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Mature rat testis contains a high molecular weight species of phosphatidylinositol transfer protein

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Immunoblot analysis of a rat testis cytosol fraction revealed two proteins which reacted with a polyclonal rabbit antibody to bovine phosphatidylinositol transfer protein. These two proteins were separated by anion exchange and molecular sieve column chromatographic procedures and shown to catalyze the transfer of phosphatidylinositol and phosphatidylcholine between populations of small unilamellar vesicles One protein was identified as the phosphatidylinositol transfer protein detectable in 16 other rat tissues and many eukaryotic species; the other phosphatidylinositol transfer protein was unique to testis. The molecular masses of the proteins, determined under denaturing electrophoretic conditions, were 35 and 41 kDa, respectively. When testis was examined in animals from birth to six weeks of age, the 35-kDa protein was present throughout, while the 41-kDa protein first appeared during week 4 and increased to adult levels by week 6, a small yet significant increase in tissue phosphatidylinositol transfer activity accompanied this expression of the testis-specific protein Selective destruction of Leydig cells by ethylene dimethanesulfonate did not cause any detectable loss of the 41-kDa phosphatidylinositol transfer protein The structural and catalytic relationships between the two testicular phosphatidylinositol transfer protein species remain to be elucidated

Introduction

The movement of phospholipid molecules between membranes in eukaryotic cells is accomplished, in part, by cytosolic proteins [1] For a wide variety of animal and plant species and cells, a number of phospholipid transfer proteins have been described [2–4], including proteins specific for the transfer of PtdCho (phosphatidylcholine), proteins which recognize and transport PtdCho and PtdIns (phosphatidylinositol), and several nonspecific lipid transfer proteins We have recently described the purification and presented some of the structural and catalytic properties of PtdIns transfer protein from rat [5] Prior to our efforts to purify this protein from brain, we undertook an extensive survey of tissues to identify and quantitative PtdIns transfer activity and protein Using a polyclonal antibody against bovine PtdIns transfer protein and the technique of immunoblotting following electrophoresis in polyacrylamide gels in the presence of sodium dodecylsulfate and 2-mercaptoethanol, we observed a common immunoreactive protein (35 kDa) in all rat tissues examined We also noted an additional immunoreactive (41 kDa) unique to testis, in 16 other male and female tissues we could not detect this high molecular weight protein We report here further characterization of the testis-specific PtdIns transfer protein, including its distribution in the male urogenital system, some molecular and catalytic properties, and developmental expression

Materials and Methods

Tissues and reagents

Pregnant female and adult male rats of the Sprague-Dawley strain were obtained from Sasco (Omaha, NE) and housed locally until use Larger quantities of rat testes were purchased from Pel-Freez (Rogers, AR) and stored at −75 °C *myo*-[2-^{3}H]Inositol (555 GBq mmol^{-1}) and [9,10-^{3}H]oleic acid (178 GBq · mmol^{-1}) were purchased from Amersham (Arlington Heights, IL), cholesteryl[1-^{14}C]oleate (2 1 GBq mmol^{-1}) was purchased from New England Nuclear (Boston, MA) Bovine plasma albumin, goat anti-rabbit antibody-al-

Abbreviations Hepes *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, LacCer, lactosylceramide, PMSF, phenylmethylsulfonyl fluoride PtdCho phosphatidylcholine, PtdIns phosphatidylinositol, PtdOH, phosphatidate

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kaline phosphatase conjugate, PMSF and pepstatin A were purchased from Sigma Chemical Co (St Louis MO) Egg PtdOH was supplied by Avanti (Birmingham, AL), and LacCer was purchased from Sigma Whatman DE-52 cellulose was obtained from Whatman LabSales (Clifton, NJ), Sephadex G-100, 40–120 μm, was purchased from Pharmacia (Piscataway, NJ), Aquacide III was a product of Calbiochem-Behring (La Jolla, CA), and nitrocellulose membranes (grade BA80) were obtained from Schleicher and Schull (Keene, NH) Ethylene dimethanesulfonate was synthesized from ethylene glycol and methanesulfonyl chloride and characterized according to Jackson and Jackson [6] A testosterone radioimmunoassay kit was generously supplied by Diagnostic Products Corporation (Los Angeles, CA) All other reagents were the highest quality available

Preparation of cytosolic fractions

Tissue samples were obtained by rapid dissection of CO_2-asphyxiated animals and placed immediately on ice. Alternatively, frozen tissue was thawed at 37°C and then placed on ice Subsequent steps of purification were performed at 4°C The tunicae vaginalis and albuginea were removed from each testis before homogenization Using a buffer of 0 25 M sucrose and 10 mM Tris-HCl (pH 7 4), containing 1 mM Na_2EDTA, 0 1 mM PMSF, and 15 μM pepstatin A, a 20% homogenate was prepared in a motor-driven Potter-Elvehjem apparatus operated at 800 rpm The homogenate and resulting supernatant fractions were subjected to centrifugation at $1000 \times g$ for 15 min (IEC swinging bucket rotor), $13\,000 \times g$ for 20 min (Sorvall GSA rotor), and, finally, $150\,000 \times g$ for 1 5 h (Beckman Ti 50 2 or Ti 75 rotor) The high-speed supernatant (cytosolic fraction) was dialyzed overnight against 5 mM sodium phosphate, 5 mM 2-mercaptoethanol, 1 mM Na_2EDTA, and 0 2 mM PMSF (pH 7 4)

Column chromatography of testis cytosolic fraction

A portion of dialyzed rat testis cytosolic fraction was applied to a column of DEAE-cellulose (1 × 20 cm) equilibrated in 5 mM sodium-phosphate containing 1 mM Na_2EDTA, 0.1 mM PMSF, and 15 μM pepstatin A (pH 7 4) After extensive washing, proteins were eluated with a linear gradient of NaCl (0–300 mM in a total volume of 350 ml) in the same buffer at a flow rate of 80 ml · h^{-1} A second portion of the cytosolic fraction was concentrated with Aquacide III and applied to column of Sephadex G-100 (2 × 95 cm) equilibrated in 50 mM sodium phosphate, 50 mM NaCl, 1 mM Na_2EDTA, 0 1 mM PMSF, 7 5 μM pepstatin A, and 0 02% NaN_3 (pH 7 4) Elution was carried out with the same buffer at a flow rate of 25 ml h^{-1}

Phospholipid transfer activity

Phospholipid transfer activity was measured between two populations of small unilamellar vesicles, as described previously [7] Donor vesicles contained 87 mol% PtdCho, 5 mol% PtdIns and 8 mol% LacCer, while acceptor vesicles contained 95 mol% PtdCho and 5 mol% PtdIns or PtdOH Either phosphatidyl[^{3}H]inositol or 2-[^{3}H]oleoyl-PtdCho was incorporated into the donor population as transferable substrates while 0 2 mol% cholesteryl[^{14}C]oleate was added to the acceptor vesicles to monitor recovery Following incubation of donor vesicles (50 nmol phospholipid) and acceptor vesicles (150 nmol phospholipid) in 0 5 ml 10 mM Hepes, 50 mM NaCl and 1 mM Na_2EDTA (pH 7 4) at 37°C for 30 min, donor vesicles were quantitatively removed by agglutination and precipitation in the presence of *Ricinus communis* agglutinin [7], and acceptor vesicles were analyzed for transferred radiolabelled PtdIns or PtdCho Control incubations were performed without addition of transfer protein Acceptor vesicle recovery was 90–95%, contamination by donor vesicles was ≤ 5% Activity was generally expressed as nmol phospholipid transferred per h

Electrophoresis and immunoblotting

Protein samples (1–200 μg) were subjected to electrophoresis on 0 75-mm gels of 12% polyacrylamide in the presence of sodium dodecylsulfate and 2-mercaptoethanol Unstained and Coomassie brilliant blue-stained molecular weight standards were applied to the gels Proteins were detected by staining with Coomassie brilliant blue For immunological detection proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose membranes for 2 5–3 h and 190 mDa at room temperature Reconstituted nonfat dried milk (22% by wt) was used in the blocking step [8] The primary antibody raised in rabbits against bovine PtdIns transfer protein and described in detail [5,9], was used in a 1 200 dilution of a 0 3 mg ml^{-1} solution The secondary antibody-enzyme conjugate was used at a 1 2500 dilution of the supplied preparation Alkaline phosphatase activity was visualized as described previously [5]

Selective destruction of Leydig cells

A group of four 8-week-old rats (225–250 g) received a single intraperitoneal injection of ethylene dimethanesulfonate (100 mg kg^{-1}) administered in 1 ml of dimethylsulfoxide/water (1 3, by vol) A second group of four animals received the vehicle alone Before treatment and four days after treatment blood samples were obtained by cardiac puncture and analyzed for serum testosterone On the fourth day the animals were killed One testis from each animal was detunicated, weighed, frozen at −75°C, and used for tissue fractionation and PtdIns transfer protein analysis, the other was fixed in situ for histological examination

Other analytical and statistical methods

Protein was estimated according to Lowry et al [10] with bovine plasma albumin as standard Lipid phosphorus was quantitated as described by Rouser et al [11] Tissue fixation and staining with hematoxylin and eosin were carried out using the method of Forssmann et al [12] Statistical comparisons of unpaired samples were based upon Student's t-test, differences were considered significant if $P < 0.05$

Results

Separation of testis PtdIns transfer proteins by ion exchange chromatography

Frozen rat testes were thawed, detunicated, homogenized, and fractionated by differential centrifugation to yield a high-speed supernatant A portion of this cytosolic fraction was dialyzed and applied to a column of DEAE-cellulose In Fig. 1 are shown the elution profiles of protein and PtdIns transfer activity Two peaks of transfer activity are apparent, one eluting at a NaCl concentration of 85 mM, which is identical to that observed for rat brain PtdIns transfer activity, and a second peak eluting at a NaCl concentration of 200 mM, in which region of the salt gradient no activity was observed in brain homogenates (Venuti, S E and Helmkamp Jr, G M, unpublished data) Two peaks of PtdCho transfer activity were also eluted from the anion exchange column (data not shown), these were essentially coincident with the two PtdIns transfer activity peaks DEAE-cellulose column chromatographic fractions were analyzed by immunoblotting A clear separation of the two transfer activities was demonstrated

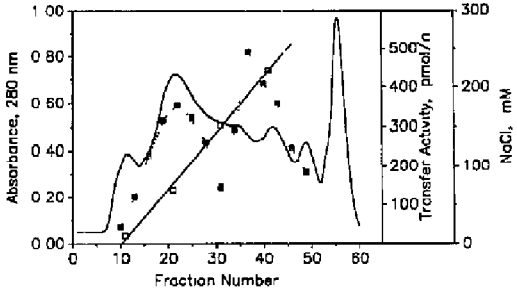


Fig 1 Anion exchange chromatography of rat testis cytosol Applied to a 1×20 cm column of Whatman DE-52 cellulose were 380 mg of cytosol protein Fractions of 8 ml were collected during elution with a linear NaCl gradient, as described in Materials and Methods Solid curve protein absorbance at 280 nm Closed squares dotted curve PtdIns transfer activity in 100 μl aliquots Open squares, solid line NaCl gradient

(Fig 2) The activity eluting at 85 mM NaCl was identified as the 35-kDa protein species, while that eluting at 200 mM NaCl corresponded with the testis-specific 41-kDa protein species

Separation of testis PtdIns transfer proteins by molecular sieve chromatography

A second portion of rat testis cytosolic fraction was concentrated and applied to a column of Sephadex G-100 In Fig 3 are illustrated the elution profiles of protein and phospholipid transfer activity As with the profile of the anion exchange column, separation of two transfer proteins was achieved Both proteins were capable of transferring PtdIns and PtdCho between phos-

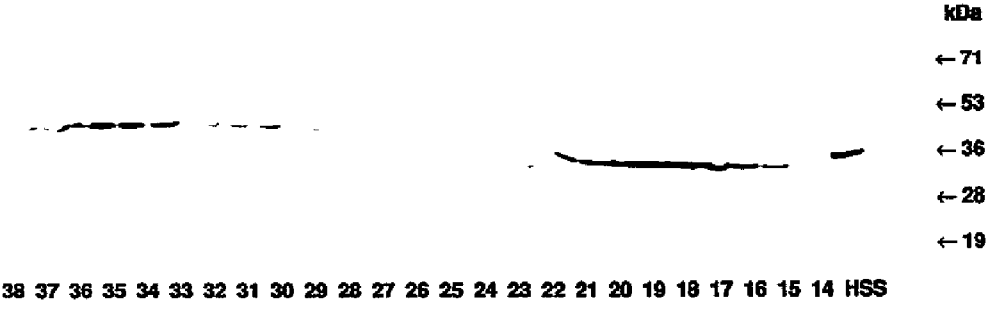


Fig 2 Immunoblot of DEAE-cellulose chromatography fractions From fractions of the anion exchange chromatographic column, 100-μl aliquots were taken to dryness in a vacuum centrifuge, redissolved in 10 μl of sample buffer containing sodium dodecylsulfate and 2-mercaptoethanol and subjected to electrophoresis on polyacrylamide gels Immunoblotting was carried out as described in Materials and Methods The actual masses of pre-stained protein standards are indicated on the right side of the gel composite The numbers refer to chromatographic fractions (Fig 1), HSS refers to the high-speed supernatant or cytosolic fraction

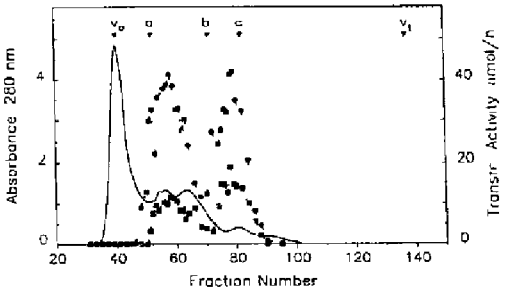


Fig. 3 Molecular sieve chromatography of rat testis cytosol After concentration to approx 3 ml, 900 mg of cytosol protein were applied to a 2×95 cm column of Sephadex G-100 Fractions of 2.8 ml were collected Solid curve protein absorbance at 280 nm Solid squares dotted curve PtdIns transfer activity in 80-µl aliquots Solid circles dotted curve PtdCho transfer activity in 100 µl aliquots Calibration of this column is indicated by the inverted triangles and was performed with the following standards bovine thyroglobulin, M_r > 600000 (v_o), bovine plasma albumin M_r 66200 (a) bovine carbonic anhydrase, M_r 31000 (b) equine myoglobin, M_r 18800 (c) and sodium dichromate, M_r < 300 (v_t)

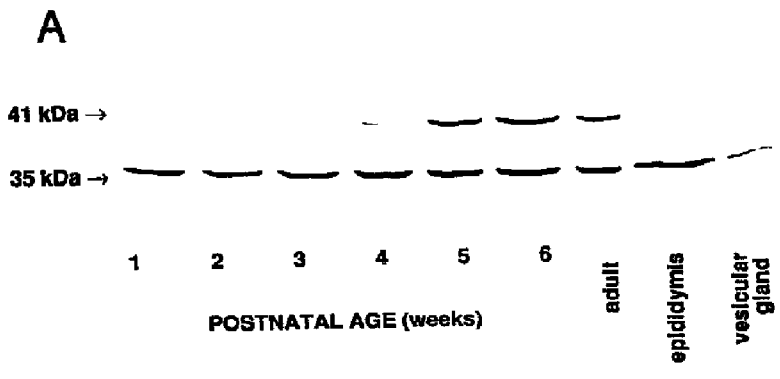
TABLE I

PtdIns transfer activity in male rat urogenital tissues

Tissues are homogenized and processed to yield 150000×g supernatant fractions Following extensive dialysis transfer activity is measured in a donor vesicle acceptor vesicle assay system using ^{3}H-labelled PtdIns Specific activity is normalized to mg of supernatant protein The values represent the mean ± S D for three determinations

Tissue	PtdIns transfer specific activity (nmol h^{-1} mg^{-1})
Testis	33 38 ± 5 70
Epididymis	13 86 ± 3 06
Vesicular gland	4 86 ± 1 06
Kidney	32 73 ± 2 10

pholipid vesicles Moreover, the ratio of the two catalytic activities was nearly identical When analyzed by immunoblotting following electrophoresis under denaturing conditions, the earlier eluting protein had a molecular mass of 41 kDa, and the later eluting protein



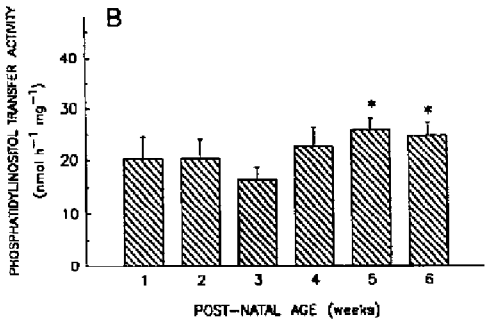


Fig. 4 Immunoreactivity and catalytic activity of rat testis cytosol during postnatal development Cytosol fractions were prepared at the indicated postnatal ages Aliquots containing 50 µg cytosolic protein were analyzed by immunoblotting (A), aliquots containing 50 and 100 µg cytosolic protein were measured for PtdIns transfer activity (B) The bar heights represent the mean ± S.D of 5–10 transfer measurements obtained with two complete series of newborn pups and developing males, an asterisk indicates a significant difference (P < 0 05) from the one-week value

had a mass of 35 kDa The molecular sieve chromatography column was calibrated with proteins of established size (Fig 3) to estimate the masses of the two testis transfer proteins under non-denaturing conditions The smaller protein, widely distributed among rat tissues, yielded a mass of 25 kDa, in good agreement with a monomeric structure of PtdIns transfer protein In contrast, the larger protein yielded a native mass of 58 kDa, a value which is considerably greater than the denatured mass and one which is, at least tentatively, consistent with a dimeric structure of the testis-specific PtdIns transfer protein

Tissue distribution of PtdIns transfer activity and proteins

Cytosolic fractions were prepared from several organs in the male rat urogenital region PtdIns transfer activity was measured between donor and acceptor populations of small unilamellar phospholipid vesicles (Table 1) Comparable specific activity was noted in testis and kidney, somewhat lower activity in the epididymis preparations, and just detectable yet still significant activity in the seminal vesicular gland When testis, epididymis, and vesicular gland were analyzed by immunoblotting, only the testis exhibited both the 35-kDa and 41-kDa bands of immunoreactive protein (Fig 4A) These two immunoreactive proteins have also been detected in mature human, bovine, buffalo, and mouse testis (data not shown) We have earlier demonstrated the absence of the 41-kDa protein in 16 other rat tissues [5]

PtdIns transfer activity in developing testis

To determine whether one or both PtdIns transfer proteins observed in adult rat testis were present at earlier stages of development, testes were obtained at weekly intervals between 1 and 6 weeks after birth For some of the earlier time points, 4–6 organs were pooled Cytosolic fractions were analyzed by immunoblotting (Fig 4A) and PtdIns transfer activity (Fig 4B) From birth through three weeks there was no detectable level of the 41-kDa protein in testis tissue The 41-kDa protein could be first detected during the fourth postnatal week, increased further during the fifth week, and reached its adult level by the sixth week, a point in development considered to be post-pubertal in the rat species PtdIns transfer activity measurements indicated a small but statistically significant increase throughout this same period ($P < 0.05$) The lower specific activities determined in this experiments may be attributed to the use of acceptor vesicles containing PtdOH rather that PtdIns, a situation which forces PtdIns transfer protein to catalyze a net transfer, rather than an exchange of PtdIns molecules [5]

TABLE II

Serum testosterone concentration in normal and ethylene dimethanesulfonate treated rats

Animals are treated with ethylene dimethanesulfonate or vehicle alone, as described in Materials and Methods Serum samples are analyzed before and four days after treatment for testosterone by radioimmunoassay The values represent the mean ± S D for four animals with determinations done in triplicate n d , not detectable

Treatment	Serum testosterone (ng ml^{-1})	
	before	after
Ethylene dimethylsulfonate in dimethylsulfoxide water	3 25 ± 0 73	n d
Dimethylsulfoxide water alone	3 52 ± 1 71	2 78 ± 0 13

Distribution of 41-kDa PtdIns transfer protein among testicular cells

Treatment of mature male rats with ethylene dimethanesulfonate selectively destroys the Leydig cell population [6] The striking decrease in serum testosterone concentration, ascertained by radioimmunoassay and summarized in Table II, confirmed the loss of these steroidogenic cells Histological examination of the fixed, vehicle-treated tissues at the light microscopic level revealed an interstitium punctuated with mature Leydig cells However, in the ethylene dimethanesulfonate-treated animals, the interstitial space was largely unpopulated with the exception of a few cells containing pycnotic nuclei, which presumably represent necrotic Leydig cells In all specimens the germinal epithelium retained its normal morphological characteristics Immunological analysis of cytosolic fractions of the ethylene dimethanesulfonate-treated testes indicated no measurable difference in the relative or absolute ratio of the 35- and 41-kDa proteins This observation was corroborated by densitometric analysis of Coomassie brilliant blue-stained polyacrylamide gels Thus, the testis-specific PtdIns transfer protein species is not unique to the Leydig cell and cannot yet be associated with a particular cell type in the testis

Discussion

Analysis of cytosolic fractions prepared from rat testis indicated a high molecular weight protein which, after electrophoresis under denaturing conditions on polyacrylamide gels, reacted with antibody to bovine PtdIns transfer protein No other tissue of the male urogenital region, nor any female reproductive tissue, exhibited this PtdIns transfer protein species The testis-specific band corresponded to a polypeptide with a mass of 41 kDa, clearly larger than the ubiquitous protein of 35 kDa seen in all other rat tissues and selected bovine and human tissues [13–15] The 35-kDa protein could be separated from the 41-kDa protein by DEAE-cellulose column chromatography PtdIns and PtdCho transfer activities were associated with both resolved proteins Of utmost significance, therefore, is

the indication that the larger protein possessed catalytic activity and could mediate the transfer of phospholipids. Both testis proteins transferred PtdIns and PtdCho between small unilamellar vesicles. Indeed, the ratio of PtdIns and PtdCho activities for the two proteins after this single step of purification was not appreciably different, suggesting a preservation of catalytic properties and substrate specificity in both transfer protein species.

Molecular sieve chromatography on a calibrated Sephadex G-100 column could also differentiate the two proteins. Under such non-denaturing conditions, the testis-specific protein exhibited a mass of 58 kDa, clearly larger than the 41 kDa determined under denaturing conditions. While the cause of this discrepancy has not been determined, it is not uncommon for molecular sieve chromatographic procedures to underestimate the apparent size of PtdIns transfer proteins from a number of species and tissues [4].

Through postnatal week 3 there is no immunological evidence of the 41-kDa isoform. Dramatically during week 4, its immunoblot intensity begins to increase and by week 6 it is comparable to that seen in the adult male. A small, but statistically significant ($P < 0.05$) increase in PtdIns transfer activity was observed between weeks 1 and weeks 5 and 6. The magnitude of this increase would suggest that the 41-kDa transfer protein is much less active than the well-characterized and ubiquitous 35-kDa species. Developmental and/or hormonal regulation of the testis-specific PtdIns transfer protein is a distinct possibility [16–18]. Increases in testis weight begin to accelerate during week 3, as does the diameter of the seminiferous tubules. These changes are accompanied by increases in the weight ratio of phospholipid to neutral lipid. In contrast, the amounts of DNA, RNA, and protein in the maturing testis actually decrease between days 5 and 60. Spermatogenesis in the testis requires the hormonal maturation and responsiveness of both Leydig and Sertoli cells, the latter cells attain adult morphology and function during week 5 [19]. The influence of pituitary peptide hormones (luteinizing hormone, follicle stimulating hormone) and testis-derived steroid hormones (testosterone, estradiol) on PtdIns metabolism and PtdIns transfer protein levels may provide additional insight into the two forms of PtdIns transfer protein in mature rat testis.

Recent attention has focussed on another lipid transfer protein in rat testis. Sterol carrier protein$_2$ or non-specific lipid transfer protein, a 14- to 15-kDa protein which has been characterized from liver and small intestine of a number of mammalian species, has been shown to catalyze the transfer of both sterols and phospholipids between membranes and to stimulate the synthesis of steroid hormone intermediates, such as pregnenolone [20–24]. Van Noort and her coworkers have described the localization of sterol carrier protein$_2$ in rat testis to Leydig cells and its absence from Sertoli and germinal cells [25]. Moreover, the cellular level of the protein and its distribution between particulate and supernatant fractions appears to be regulated by luteinizing hormone over the concentration range 0.1–1 $\mu g\ ml^{-1}$ [26,27].

Our results with the selective destruction of steroidogenesis and retention of the 41-kDa PtdIns transfer protein rule out a unique Leydig cell expression of the testis-specific PtdIns transfer protein. Clearly, additional experimentation is required before further commenting on which of the other testis cells may express this unusual protein. It should be pointed out that preliminary immunocytological comparisons of immature and mature rat testis, using rabbit antibody against bovine PtdIns transfer protein have been inconclusive (Wendelburg, B.E. and Helmkamp, Jr., G.M., unpublished data). It is noteworthy that a polyclonal affinity-purified antibody raised against rat liver sterol carrier protein$_2$ detected an immunologically cross-reactive, 58-kDa protein in rat liver homogenates [28]. Although no catalytic activity was attributed to the larger liver protein, it appeared to be localized to the peroxisomes. As with the small and large liver proteins, structural relationships between the two testis PtdIns transfer proteins have not yet been explored.

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