

References

1. Than, G.N., Bohn, H. & Szabo, D.G. (1993) *Advances in Pregnancy-Related Protein Research*, 1st edn. pp. 1–333. CRC Press, Boston.
2. Bohn, H., Kraus, W. & Winckler, W. (1983) Purification and characterization of two new soluble placental tissue proteins (PP13 and PP17). *Oncodev. Biol. Med.* **4**, 343–350.
3. Than, N.G., Sümegi, B., Than, G.N., Berente, Z. & Bohn, H. (1999) Isolation and sequence analysis of a cDNA encoding human placental tissue protein 13 (PP13), a new lysophospholipase, homologue of the human eosinophil Charcot–Leyden crystal protein. *Placenta* **20**, 703–710.
4. Admon, A., Paltieli, Y., Slotky, R. & Mandel, S. (1999) Placental Protein P3 (PP13), Patent: IL (WO/99/38970)-A.
5. Ackerman, S.J., Corrette, S.E., Rosenberg, H.F., Bennett, J.C., Mastrianni, D.M., Nicholson-Weller, A., Weller, P.F., Chin, D.T. & Tenen, D.G. (1993) Molecular cloning and characterization of human eosinophil Charcot–Leyden crystal protein (lysophospholipase). Similarities to IgE binding proteins and the S-type animal lectin superfamily. *J. Immunol.* **150**, 456–468.
6. Barondes, S.H., Cooper, D.N.W., Gitt, M.A. & Leffler, H. (1994) Galectins. Structure and function of a large family of animal lectins. *J. Biol. Chem.* **269**, 20807–20810.
7. Visegrady, B., Than, N.G., Kilar, F., Sumegi, B., Than, G.N. & Bohn, H. (2001) Homology modelling and molecular dynamics studies of human placental tissue protein 13 (galectin-13). *Prot. Eng.* **14**, 875–880.
8. Yang, Q.S., Ying, K., Yuan, H.L., Chen, J.Z., Meng, X.F., Wang, Z., Xie, Y. & Mao, Y.M. (2002) Cloning and expression of a novel human galectin cDNA, predominantly expressed in placenta. *Biochim. Biophys. Acta* **1574**, 407–411.
9. Cooper, D.N.W. (2002) Galectinomics: finding themes in complexity. *Biochim. Biophys. Acta* **1572**, 209–231.
10. Ackerman, S.J., Liu, L., Kwatia, M.A., Savage, M.P., Leonidas, D.D., Swaminathan, G.J. & Acharya, K.R. (2002) Charcot–Leyden Crystal Protein (Galectin-10) is not a dual function galectin with lysophospholipase activity but binds a lysophospholipase inhibitor in a novel structural fashion. *J. Biol. Chem.* **277**, 14859–14868.
11. Silberman, M. (1993) Method for the detection of pregnancy disorders. United States Patent 5,198,366.
12. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.
13. Thompson, J.D., Higgins, J.D. & Gibbons, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680.
14. Falquet, L., Pagni, M., Bucher, P., Hulo, N., Sigrist, C.J., Hofmann, K. & Bairoch, A. (2002) The PROSITE database, its status in 2002. *Nucleic Acids Res.* **30**, 235–238.
15. Blom, N., Gammeltoft, S. & Brunak, S. (1999) Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.* **294**, 1351–1362.
16. Sayle, R.A. & Milner-White, E.J. (1995) RASMOL: biomolecular graphics for all. *Trends Biochem. Sci.* **20**, 374–376.
17. Frohman, M.A. (1990) *PCR Protocol: a Guide to Methods and Applications* In (Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J., eds), p28. Academic Press, San Diego.
18. Graham, A., Hopkins, B., Powell, S.J., Danks, P. & Briggs, I. (1991) Isolation and characterisation of the human lung NK-2 receptor gene using rapid amplification of cDNA ends. *Biochem. Biophys. Res. Commun.* **177**, 8–16.
19. Kozak, M. (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nuclelic Acids Res.* **15**, 8125–8148.
20. Rosenfeld, J., Capdevielle, J., Guillemot, J.C. & Ferrara, P. (1992) In-gel digestion of protein for internal sequence analysis after one- or two-dimensional gel-electrophoresis. *Anal. Biochem.* **203**, 173–179.
21. Eshhar, Z., Blatt, C., Bergman, Y. & Haimovich, J. (1979) Induction of secretion of IgM from cells of the B cell line 38C-13 by somatic cell hybridization. *J. Immunol.* **122**, 2430–2434.
22. Lee, R.T., Ichikawa, Y., Allen, H.J. & Lee, Y.C. (1990) Binding characteristics of galactoside-binding lectin (galaptin) from human spleen. *J. Biol. Chem.* **265**, 7864–7871.
23. Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850–858.
24. Clauser, K.R., Baker, P.R. & Burlingame, A.L. (1999) Role of accurate mass measurement (± 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal. Chem.* **71**, 2871–2882.
25. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
26. Bratthauer, G.L. & Adams, L.R. (1994) Immunohistochemistry: antigen detection in tissue. In *Advanced Laboratory Methods in Histology and Pathology* (Mikel, U.V., ed.), pp. 1–40. Armed Forces Institute of Pathology and American Registry of Pathology, Washington DC.

27. Madsen, P., Rasmussen, H.H., Flint, T., Gromov, P., Kruse, T.A., Honore, B., Vorum, H. & Celis, J.E. (1995) Cloning, expression, and chromosome mapping of human galectin-7. *J. Biol. Chem.* **270**, 5823–5829.
28. Mastrianni, D.M., Eddy, R.L., Rosenberg, H.F., Corrette, S.E., Shows, T.B., Tenen, D.G. & Ackerman, S.J. (1992) Localization of the human eosinophil Charcot–Leyden crystal protein (lysophospholipase) gene (CLC) to chromosome 19 and the human ribonuclease 2 (eosinophil-derived neurotoxin) and ribonuclease 3 (eosinophil cationic protein) genes (*RNS2* and *RNS3*) to chromosome 14. *Genomics* **13**, 240–242.
29. Rechreche, H., Mallo, G.V., Montalto, G., Dagorn, J.C. & Iovanna, J.L. (1997) Cloning and expression of the mRNA of human galectin-4, an S-type lectin down-regulated in colorectal cancer. *Eur. J. Biochem.* **248**, 225–230.
30. Plückthun, A. & Dennis, E.A. (1982) Acyl and phosphoryl migration in lysophospholipids: importance in phospholipid synthesis and phospholipase specificity. *Biochemistry* **21**, 1743–1750.
31. Selle, H., Chapman, B.E. & Kuchel, P.W. (1993) Glycerophosphocholine release in human erythrocytes. ^{1}H spin-echo and ^{31}P-NMR evidence for lysophospholipase. *Eur. J. Biochem.* **212**, 411–416.
32. Loo, R.W., Conde-Frieboes, K., Reynolds, L.J. & Dennis, E.A. (1997) Activation, inhibition and regiospecificity of the lysophospholipase activity of the 85-kDa group IV cytosolic phospholipase A_2. *J. Biol. Chem.* **272**, 19214–19219.
33. Wang, A., Yang, H.C., Friedman, P., Johnson, C.A. & Dennis, E.A. (1999) A specific human lysophospholipase: cDNA cloning, tissue distribution and kinetic characterization. *Biochim. Biophys. Acta* **1437**, 157–169.
34. Jarvis, A.A., Cain, C. & Dennis, E.A. (1984) Purification and characterization of a lysophospholipase from human amnionic membranes. *J. Biol. Chem.* **259**, 15188–15195.
35. Inagaki, Y., Sohma, Y., Horie, H., Nozawa, R. & Kadoya, T. (2000) Oxidized galectin-1 promotes axonal regeneration in peripheral nerves but does not possess lectin properties. *Eur. J. Biochem.* **267**, 2955–2964.
36. Plager, D.A., Adolphson, C.R. & Gleich, G.J. (2001) A novel human homolog of eosinophil major basic protein. *Immunol. Rev.* **179**, 192–202.
37. Soilleux, E.J., Barten, R. & Trowsdale, J. (2000) *DC-SIGN*; a related gene, *DC-SIGNR*; and *CD23* form a cluster on 19p13. *J. Immunol.* **165**, 2937–2942.
38. Soilleux, E.J. (2003) *DC-SIGN* (dendritic cell-specific ICAM-grabbing non-intergrin) and DC-SIGN-related (*DC-SIGNR*): friend or foe? *Clin. Sci.* **104**, 437–446.
39. Konska, G., Zamorska, L., Pituch-Noworolska, A., Szmaciarz, M. & Guillot, J. (2003) Application of fluorescein-labelled lectins with different glycan-binding specificities to the studies of cellular glycoconjugates in human full-term placenta. *Folia Histochem. Cytobiol.* **41**, 155–160.
40. Barondes, S.H. (1984) Soluble lectins: a new class of extracellular proteins. *Science* **223**, 1259–1264.
41. Hirabayashi, J. & Kasai, K. (1993) The family of metazoan metal-independent β-galactoside-binding lectins: structure, function and molecular evolution. *Glycobiology* **3**, 297–304.
42. Leonidas, D.D., Elbert, B.L., Zhou, Z., Leffler, H., Ackerman, S.J. & Acharya, K.R. (1995) Crystal structure of human Charcot–Leyden crystal protein, an eosinophil lysophospholipase, identifies it as a new member of the carbohydrate-binding family of galectins. *Structure* **3**, 1379–1393.
43. Liao, D.I., Kapadia, G., Ahmed, H., Vasta, G.R. & Herzberg, O. (1994) Structure of S-lectin, a developmentally regulated vertebrate β-galactoside-binding protein. *Proc. Natl Acad. Sci. USA* **91**, 1428–1432.
44. Lobsanov, Y.D., Gitt, M.A., Leffler, H., Barondes, S.H. & Rini, J.M. (1993) X-ray crystal structure of the human dimeric S-Lac lectin, L-14-II, in complex with lactose at 2.9-Å resolution. *J. Biol. Chem.* **268**, 27034–27038.
45. Leonidas, D.D., Vatzaki, E.H., Vorum, H., Celis, J.E., Madsen, P. & Acharya, K.R. (1998) Structural basis for the recognition of carbohydrates by human galectin-7. *Biochemistry* **37**, 13930–13940.
46. Mazurek, N., Conklin, J., Byrd, J.C., Raz, A. & Bresalier, R.S. (2000) Phosphorylation of the β-galactoside-binding protein galectin-3 modulates binding to its ligands. *J. Biol. Chem.* **275**, 36311–36315.
47. Yoshii, T., Fukumori, T., Honjo, Y., Inohara, H., Kim, H.-R.C. & Raz, A. (2002) Galectin-3 phosphorylation is required for its anti-apoptotic function and cell cycle arrest. *J. Biol. Chem.* **277**, 6852–6857.
48. Maquoi, E., van den Brule, F.A., Castronovo, V. & Foidart, J.M. (1997) Changes in the distribution pattern of galectin-1 and galectin-3 in human placenta correlates with the differentiation pathways of trophoblasts. *Placenta* **18**, 433–439.
49. Ozeki, Y., Yokota, Y., Kato, K.H., Titani, K. & Matsui, T. (1995) Developmental expression of D-galactoside-binding lectin in sea urchin (*Anthocidaris crassispina*) eggs. *Exp. Cell Res.* **216**, 318–324.
50. Joubert, R., Caron, M., Avellana-Adalid, V., Mornet, D. & Bladier, D. (1992) Human brain lectin: a soluble lectin that binds actin. *J. Neurochem.* **58**, 200–203.
51. Hughes, R.C. (1999) Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim. Biophys. Acta* **1473**, 172–185.
52. Mehul, B. & Hughes, R.C. (1997) Plasma membrane targetting, vesicular budding and release of galectin-3 from the cytoplasm of mammalian cells during secretion. *J. Cell Sci.* **110**, 1169–1178.
53. Aarli, A., Kristoffersen, E.K., Jensen, T.S., Ulvestad, E. & Matre, R. (1997) Suppressive effect on lymphoproliferation in vitro by soluble annexin II released from isolated placental membranes. *Am. J. Reprod. Immunol.* **38**, 313–319.
54. Kaczan-Bourgois, D., Salles, J.P., Hullin, F., Fauvel, J., Moisand, A., Duga-Neulat, I., Berrebi, A., Campistron, G. & Chap, H. (1996) Increased content of annexin II (p36) and p11 in human placenta brush-border membrane vesicles during syncytiotrophoblast maturation and differentiation. *Placenta* **17**, 669–676.
55. Hajjar, K.A. & Acharya, S.S. (2000) Annexin II and regulation of cell surface fibrinolysis. *Ann. N.Y. Acad. Sci.* **902**, 265–271.
56. Fitzpatrick, S.L., Kassam, G., Choi, K.S., Kang, H.M., Fogg, D.K. & Waisman, D.M. (2000) Regulation of plasmin activity by annexin II tetramer. *Biochemistry* **8**, 1021–1028.
57. MacLeod, T.J., Kwon, M., Filipenko, N.R. & Waisman, D.M. (2003) Phospholipid-associated annexin A2-S100A10 heterotetramer and its subunits: Characterization of the interaction with tissue plasminogen activator, plasminogen, and plasmin. *J. Biol. Chem.* **278**, 25577–25584.