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Biochemistry

PP1γ2, a testis-specific protein-serine/threonine-phosphatase type 1 catalytic subunit, is associated with a protein having high sequence homology with the 78-kDa glucose-regulated protein, a member of the 70-kDa heat shock protein family

(purification/holoenzyme)

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ABSTRACT **Protein phosphatase 1γ2 (PP1γ2) is a testis-specific isotype of the protein-serine/threonine-phosphatase type 1 catalytic subunit. Three native forms of PP1γ2 were detected in a crude fraction of rat testis by electrophoresis in a nondenaturing polyacrylamide gel. We purified a major native form of PP1γ2 to homogeneity by successive column chromatography on Mono Q-Sepharose, EAH-agarose, protamine-agarose, and G3000SW and by electrophoresis in a nondenaturing polyacrylamide gel. The G3000SW-purified PP1γ2 native form had an apparent molecular mass of 170 kDa. The purified holoenzyme from nondenaturing polyacrylamide gel was composed of the catalytic subunit and two noncatalytic subunits, of 78 kDa and 55 kDa. Partial amino acid sequence analysis of the 78-kDa protein suggested that it is the 78-kDa glucose-regulated protein, a member of the 70-kDa heat shock protein family. The 78-kDa protein may possibly function as a chaperone or by confining substrate specificity of PP1γ2.**

The phosphorylation of proteins is an important mechanism for the control of various cellular events in eukaryotic cells. Protein phosphatase type 1 (PP1) is one of the serine/threonine-specific protein phosphatases of eukaryotic cells and is distinguished from PP2 by its specific activity on the β subunit of phosphorylase kinase and sensitivity to several inhibitors (1, 2). One of the most highly conserved enzymes in eukaryotes is PP1, and its conservation suggests that PP1 plays essential roles in cellular physiology. The existence of four isoforms of the PP1 catalytic subunit in rat tissues was proven by cDNA cloning and immunoreactivities of specific antibodies against each isoform (3, 4). PP1γ2 is one of the four PP1 catalytic subunit isoforms. PP1γ2, which corresponds to dis2m1 cloned from a mouse fetal brain cDNA library (5), was found to be encoded by an alternatively spliced form of PP1γ1 mRNA and expressed specifically in the testis of adult rats (3).

Mutations in putative PP1 genes of lower eukaryotic organisms cause mitotic defects in chromosome disjunction and separation of nuclei during anaphase (5). There is a report that PP1, a cytoplasmic protein in G_1- and S-phase cells, progressively accumulates in the nucleus as cells progress through G_2 phase and into mitosis. Such nuclear PP1 is tightly associated with condensed chromosomes during all stages of mitosis in mammalian cells (6). Retinoblastoma (RB) protein, a nuclear tumor-suppressor protein, is associated with PP1 catalytic subunit from mitosis to early G_1 phase (7). One function of PP1 in cell cycle regulation may be to regulate the phosphorylation status of the RB protein. In adult rats, PP1γ2 is expressed only in the testis, where meiotic division occurs and is expressed in spermatocytes at high levels and also in spermatids (8). Given these pieces of information, it is very plausible that PP1γ2 plays important roles in meiosis of male germ cells. PP1α, PP1γ1, and PP1δ are also expressed in testis, but these expression levels were high before weaning and decreased to low steady levels that were maintained (9). Some isoforms are likely to be involved in mitosis. All four isotypes of the PP1 catalytic subunit purified from *Escherichia coli* transformed with rat recombinant cDNAs have similar phosphatase activities on several substrates *in vitro* (10).

PP1 catalytic subunits are often detected as high molecular weight complexes, containing other "subunits," which may play at least three distinct roles: (*i*) targeting of catalytic subunits to the required intracellular location, (*ii*) modification of the substrate specificity of the catalytic subunit, and (*iii*) allowing the activity of the catalytic subunit to respond to hormones (11, 12). In several instances, substrate specificity of PP1 seems to be imparted by subunits which specifically target the phosphatase to its particular substrate (12, 13). These findings have provided important clues into elucidating the physiological roles of PP1 in holoenzyme forms. However, only three targeted forms of PP1 have been purified to homogeneity so far: PP1G (14), smooth-muscle PP1M (15), and skeletal-muscle PP1M (16).

An additional form of PP1, termed PP1I, has been identified as a heterodimer composed of the catalytic subunit PP1C and inhibitor 2 (17). Recently the role of inhibitor 2 was proposed to be that of a chaperone, folding the PP1 catalytic subunit into a conformation with specific affinities for regulatory subunits and thereby conferring substrate specificities (18).

A major challenge for study of PPs is to determine how many regulatory subunits exist and also what roles the subunits play in the physiological function of these enzymes. In this study, we purified a major form of PP1γ2 holoenzyme from rat testis and found that the PP1γ2 catalytic subunit formed a complex with 55-kDa and 78-kDa proteins and that the latter had high sequence homology with 78-kDa glucose-regulated protein (GRP-78) (19), a member of the 70-kDa heat shock protein (HSP70) family.

MATERIALS AND METHODS

Columns and Antibodies. Mono Q-Sepharose and EAH-agarose were purchased from Pharmacia, and protamine-agarose from Sigma. A rabbit anti-rat PP1γ2 (anti-PP1γ2) raised by us against the C-terminal peptide of PP1γ2 (8) was used for detection of PP1γ2. A rabbit anti-rat GRP-78 anti-

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Abbreviations: PP, protein phosphatase; GRP, glucose-regulated protein; HSP, heat shock protein.

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body (anti-GRP-78) raised against the C-terminal peptide (KEEDTSEKDEL) of rat GRP-78 was purchased from StressGen Biotechnologies (Victoria, BC, Canada). No appreciable cross reaction occurred with these two antibodies.

Purification of PP1γ2 Holoenzyme from Rat Testis. Samples (1 kg) of testes of 6-week-old Sprague Dawley rats were homogenized in 3 volumes of buffer A (10 mM Tris·HCl, pH 7.4/0.1 M NaCl/1 mM benzamidine/0.1 mM phenylmethanesulfonyl fluoride/14 mM 2-mercaptoethanol with leupeptin at 10 μg/ml). Unless otherwise stated all procedures were carried out at 4°C. The homogenate was centrifuged at 16,000 × *g* for 20 min and the resulting supernatant (crude fraction) was then made 30–60% saturated with ammonium sulfate by addition of the solid salt. The ammonium sulfate-precipitated pellet was dissolved in and dialyzed against buffer A. The retentate was centrifuged at 16,000 × *g* for 30 min to remove precipitate and loaded on a Mono Q column (5 cm × 100 cm) equilibrated with buffer A. After the column was washed with 3 liters of buffer A, the proteins adsorbed were eluted with a linear gradient of 0.1–0.6 M NaCl in buffer A at a flow rate of 1 ml/min over 20 min. SDS/PAGE followed by immunoblotting was done to distinguish PP1γ2 holoenzymes from the other phosphatases. Immunoblotting after nondenaturing PAGE was also performed on the pool of immunopositive fractions to confirm maintenance of the original structure of B1 (see *Results*), with the crude fraction as standard. Immunoblotting after SDS and nondenaturing PAGE were used in further purification steps. Immunopositive fractions diluted 5-fold in buffer A without NaCl were applied to an EAH column (5 cm × 50 cm) equilibrated with the same buffer. The column was washed with 2 liters of buffer A and proteins eluted from the column with 2 liters of a 0.1–1 M NaCl linear gradient in buffer A were collected in 20-ml fractions at a flow rate of 1 ml/min over 20 min. The pool of immunopositive fractions was poured into the protamine column (5 cm × 10 cm) and the column was washed with 500 ml of buffer A. The column was developed with 2 liters of a 0.3–1.5 M NaCl linear gradient in buffer A, and 20-ml fractions were collected at a flow rate of 1 ml/min. The immunopositive fractions were pooled and dialyzed against 0.15 M sodium phosphate, pH 7.0/0.1 mM phenylmethanesulfonyl fluoride/1 mM benzamidine/14 mM 2-mercaptoethanol with leupeptin at 10 μg/ml. The sample was then concentrated (Amicon YM10 membrane) to its proper volume for HPLC on a Tosoh TSK-gel G3000SW column (5.5 mm × 30 cm) at a flow rate of 0.5 ml/min. Fractions of 0.2 ml were collected. Immunopositive fractions were pooled, dialyzed against buffer A, and centrifuged to remove precipitates.

Gel Electrophoresis. A crude fraction and collected fractions at each purification step were subjected to PAGE. Nondenaturing PAGE was performed at constant current (12 mA per plate) with a buffer system of 25 mM Tris, 5 mM dithiothreitol, and 0.19 M glycine at pH 8.0, in a 10% resolving gel with a 4.5% stacking gel. SDS/PAGE was performed at constant voltage (20 V per plate) with the buffer system of Laemmli (20) in a 10% resolving gel with an 8% stacking gel.

Immunoblot Analysis. Proteins separated in nondenaturing or SDS/polyacrylamide gels were electrotransferred to an Immobilon membrane (Millipore). The membrane was incubated with anti-PP1γ2 (1:400 dilution) or anti-GRP-78 (1:200) and then with ^{125}I-labeled protein A (1:400) (Amersham). The densities of the bands were analyzed with an Image analyzer (Fuji). PP1γ2 purified from *E. coli* cells which were transformed with a recombinant rat PP1γ2 cDNA (10) was used as a standard protein for determining migration distance.

Identification of Components of PP1γ2 Holoenzyme. The G3000SW-purified protein was subjected to nondenaturing PAGE and stained with Coomassie brilliant blue (no fixation step) to confirm its purity. A part of the gel was immunoblotted to confirm the protein band of PP1γ2. The protein band identified as PP1γ2 holoenzyme was cut out of the stained gel. The proteins eluted with Laemmli sample buffer were subjected to SDS/PAGE. The gel was cut into two strips and subjected to immunoblotting and silver staining.

N-Terminal Amino Acid Sequencing. A 40-μg sample of the G3000SW-purified protein was subjected to SDS/PAGE and electroblotted onto ProBlott (Applied Biosystems). The 78-kDa protein band stained with Coomassie blue was cut out and loaded onto a model 477A sequencer (Applied Biosystems) for analysis of the N-terminal amino acid sequence.

Peptide Purification and Amino Acid Sequence. A 400-μg sample of the G3000SW-purified protein was subjected to large-scale SDS/PAGE. The portion corresponding to the 78-kDa protein was excised and subjected to in-gel digestion with a lysyl endopeptidase, *Achromobacter* protease I, as described (21, 22). This digested protein was eluted from the gels and the peptide solution was chromatographed on a cartridge column (Aquapore RP-300, 4.6 mm × 3 cm) equilibrated with 0.1% trifluoroacetic acid, with an increase in acetonitrile concentration of 0.87% by volume per minute. The flow rate was 0.2 ml/min; peak fractions monitored at 210 nm were collected manually. One fraction with a sharp peak was analyzed on an Applied Biosystems model 477A sequencer.

Immunoprecipitation of PP1γ2 Holoenzyme. The crude fraction was incubated with anti-PP1γ2 or anti-GRP-78 at 4°C for 2 hr with gentle end-over-end agitation. Protein A-Sepharose was then added to these mixtures and further incubated for 1 hr. The incubates were centrifuged, and the pellets obtained were washed with phosphate-buffered saline five times, dissolved in Laemmli sample buffer, and subjected to immunoblotting with two antibodies after SDS/PAGE. Preimmune serum was used as a negative control.

RESULTS

Native Forms of PP1γ2 in Crude Fraction. Three native forms of PP1γ2—B1, B2, and B3—were detected in the crude fraction of adult rat testis with nondenaturing PAGE (Fig. 1*a*). B1 and B2 native forms extracted from the gel were further electrophoresed in an SDS/polyacrylamide gel; the migration distance of their catalytic subunit, PP1γ2, was observed to be identical to that of purified *E. coli*-expressed PP1γ2 (10) (Fig. 1*b*). The expression level of B3 was very low; the presence of 39-kDa PP1γ2 in B3 was not confirmed by SDS/PAGE. It is, however, inferred that B3 is a minor form of PP1γ2 in rat testis, based on a very high specificity of anti-PP1γ2 and appearance of the B3 band coinciding with those of B1 and B2 during rat developmental stages (unpublished results).

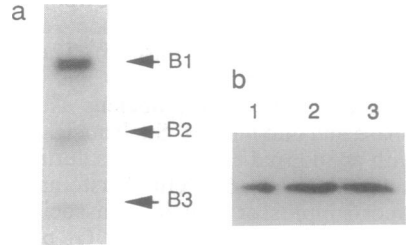

FIG. 1. Anti-PP1γ2 immunoblotting of crude extract of rat testis after nondenaturing or SDS/PAGE. (*a*) Immunoblotting of crude extract after nondenaturing PAGE of 50 μg of total protein. Three forms—B1, B2, and B3—were detected. (*b*) Immunoblotting of B1 and B2 proteins after SDS/PAGE. Several gel pieces of each band were pooled and the extract was subjected to SDS/PAGE. Lane 1, 20 ng of purified *E. coli*-expressed PP1γ2; lane 2, B1; lane 3, B2.

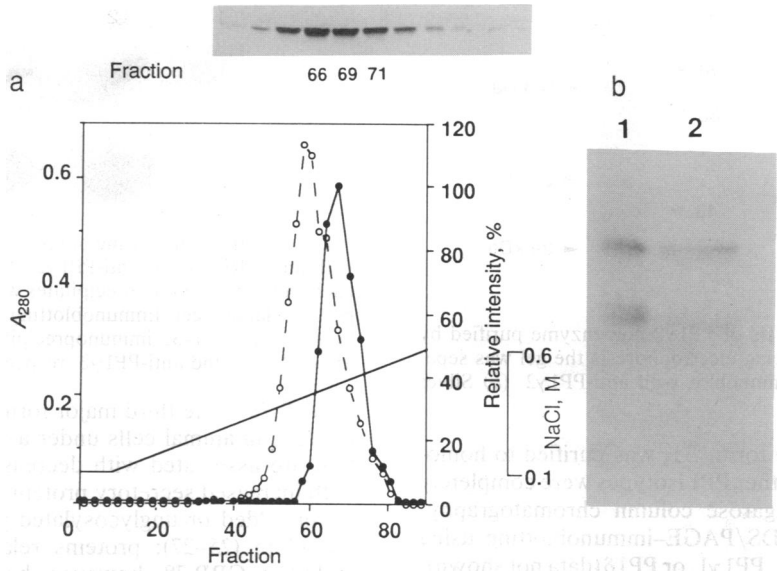

FIG. 2. Purification of a PP1γ2 holoenzyme by Mono Q column chromatography. (*a*) Elution profile shown by anti-PP1γ2 immunoblot after SDS/PAGE (*Upper*) as a plot of intensity of each immunoblot band (●) and absorbance at 280 nm (○). (*b*) Immunoblot of the pool of immunopositive fractions in *a* with anti-PP1γ2 after nondenaturing PAGE. Lane 1, crude extract; lane 2, pool of positive fractions.

Purification of Holoenzyme of PP1γ2. B1, the major native form, was purified to homogeneity by successive column chromatography on Mono Q, EAH, protamine, and G3000SW. Throughout the purification steps, immunoblotting after SDS/PAGE was used to distinguish PP1γ2 enzyme from other phosphatases instead of measuring phosphatase activity, as illustrated in Fig. 2*a* for the fractions from Mono Q column chromatography. Immunoblotting after nondenaturing PAGE was also performed on the pool of immunopositive fractions from SDS/PAGE to confirm maintenance of the original structure of B1, using crude fraction as standard (Fig. 2*b*). In the preparations obtained after protamine-agarose column chromatography, B2 and B3 were not detected. After nondenaturing PAGE, the G3000SW-purified protein showed a single band by Coomassie blue staining (Fig. 3*a*), and the presence of PP1γ2 in this fraction was demonstrated by immunoblotting (Fig. 3*b*).

The purification of the PP1γ2 native form B1 is summarized in Table 1. The fold purification and yield were quantified by comparison with immunoreactivity of recombinant PP1γ2 purified from *E. coli*.

Composition of PP1γ2 Holoenzyme. The G3000SW-purified protein was further purified by nondenaturing PAGE. The protein eluted from the gel was analyzed by SDS/PAGE. Three components were detected by silver staining. One was the 39-kDa catalytic subunit, showing identical migration with purified *E. coli*-expressed PP1γ2 (data not shown). Apparent molecular masses of the other two (noncatalytic) subunits were estimated to be 78 kDa and 55 kDa (Fig. 4*b*).

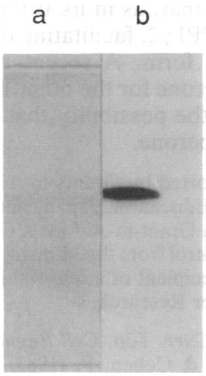

FIG. 3. Nondenaturing PAGE of G3000SW-purified PP1γ2 holoenzyme. (*a*) The gel stained with Coomassie blue. (*b*) Anti-PP1γ2 immunoblot of a part of the gel.

Amino Acid Sequence Analysis of 78-kDa Subunit. Two different portions of the 78-kDa protein were analyzed. An N-terminal 17-amino acid sequence was identical to that of rat GRP-78 (19). A 27-amino acid sequence of a peptide from the enzyme digest was the same as that of the middle portion of GRP-78. Amino acid sequences of these two separated portions of the protein, 44 amino acids in all, showed 100% identity with rat GRP-78 (Fig. 5).

Immunoprecipitation of PP1γ2 Holoenzyme. GRP-78 was coimmunoprecipitated with anti-PP1γ2. Likewise, PP1γ2 was also detected in the immunoprecipitates by anti-GRP-78. These proteins were not immunoprecipitated by preimmune serum (Fig. 6).

DISCUSSION

We demonstrated by immunoblotting that three native forms of PP1γ2 were present in the crude extract of adult rat testis (Fig. 1*a*); two of these forms were confirmed to have PP1γ2 as a component by SDS/PAGE using purified PP1γ2 expressed in *E. coli* as a molecular weight standard (Fig. 1*b*).

Table 1. Purification of PP1γ2 holoenzyme

Fraction	Total protein, mg	PP1γ2,* mg	Yield, %	Fold purification†
Crude	49,900	20.26		
Amonium sulfate	19,000	6.22	100	1
Mono Q-Sepharose	2,180	4.41	71	6
EAH-agarose	144	2.33	38	49
Protamine-agarose	15	1.31	21	267
G3000SW	0.94	0.19	3	617

*PP1γ2 protein was quantified by measuring the intensity of immunostaining after SDS/PAGE with an Image analyzer (Fuji); purified *E. coli*-expressed PP1γ2 served as standard. A linear dose–response of the purified PP1γ2 from *E. coli* was observed at least between 10 ng and 50 ng in image analysis.

†Calculated on the basis of PP1γ2 protein.

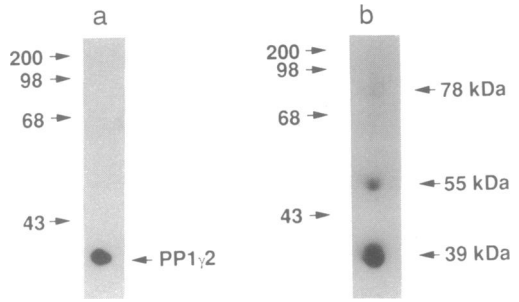

FIG. 4. SDS/PAGE analysis of PP1γ2 holoenzyme purified by nondenaturing PAGE. Following electrophoresis the gel was separated into two strips. (*a*) Immunoblot with anti-PP1γ2. (*b*) Silver staining.

The major PP1γ2 native form, B1, was purified to homogeneity. Holoenzymes of other PP1 isotypes were completely separated by protamine-agarose column chromatography, judging from results of SDS/PAGE–immunoblotting using antibodies specific to PP1α, PP1γ1, or PP1δ (data not shown). The G3000SW-purified PP1γ2 holoenzyme showed phosphatase activity on ^{32}P-labeled phosphorylase *a*, and this activity was inhibited by the addition of inhibitor 2, a PP1-specific inhibitor, and also by okadaic acid (data not shown).

The purified holoenzyme was shown to be composed of 39-kDa PP1γ2 and 78-kDa and 55-kDa proteins (Fig. 4). A 1:1:1 stoichiometry of these components in the holoenzyme is likely, as was determined from comparisons of the molecular size of holoenzyme estimated from the G3000SW gel filtration (≈170 kDa) and the total molecular mass of the three components; the most probable structure is a heterotrimer. Nevertheless, despite its molecular mass, the 78-kDa protein showed weak intensity on silver staining, which may have been due to its physicochemical properties and low elution efficiency from the nondenaturing gel.

Partial amino acid sequences of the 78-kDa protein were demonstrated to be identical to sequences in GRP-78, an HSP70 family member (Fig. 5). The N-terminal sequence of the 78-kDa protein started after the signal sequence of GRP-78. Anti-GRP-78 which recognizes the C-terminal part of rat GRP-78 reacted with the 78-kDa protein, indicating that the C-terminal region of GRP-78 and the 78-kDa protein should be in common. The anti-GRP-78 reacted with a protein of 78 kDa in several rat tissues (data not shown). It is not known whether the 78-kDa protein is wholly the same as the GRP-78 reported (19). The interaction mode of these three subunits has not been undetermined. After cDNA cloning of the 55-kDa protein, further study will be necessary to evaluate the functional roles of these noncatalytic subunits and the substrate specificities of this holoenzyme.

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<----------------------------->
MKFTVVAAALLLLCAVRAEEEDKKEDVGTVVGIDLGTTYS      1-40
                 ****************
CVGVFKNGRVEIIANDQGNRITPSYVAFTPEGERLIGDAA      41-80
KNQLTSNPENTVFDAKRLIGRTWNDPSVQQDIKFLPFKVV      81-120
EKKTKPYIQVDIGGGQTKFTAPEEISAMVLTKMKETAEAY      121-160
LGKKVTHAVVTVPAYFNDAQRQATKDAGTIAGLNVMRIIN      161-200
                          ****************
EPTAAAIAYGLDKREGEKNILVFDLGGGTFDVSLLTIDNG      201-240
**********
VFEVVATNGDTHLGGEDFDQ — — — — — — — —          -654
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FIG. 5. Results of partial amino acid sequence analysis of the purified 78-kDa protein. The N-terminal half (residues 1–260) of the sequence previously reported for rat GRP-78 is shown (19). Double-headed arrow indicates the reported signal sequence of rat GRP-78. Stars indicate amino acids identical to those of the purified 78-kDa protein detected in the N-terminal region and a peptide obtained by enzyme digestion. The signal peptide of rat GRP-78 was not detected at the N terminus of the 78-kDa protein.

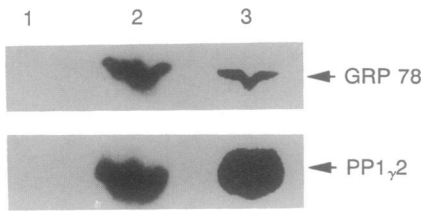

FIG. 6. Immunoblotting of immunoprecipitates of crude fraction with anti-GRP-78 and anti-PP1γ2. After immunoprecipitation with each antibody, immunoprecipitates were electrophoresed in an SDS/polyacrylamide gel. Immunoblotting was performed with both antibodies. Lanes 1–3, immunoprecipitates with preimmune serum, anti-GRP-78, and anti-PP1γ2, respectively.

GRP-78 is the third major form in the HSP70 family. It is induced in animal cells under a variety of stress conditions that are associated with decreased glucose levels (23) and with increased secretory protein traffic (24) or accumulation of misfolded or unglycosylated protein in the endoplasmic reticulum (25–27); proteins related to the HSP70 family including GRP-78, however, have been found in normal, unstressed cells (28, 29). GRP-78 shares about 60% identity in its amino acid sequence with HSP70 (19). It is highly conserved among species, and rat and hamster GRP-78 proteins display 99.4% identity in amino acid sequence (19, 30). The evolutionary conservation of these proteins suggests that they play important roles in cellular physiology.

GRP-78 is identical with the immunoglobulin-binding protein BiP and known to form transient complexes with newly synthesized immunoglobulin chains before their assembly into the final oligomeric structure (31). Although a number of studies have suggested roles of GRP-78 in facilitating the import of proteins into the endoplasmic reticulum and subsequent folding and assembly, the specific functions of the GRPs are still unclear. It is also possible that GRP-78 in testis modulates the phosphatase activity of PP1γ2, similar to HSP90 family involvement in modulation of steroid receptor action and of the α subunit of eukaryotic translation initiation factor (eIF-2α) kinase (32). Recent information that purified mammalian HSP70 activates PPs *in vitro* (33) also supports the possibility that GRP-78 is a modulator of PP1γ2 activity.

The 78-kDa subunit was also detected in the nuclei of rat spermatocytes and spermatids, but not in spermatogonia; PP1γ2 and 78-kDa protein showed a similar localization pattern in rat testis (unpublished result). The B1 holoenzyme may be present in nuclei of male meiotic cells. Even though the function of each noncatalytic subunit is still unclear, the 78-kDa protein may possibly target the catalytic subunit to specific organelles especially the nucleus in testis, because PP1γ2 has no nuclear targeting signal. Considering the well-known functions of GRP-78 as a molecular chaperone (31), there is a possibility that, as in its BiP function, the 78-kDa protein chaperones PP1γ2, facilitating its folding and assembly into the mature form. A recent report has proposed inhibitor 2 as a chaperone for the other PP1 isotypes (18) and this report suggests the possibility that PP1γ2 is associated with its specific chaperone.

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