Prolin-Rich Tyrosine Kinase 2 (PYK2) Expression and Localization in Mouse Testis

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ABSTRACT Prolin-rich kinase 2 (PYK2) is a nonreceptor tyrosine kinase related to the focal adhesion kinase (FAK) $p125^{FAK}$. PYK2 is rapidly phosphorylated on tyrosine residues in response to various stimuli, such as tumor necrosis factor-α (TNF-α), changes in osmolarity, elevation in intracellular calcium concentration, angiotensin, and UV irradiation. PYK2 has ligand sequences for Src homology 2 and 3 (SH-2 and SH-3), and has binding sites for paxillin and $p130^{cas}$. Activation of PYK2 leads to modulation of ion channel function, phosphorylation of tyrosine residues, and activation of the MAP kinase signaling pathways. Immunocytochemistry shows that PYK2 is present in mouse germinal and Sertoli cells (ser). Northern blot and immunoprecipitation analysis demonstrate that, among germinal cells, PYK2 is more abundant in spermatocytes (spc) and spermatids (spt); in addition, immunofluorescence analysis clearly shows that the diffuse cytoplasmic localization of PYK2 changes in a specific cellular compartment in spt and spermatozoa. *Mol. Reprod. Dev. 65: 330–335, 2003.*

Key Words: spermatogenesis; focal adhesion kinase; phosphorylation

INTRODUCTION

The activation of protein-tyrosine kinases (PTKs) is one of the most common signal transduction mechanisms directly coupled to receptor activation by external signals. The PTKs that do not span the plasma membrane (the so-called nonreceptor PTKs) have been classified into different subclasses (subfamilies) (Robinson et al., 2000).

Protein tyrosine kinases have been well documented to be involved in the regulatory pathways in the cellular proliferation and differentiation (Dimecky et al., 1990). Some of the PTKs are involved in the spermatogenesis (Sorrentino et al., 1991; Yoshinaga et al., 1991), which is a well characterized developmental process from spermatogonia (spg) to mature sperm, providing an useful model systems to clarify the gene expression in cellular proliferation and differentiation (McCarrey, 1993).

Prolin-rich tyrosine kinase 2 (PYK2, also known as CAKB, RAFTK, FAK2, and CADTK) is a nonreceptor PTK that is closely related to the focal adhesion kinase (FAK) $p125^{FAK}$ (Avraham et al., 1995; Lev et al., 1995). The cDNAs of the protein have been cloned from rat (Sasaki et al., 1995), mouse (Avraham et al., 1995), and human (Lev et al., 1995; Herzog et al., 1996). PYK2 and FAK share a similar structural organization with a tyrosine kinase domain flanked by noncatalytic domains at both N- and C-termini. Both proteins have ligand sequence for Src homology 2 and 3 (SH-2 and SH-3) domain (Dikic et al., 1996) but do not have SH-2 and SH-3 domains themselves. It has been shown a constitutive association of PYK2 with paxillin (Li and Earp, 1997) and $p130^{cas}$ (Astier et al., 1997), two other FAK-binding proteins (Guan, 1997). PYK2 mRNA is less evenly expressed in a variety of organs than FAK mRNA (Sasaki et al., 1995). PYK2 is highly expressed in the central nervous system (Lev et al., 1995), in cells and tissue derived from hematopoietic lineages (Dikic et al., 1998), and in the prostatic epithelium (Stanzione et al., 2001; Picascia et al., 2002).

PYK2 is activated by various extracellular signals including inflammatory cytokines, TNF-α, angiotensin, UV irradiation, changes in osmolarity, and increase in intracellular calcium concentration. PYK2 can function as an upstream mediator of the JNK signaling pathway via a mechanism currently unknown (Tokiwa et al., 1996). In addition, PYK2 can activate the Ras-MAPK signaling pathway by both direct and indirect recruitment of the adapter protein Grb2 and Shc (Dikic et al., 1996), appearing to be a key mediator of intracellular signaling linking a variety of extracellular stimuli (Guo et al., 1998). Thus, PYK2 could be the cross step linking the variously activated pathways controlling cell growth and differentiation.

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In the present paper, we analyze the expression, the localization, and the activation of PYK2 in mouse testis. The results reported in this study indicate the presence of PYK2 in the germinal epithelium suggesting its potential role in male germ cell proliferation and differentiation.

MATERIALS AND METHODS

Preparation of Testicular Cells

Spermatocytes (spc) and spermatids (spt) cells were prepared from CD1 mice (Charles River, Italy) as previously described (Sette et al., 1999). Briefly, after dissection of albuginea membrane, testes were digested for 15 min in 0.25% (w/v) collagenase (type IX, Sigma Chemical Corporation, St. Louis, MO) at room temperature under constant shaking. Digestion was followed by two washes in minimum essential medium (Life Technologies, Inc., Paisley, UK), hence seminiferous tubules were cut in pieces using a sterile blade and further digested in minimum essential medium containing 1 mg/ml trypsin for 30 min at 30°C. Digestion was stopped by adding 10% fetal calf serum and the released germ cells were collected after sedimentation (10 min at room temperature) of tissue debris. Germ cells were centrifuged for 13 min at 1,500 rpm at 4°C and the pellet resuspended in 20 ml of elutriation medium (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM MgS_4 (7 H_2O), 1.3 mM $CaCl_2$, 11 mM glucose, 1 × essential amino acid (Life Technologies, Inc.), penicillin, streptomycin, 0.5% bovine serum albumin (BSA). Germ cells at pachytene spc, and spt steps were obtained by elutriation of the unfractionated single cell suspension as described previously (Meistrich, 1977). Homogeneity of cell populations, ranged between 80 and 85% (pachytene spc) and 95% (spt), was routinely monitored morphologically. Mature spermatozoa were obtained from the cauda of the epididyms mature mice as described previously (Sette et al., 1997). Spg and Sertoli cells (ser) were obtained from prepuberal mice as previously described (Grimaldi et al., 1993; Rossi et al., 1993).

The human prostate cancer cell line PC3 used in these studies were grown in Dulbecco Modified Eagle Medium supplemented with 5% fetal calf serum (Kaingn et al., 1979).

Antibodies

Antibodies polyclonal rabbit anti-PYK2 antisera #600, #602, and #638 were kindly provided by Prof. Joseph Schlessinger and by Dr. Ivan Dikic and described previously (Lev et al., 1995; Dikic et al., 1998); polyclonal anti-PYK2 [pY^{402}] raised in rabbit (no. 44–618; Biosource International, Inc., Camarillo, CA).

Immunocytochemistry

Mouse testes rapidly removed and fixed in Bouin's fluid, were serially dehydrated in ethanol and cleared in xylene. For each paraffin embedded sample, 4 μm serial sections, mounted on slides, were dewaxed in xylene, and brought through ethanols to deionized distilled water. Ten sections/animal/month were examined. The endogenous peroxidases were quenched by incubation of sections in 0.1% sodium azide with 0.3% hydrogen peroxide for 30 min at 22°C; nonspecific binding were blocked by incubation with nonimmune serum (1% Tris-bovine albumin for 15 min at 22°C).

All sections were pre-treated with 0.5% trypsin in 0.1% hydrochloric acid for 30 min at 37°C to unmask antigen. Before performing the immunohistochemical staining sections were further incubated in a 750 W microwave oven for 15 min (3 cycles of 5 min) in 10 mM buffered citrate, pH 6.0, in order to complete antigen unmasking.

The standard avidin–biotin-peroxidase complex (ABC) procedure was used (Hsu et al., 1981). Anti PYK2 antibody (#638) were utilized at a dilution 1:400. Control experiments were performed using the secondary antibody alone or preabsorbed the primary antibody with excess of the cognate peptide (10^{-6} M).

The peroxidase activity was developed with the use of a filtered solution of 5 mg of 3-3′-diaminobenzideine tetrahydrochloride (dissolved in 10 ml of Tris-buffer 0.05 M, pH 7.6) and 0.03% H_2O_2. For nuclear counterstaining, Mayer's hematoxylin was employed. Sections were mounted with a synthetic medium.

Immunofluorescence

Cells were spotted on poly-L-lisine-coated glass slides and fixed either at room temperature for 15 min in PBS containing 0.1% paraformaldheyde (PFA) or at −20°C for 5 min in methanol acetone 1:1. PFA treated cells were permeabilized for 5 min in PBS containing 0.1% Triton X-100. Fixed cells were incubated for 30 min at room temperature with 10% goat serum in PBS and after three washes with 1.5% goat serum in PBS, were incubated for 1 hr at room temperature with either polyclonal PYK2 antibodies #602 or #638 (diluted 1:200 in 1.5% goat serum in PBS), as primary antibody. Following five washes (10 min in 1.5% goat serum in PBS), cells were incubated for 1 hr at room temperature with cyanin 3-conjugate anti-rabbit IgGs (Chemicon, #AP132C, diluted 1:400) as secondary antibody. After five more washes (10 min in PBS), slides were mounted in 50% glycerol in PBS and immediately examined by fluorescence microscopy; to stain DNA, 0.1 mg/ml Hoechst dye (Sigma) was added to the first wash. Control experiments were performed using the secondary antibody alone or preabsorbed the primary antibody with excess of the cognate peptide (10^{-6} M) (data not shown).

Sperm Preparation and Capacitation

Uncapacitated caudal epidymal sperm collected from CD1, was accomplished by the method of Visconti et al. (1995). Then, washed sperm cells (10^6 cells/ml) were capacitated by adding to medium Ca^{2+} (2 mM), pyruvate (1 mM), and BSA (3 mg/ml), followed by incubation at 37°C for 30 min under 5% CO_2 in air.

Capacitated mouse sperm (10^6 cells/ml) were incubated with ionophore A23187 (10 μM, Sigma) for 1 hr at

37°C. The status of the sperm acrosome was determined by a staining technique (Feng et al., 1998). Briefly spermatozoa were air-dried on glass slides, fixed with 5% paraformaldehyde in PBS (pH 7.4) and stained with 0.04% Coomassie G-250 (in 3.5% perchloric acid) for 5 min, and then rinsed with H_2O.

Acrosome Staining Using Comassie Brillant Blue

Acrosomal integrity was determined by a modified Comassie brillant blue staining technique (Moller et al., 1990). Spermatozoa were air-dried on polilisynated glass slides, fixed with 4% paraformaldehyde in phosphate buffer saline (PBS, 0.1 M, pH 7.4) for 15 min, and washed once in PBS. The slides were stained for 5 min with 0.25% Commassie brillant blue made with 10% glacial acetic acid and 25% methanol in H_2O, and then rinsed with H_2O and cover-slipped under 50% glycerol in PBS. This method stains the acrosomal cap blue in acrosome-intact sperm but does stain the acrosome region in acrosome reacted sperm (Feng et al., 1997).

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from cells and tissues using the Trizol Reagent (Gibco-BRL, Paisley, UK) and following the manufacture's instructions. Twenty micrograms of total RNA extracted were fractionated on a 1.2% agarose/formaldehyde gel and blotted onto nylon membrane (Hybond-N, Amersham, UK) with 10 × SSC. The membrane was prehybridized at 42°C for 4 hr in a solution containing 50% formamide, 3 × SSC, 60 mM phosphate buffer (pH 6.8), 10 mM EDTA (pH 7.2), 0.2% SDS, and 5 × Denhardt solution. Hybridization was carried out overnight under the same conditions with Pyk2-cDNA clone radiolabeled with [α^{32}P] dATP by random examiner labeling (Feinberg and Volgelstein, 1984; Sambrook et al., 1989).

The membranes was washed once with 1 × SSC, 0.1% SDS at 42°C before autoradiography.

Protein Extraction

Tissues and cells were homogenized directly into lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100 (1:2 weight/volume), 1 mM phenylmethylsulfonyl fluoride (PMSF) 1 μg aprotinin, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate, (Sigma), and clarified by centrifugation at 14,000*g* for 10 min. Protein concentrations were estimated using a modified Bradford assay (Bio-Rad, Melville, NY).

Immunoprecipitation and Western Blot Analysis

Fifty microliters of protein A-Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK) were incubated with 5 μl of rabbit anti PYK2 antibody (#600) for 1 hr at 4°C, and subsequently incubated with 500 μg of protein total lysates for 2 hr at 4°C. Immunoprecipitates were washed four times with HNTG (20 mM HEPES, 15 mM NaCl, 0.1% Triton X-100, 5 or 10% glycerol) and boiled in Laemmli buffer (Tris-HCl, pH 6.8, 0.125 M, SDS 4%, glycerol 20%, 2-mercaptoethanol 10%, bromophenol blue 0.002%) for 5 min before electrophoresis (Tokiwa et al., 1996). Immunoprecipitates were subjected to SDS–PAGE (7.5% polyacrylamide) under reducing condition. After electrophoresis, proteins were transferred to nitrocellulose membrane (Immobilon Millipore Corporation); complete transfer was assessed using pre-stained protein standards (Bio-Rad, Hercules, CA). After blocking with TBS–BSA (25 mM Tris, pH 7.4, 200 mM NaCl, 5% BSA), the membrane was incubated with the primary antibody against PYK2 (#600 or #602, diluted 1:500) in TBS for 1 hr (at room temperature). All the primary antibodies used were rabbit polyclonal IgG raised against the peptide sequence. Membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:2,000) for 45 min (at room temperature) and the reaction was detected with ECL system (Amersham Life Science, Bucks, UK).

RESULTS

Expression and Localization of PYK2 in the Mouse Testis

Immunocytochemistry was performed on serial testis sections using antibody against N-terminal region of PYK2 (#638). The protein was found in the germinal epithelium in the cytoplasm of spg, spc, spt, and in ser (Fig. 1A). The antiserum used in this study fulfils the criteria of specificity. In particular, immunoadsorption tests revealed that the labeling was totally blocked preincubating antibody with 10^{-6} M of the cognate peptide (Fig. 1B).

We evaluated PYK2 expression in various testis cells using Northern blot hybridization with PYK2 specific probe. PYK2 mRNA was expressed as a single band of 4.5 kb in germ cells during the progression from spg to spt, as compared with the prostatic PC3 cells positive control (Fig. 2). In the germinal epithelium cells, the transcript was more abundant in spc, spt, present in ser, and it was almost absent in the spg and interstitial cells (Fig. 2).

Immuprecipitation and Western blot analysis of whole mouse testis and cell extracts from fractionated adult testis cells showed a single product migrating as a 110 kDa protein as compared with the prostatic PC3 cells positive control (Fig. 3). Among germ cells, it was present in spg, abundant in spc, spt, and spermatozoa. PYK2 protein was present in ser and almost absent in the interstitial cells (Fig. 3); the presence of a specific weak band in the interstitial extract cells is, probably, due to the ematopoietic cells contamination that express PYK2 protein (Dikic et al., 1998).

Immunofluorescence Analysis of PYK2 in Mouse Germinal Cells

The localization of PYK2 in germinal cells were investigated by immufluorescence analysis, using either polyclonal rabbit anti-PYK2 antisera #602 or #638.

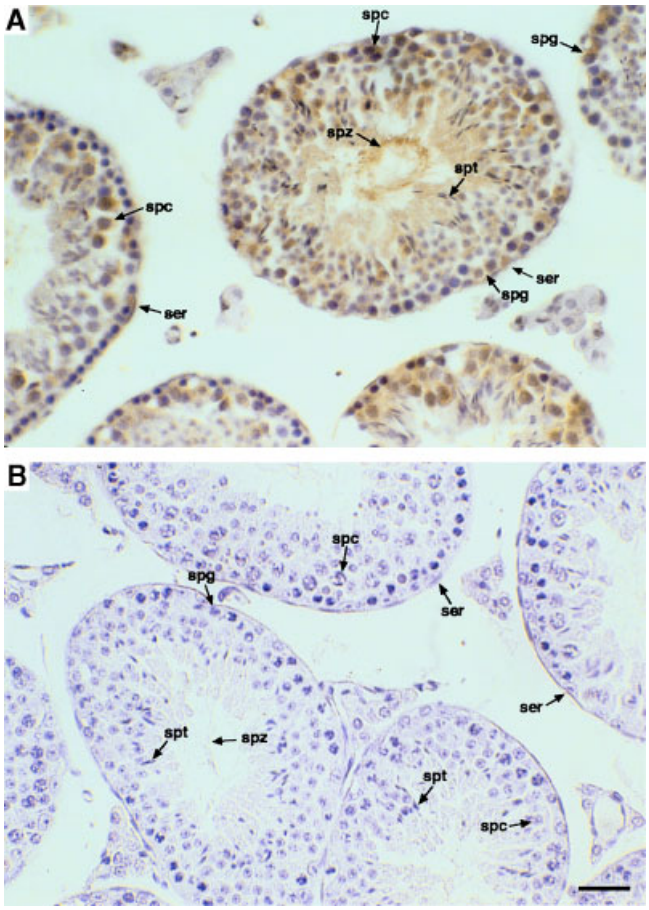

Fig. 1. A: Localization of the prolin-rich tyrosine kinase 2 (PYK2) protein in sections of adult mouse testis by immunocytochemistry. Representative seminiferous tubules showing staining in the cytoplasm of spermatogonia (spg), spermatocytes (spc), spermatids (spt), and Sertoli cells (ser). **B**: Control section using the antibody preadsorbed with the cognate peptide (10^{-6} M); symbols are as indicated above. Bar = 50 μm.

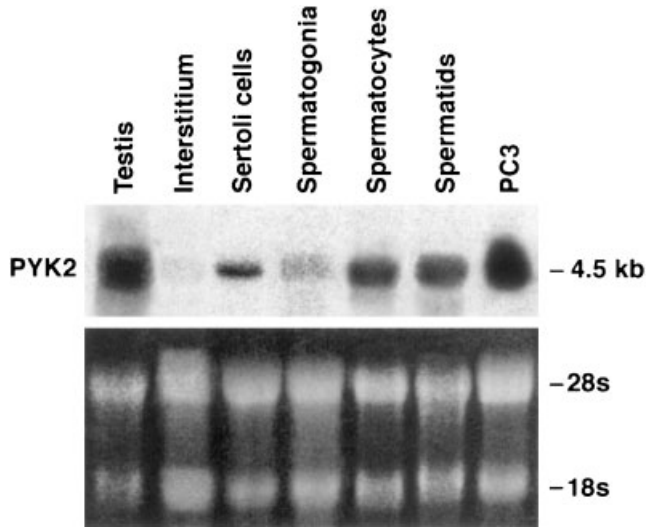

Fig. 2. Expression of Pyk2 mRNA in mouse testis. Northern Blot analysis of Pyk2 mRNA in adult mouse testis (**lane 1**), interstitial tissue (**lane 2**), ser (**lane 3**), in normal freshly isolated testicular cell populations (**lanes 4–6**), and PC3 cells positive control (**lane 7**). Each lane contained 20 μg of total RNA. All blots were probed with Pyk2-c-DNA. The integrity and relative abundance of RNA samples were determined by ethidium bromide staining of the filter (**lower frame**).

The cells were fixed with methanol acetone and stained using PYK2 #602 antibody. Stained cells showed a diffuse distribution of PYK2 protein with prevalent distribution in the cytoplasm region. In both 7–8 days spg (Fig. 4A) old mice and adult mice spc (Fig. 4B), PYK2 immunostaining was observed in the cytoplasm with no localization in the nucleus. Similar results were obtained using either PFA or methanol acetone fixation with both antibody #602 and #638. Like in both spg and spc, in adult mice round spt, PYK2 showed a cytoplasmatic distribution (Fig. 4C). However, in round spt PYK2 staining was localized in a polar side of the cells which correspond in elongated spt to the acrosome region (Fig. 4D). In adult mice spermatozoa, PYK2 staining was localized in both acrosome region and tail (Fig. 4E). The specific acrosomal distribution was also demonstrated in Figure 4F in which acrosome reacted spermatozoa were stained using PYK2 antibody #638. In these cells, we observed a tail immunopositive staining but we never observed positive staining in the acrosomal region. Acrosomal integrity was determined using Commassie brillant blue acrosome staining (data not shown).

PYK2 Activation in Mouse Spermatozoa

In order to investigate the activation status of PYK2, mouse spermatozoa were maintained in serum free medium (for 24 hr) and in presence of $CaCl_2$. Then spermatozoa were treated with the ionophore A23187 (10 μM, Sigma). A consistent activation of PYK2 (phosphorylation of the tyrosine 402) was detectable within 2.5 min after A23187 stimulation (Fig. 5) and declined thereafter. The phosphorylation status was confirmed observed the equal amount of PYK2 protein (Fig. 5).

DISCUSSION

This study was initiated to investigate the involvement in the testis of the nonreceptor tyrosine kinase PYK2 having a specific kinase activity on tyrosine residues (Avraham et al., 1995; Lev et al., 1995). PYK2

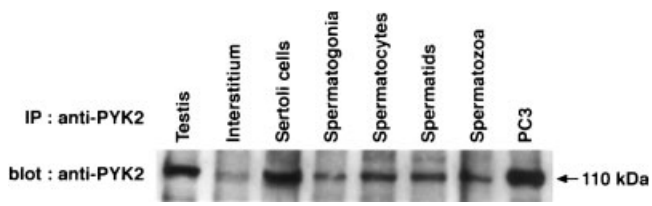

Fig. 3. The expression of PYK2 proteins in mouse adult testis (**lane 1**), interstitium (**lane 2**), ser (**lane 3**), normal mouse testis germ cells (**lanes 4–7**), and PC3 cells positive control (**lane 8**). Total lysates (500 μg) for each sample were subjected to immunoprecipitation and immunoblot with antibody to PYK2 (#600). A specific band was observed at 110 kDa by comparison with co-migrating size markers.

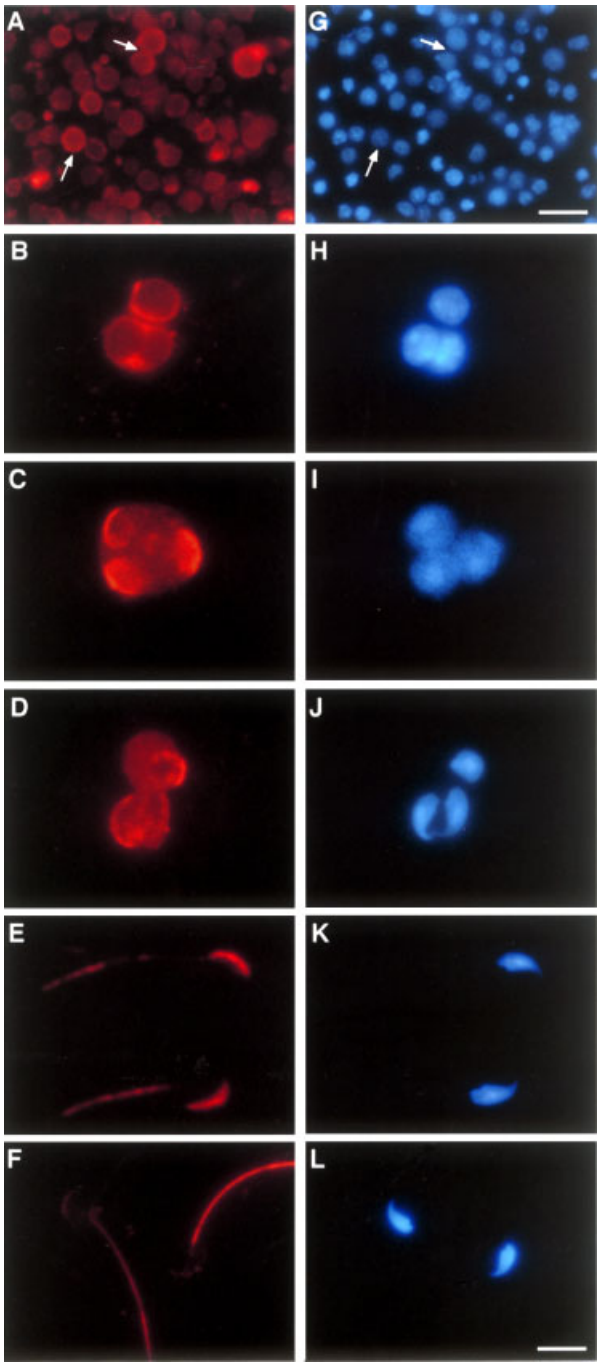

Fig. 4. Fluorescence microphotographs of (**left panel**) spg (**A**, arrows), spc (**B**), multinucleated round spermatid (**C**), elongated spt (**D**), spermatozoa (**E**), and acrosoma reacted spermatozoa (**F**) subjected to indirect immunofluorescence analysis with rabbit polyclonal anti PYK2 (#638) antibody. All samples were also stained with Hoechst for the visualization of chromatin (**right panel**) spg (**G**, arrows), spc (**H**), round spermatid (**I**), elongated spt (**J**), spermatozoa (**K**), and acrosoma reacted spermatozoa (**L**). Magnification: bar = 50 μm (A, G); bar = 15 μm (B, C, D, E, F, H, I, J, K, L).

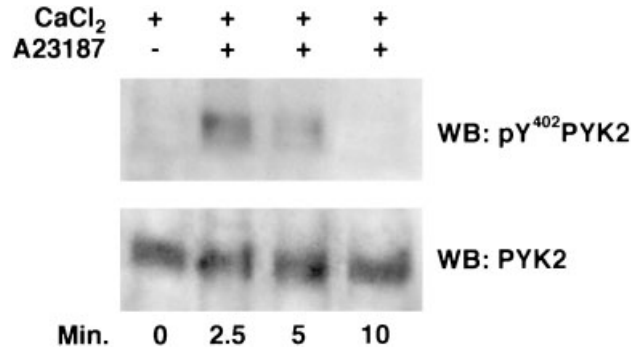

Fig. 5. Western blot detection of PYK2 proteins in the spermatozoa mouse extracts. The cells were incubated with $CaCl_2$ and with the ionophore A23187 (10 μM, Sigma Chemical Corporation, St. Louis, MO) at different time. Proteins (50 μg per lane) were resolved by SDS–PAGE, transferred to nitrocellulose membranes and then incubated with antibody raised against phospho-Y^{402} PYK2 and PYK2 (#600) protein. A specific band was observed at 110 kDa by comparison with co-migrating size marker.

activation appears to be linked to neurotransmitters, growth factors, and inflammatory cytokines that induce alterations in calcium-dependent signaling pathways (Lev et al., 1995). PYK2 could thus take part in the complex network of signal transduction pathways controlling both proliferation and differentiation. This may possibly occur via the activation of MAPK and/or JNK pathways (Lev et al., 1995; Dikic et al., 1996). It has been demonstrated that PYK2 associates with both microfilaments and microtubules, and play important roles for cellular movements such as membrane ruffflings, cellular attachments, and cytokinesis (Aoto et al., 2000). It is well documented that PYK2 is highly expressed in brain (Avraham et al., 1995), hematopoietic (Dikic et al., 1998), and prostatic (Stanzione et al., 2001; Picascia et al., 2002) tissues, and in a variety of cell lines (e.g., PC12) (Lev et al., 1995). However, the localization and the role of PYK2 in testes has not been elucidated yet.

In the present study, we show that the expression of PYK2 varies in amount in the different cellular types of mouse testis. In fact, Northern blot and immunoprecipitation analysis demonstrate that PYK2 is more abundant in spermacytes and spt among germinal epithelium lineages, and in ser. In addition, the immunofluorescence analysis clearly show that the diffuse cytoplasmic PYK2 immunoreactivity present in spg and spc involves in a specific cellular compartment in round spt which correspond in elongated spt to the acrosome region; subsequently, in the mature spermatozoa PYK2 is detected with a specific localization in the acrosome region and in the tail. We could hypothesized a possible implication of PYK2 in the capacitation process of spermatozoa; in fact, it is well known that in human and mouse spermatozoa the calcium is necessary for the capacitation mechanism and that during this process it is present a strong increase of phosphorylation on tyrosine residues of many proteins (Visconti et al., 1995; Visconti and Kopf, 1998).

Different PTKs expressing in testis have been identified by molecular cloning. For example, c-kit, a transmembrane *PTK* gene, is well documented to be involved in germ cell development (Sorrentino et al., 1991; Yoshinaga

et al., 1991). However, the relationship between PYK2 and the spermatogenesis has not been reported so far. To our knowledge, this is the first report showing the involvement of PYK2 in the spermatogenesis.

CONCLUSIONS

In conclusion, we have shown the involvement of PYK2 in mouse spermatogenesis by using different technical approaches. Our findings reported here support the idea that PYK2 activation could take part in a variety of cellular processes such as proliferation, differentiation, and spermatozoa capacitation. This study may give a clue to a better understanding the role of this tyrosine kinase product.

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