PAWP, a Sperm-specific WW Domain-binding Protein, Promotes Meiotic Resumption and Pronuclear Development during Fertilization*§1

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We report a novel alkaline extractable protein of the sperm head that exclusively resides in the post-acrosomal sheath region of the perinuclear theca (PT) and is expressed and assembled in elongating spermatids. It is a protein that shares sequence homology to the N-terminal half of WW domain-binding protein 2, while the C-terminal half is unique and rich in proline. A functional PPXY consensus binding site for group-I WW domain-containing proteins, and numerous unique repeating motifs, YGXPPXG, are identified in the proline-rich region. Considering these molecular characteristics, we designated this protein PAWP for postacrosomal sheath WW domain-binding protein. Microinjection of recombinant PAWP or alkaline PT extract into metaphase II-arrested porcine, bovine, macaque, and Xenopus oocytes induced a high rate of pronuclear formation, which was prevented by coinjection of a competitive PPXY motif containing peptide derived from PAWP but not by coinjection of the point-mutated peptide. Intracytoplasmic sperm injection (ICSI) of porcine oocytes combined with coinjection of the competitive PPXY peptide or an anti-recombinant PAWP antisemur prevented pronuclear formation and arrested fertilization. Conversely, coinjection of the modified PPXY peptide, when the tyrosine residue of PPXY was either phosphorylated or substituted with phenylalanine, did not prevent ICSI-induced fertilization. This study uncovers a group I WW domain module signal transduction event within the fertilized egg that appears compulsory for meiotic resumption and pronuclear development during egg activation and provides compelling evidence that a PPXY motif of sperm-contributed PAWP can trigger these events.

The perinuclear theca (PT)§ of the mammalian sperm head is a condensed cytosolic structure layered between the sperm acrosome and nucleus and, continuing caudally, between the plasmalemma and nucleus. On a compositional basis, the PT can be subdivided into three structurally continuous regions, the subacrosomal layer, the outer periacrosomal layer on the outer aspect of the equatorial segment and the post-acrosomal sheath (PAS) (1, 2). Traditionally, PT has been considered as a cytoskeletal scaffold responsible for maintaining the overall architecture of the mature sperm head. However, recent studies indicate that the bulk of proteins making up the PT are not traditional cytoskeletal proteins but rather a variety of cytosolic proteins linked together and susceptible to extraction under different regimens (3). For example, alkaline extractable SubH2Bv, exclusive to the subacrosomal layer, is implicated in acrosome-nuclear docking during spermiogenesis (1). Salt-extractable non-nuclear core histones, residing in the PAS, may be involved in stabilizing the chromatin of the decondensing sperm nucleus soon after oocyte entry (4, 5). The DTT salt or alkaline extractable calcin and cyclin II share a basic pl with the histones and bind to actin in vitro (6–8). Detergent- and salt-resistant fraction, “calyx fraction” of the PT, contains novel actin related proteins Arp-T1 and Arp-T2, which share a 60% sequence similarity to β-actin and may interact with calcin and cyclin in situ (7–9). The DTT-cetyltrimethylammonium bromide-extractable Stat6, a transcription factor, presumably found throughout the PT (10), can contribute to the zygotic development after fertilization. Together, these data imply that the PT is an assembly of specialized proteins that serve multiple functions in both spermiogenesis and fertilization. Based on the relative ease of solubilization of the PAS in

* This work was supported by Canadian Institutes of Health Research Grant MOP-62706 (to R. O.), National Research Initiative Competitive Grants 99-35203-7785 and 2002-35203-12237 from the United States Department of Agriculture Cooperative State Research, Education and Extension Service (to P. S.), by Food for the 21st Century Program of the University of Missouri–Columbia (to P. S.), by the Post-doctoral Fellowship Program of Korea Science and Engineering Foundation (KOSEF; to Y.-J. Y.), and by the National Institutes of Health R 21 Opportunities for Research at Regional Primate Research Centers (to R. O., P. S., and G. Schatten). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† This article was selected as a Paper of the Week.

§ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) AF393575 (human) and AF322215 (bull).

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

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the oocyte cytoplasm and the signaling nature of some of its constituents (2), the PAS is the most likely region to harbor sperm proteins regulating oocyte activation and early stages of zygotic development after fertilization.

Several hypotheses have been proposed to account for how oocyte activation is achieved by the spermatozoon during mammalian fertilization. The currently favored cytosolic factor model postulates that upon sperm-oocyte fusion, signaling molecules are released into the egg cytoplasm that activate the dormant egg by eliciting intracellular Ca\(^{2+}\) oscillations, which serve as a secondary messenger for downstream effectors of zygotic development (reviewed in Refs. 11 and 12). Thus far, two sperm molecules, tr-kit (truncated c-Kit tyrosine kinase) and phospholipase C\(\gamma\), have been shown to parthenogenetically trigger oocyte activation leading tocleaved mouse embryos. tr-kit appears to activate the oocyte via a Fyn-phospholipase C\(\gamma\)-mediated signaling pathway (13, 14); a similar pathway has also been established in echinoderm and ascidian eggs (15). The discovery of the sperm-specific phospholipase C\(\gamma\), on the other hand, offered the possibility that a phospholipase C protein of paternal origin was directly involved in triggering Ca\(^{2+}\) oscillations during oocyte activation (16, 17).

The role of sperm perinuclear theca during fertilization can be best appreciated from the observations that oocyte activation can be fully triggered by introducing a sperm head with only the nucleus and PT present via intracytoplasmic sperm injection (18). Furthermore, local solubilization of the PAS-PT is sufficient to elicit full oocyte activation in the absence of complete sperm incorporation into the ooplasm (2, 19). Kurokawa et al. (12) showed that three supernatants of successive sperm extractions (i.e., sonication, Triton X-100, and alkaline carbonate, pH 11.5) were all able to trigger Ca\(^{2+}\) oscillations comparable with those observed at natural fertilization. These investigators emphasized that only in the case of high pH treatment was the Ca\(^{2+}\) releasing activity fully released indicating that the factor was most likely a PT component according to our definition (4, 20). Perry et al. (21) proposed a so-called trans-complementation scheme of sperm-borne oocyte activating factors, detailing that full oocyte activation and embryo development can be achieved only with the combination of a DTT-soluble fraction obtained from Triton-demembranated mouse sperm head and the heat-stable component of the head after DTT extraction. Their observation indicated that at least two different sperm factors are involved during oocyte activation. Collectively, these observations corroborate our hypothesis that PAS-PT is the site housing sperm oocyte activating factor(s). In this context, we report the characterization of a novel, sperm-specific PAS-PT protein, PAWP, which appears to promote meiotic resumption and pronuclear formation, mediated by an unprecedented WW domain-signaling pathway during fertilization.

**EXPERIMENTAL PROCEDURES**

**Antibody Preparation and Immunoblotting**—Immune sera were raised in rabbits against the bull PT alkaline extract (anti-PT serum) as described previously (22). Once PAWP was cloned, polyclonal antibodies were raised in rabbits against two synthetic oligopeptides from both N- and C-terminal ends of its open reading frame, termed anti-PAWP\_N and anti-PAWP\_C, respectively. Immune serum was also produced in rabbits against the entire bull recombinant PAWP protein (anti-rec-PAWP). The four antibodies were affinity purified against rec-PAWP using a standard procedure (20). SDS-PAGE analyses in this study were performed on 4.5% stacking and 12% polyacrylamide gels. For the Western bloting procedure, either polyvinylidene difluoride microporous membrane (0.45 \(\mu\)m pore size, Millipore, Mississauga, Ontario, Canada) or nitrocellulose membrane (0.45 \(\mu\)m pore size, Schleicher & Schuell, Dassel, Germany) was used in protein transfer using a Hoefer Transphor apparatus (Hoefer Scientific Instruments). Immunoblot detection reaction was visualized by SuperSignal\textsuperscript{®} West Pico chemiluminescent substrate (Biolynx, Brockville, Ontario, Canada).

**Differential Sperm Head Treatments**—Isolated bull sperm heads underwent two cycles of freeze-thaw followed by centrifugation (14,000 \(\times\) g). The combined supernatants were then dialyzed and lyophilized for Western blot analysis along with the pellet. Isolated bull sperm heads were also treated with 0.1% Triton X-100 at 4 °C for 1 h and washed three times with PBS. The pellet was re-suspended in 15 mM DTT at 4 °C for 1 h. The suspension was then separated into supernatant and pellet by centrifugation for immunoblot analysis (20).

**Molecular Cloning of cDNA Encoding PAWP**—Anti-PT molecular cloning was used to screen a bull testicular Stratagene ZAP Express\textsuperscript{TM} cDNA library as described (1). One of six clones was chosen for full-length sequencing (Cortech, Queen’s University, Kingston, Ontario, Canada) and the sequence of both its nucleotide strands was determined.

For obtaining the human homologue of PAWP, primers were designed according to the draft sequence (GenBank\textsuperscript{TM} accession number XM\_001168) and used for standard reverse transcription-PCR followed by cDNA amplification (Qiagen, Mississauga, Ontario, Canada) with upstream primer (5'-ATG CCA TTT GAT CTG ATG-3') and downstream primer (5'-TAC CTC ATT GTC AGG TAG-3') at \(T_m\) 52 °C for 34 cycles. The human cDNA was inserted into pCR\textsuperscript{®} II-TOPO\textsuperscript{®} vector within an EcoRI restriction site (Invitrogen), sequenced, and deposited at GenBank\textsuperscript{TM} (accession no. AF393575). The mouse version of PAWP was cloned and amplified in the same fashion, with upstream primer (5'-GAG CTC GAT GCC ACT GAA CCA G-3') and downstream primer (5'-GGC GCC GCC AAG GTT AAC ATC TTA GAG C-3' (the underlined sequences represent SacI and NotI restriction sites, respectively) at \(T_m\) 52 °C for 34 cycles.

**Construction, Expression, and Purification of Bovine recPAWP**—Full-length PAWP cDNA was subcloned into the pET28b vector (Novagen, Madison, WI) between the HindIII and XhoI sites and sequenced for verification. The construct was then transformed into *Escherichia coli* BL21 DE3(PlyS) cells (Novagen) for expression and the subsequent nickel column purification of His-tagged recPAWP, using the protocol provided by the vendor (Qiagen). The His tag was removed using thrombin (Sigma).

**PAWP Sperm Equivalent Estimation**—The amount of PAWP present in a single spermatozoon was calculated based upon densitometric comparison of known concentrations of rec-
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PAWP with known amounts of bovine spermatozoa by utilizing anti-recPAWP antiseraum probed immunoblots. The reactive bands of the immunoblots were analyzed by using Scion Image Beta 4.02 Acquisition and Analysis software (Scion Corp., Frederick, MA). The standard curve generated from recPAWP (ranged from 0.025 to 0.2 μg) by using Microsoft Excel Data Analysis software was used for extrapolation of PAWP protein concentration present in a single spermatozoon.

Northern Blotting and Chemiluminescent Detection—Total RNA from various bovine and rat tissues was isolated by using RNeasy mini-preparation kit (Qiagen), separated by agarose gel electrophoresis and transferred to and UV-cross-linked to positively charged nylon membrane (0.45 μm pore size, Roche Applied Science). DIG-labeled sense and antisense riboprobes were synthesized and used for Northern blot hybridization according to the DIG System User’s Guide for Filter Hybridization (Roche Applied Science).

Ultrastructural Immunocytochemistry—The LR-white-embbeded bovine testicular and epididymal tissues were processed for immunogold labeling by using previously described procedures (5). Ultrathin sections were mounted on Formvar-coated nickel grids and blocked with 10% normal goat serum prior to overnight incubation of the primary antibodies at 4 °C. The sections were then washed and incubated with goat anti-rabbit secondary antibody conjugated to 10-nm gold particles (1:20; Sigma) followed by counterstaining with uranyl acetate and lead citrate. The final sections were analyzed by transmission electron microscope (Hitachi 7000).

Verification of Functional PPXY Motifs in PAWP—The binding the two putative PPXY motifs of recPAWP was examined by using GST-fused wild-type WW domains of YAP and Nedd4 (referred to as YAP and Nedd4). To show the specificity of the binding, two YAP WW domain mutants were used, YAPH129F and YAPP202A (all WW domain proteins, their respective mutants, and ligands were generous gifts from Dr. Marius Sudol) (23–25). Approximately equal amounts (5 μg) of purified GST-fused WW domains were separated by SDS-PAGE and transferred to nitrocellulose membrane for both positive binding and competitive inhibition assays. For positive binding assay, blots were blocked with 2% milk in PBS (pH 7.4, 0.05% Tween) and incubated with recPAWP (0.1 μg) in same buffer at 4 °C overnight followed by anti-recPAWP antibody detection. A synthetic peptide, Ac-PPVRYGSPPYEAPT-CONH2 (bold fonts indicate the PPXY motif overlapping with the underlined YGPPPXY motif) was designed as a competitive inhibitor to recPAWP by comparing bull, human, and mouse amino acid sequences. (Fig. 2b, inset). The above peptide was modified to demonstrate the specificity and the importance of the PPXY motif. In one modification, the tyrosine residue was constitutively phosphorlyated (PPGY), while in the other the tyrosine residue was substituted with phenylalanine, PPGF. A synthetic peptide Ac-GTGTKPRRTL-CONH2, matching amino acid sequence of a common bovine sperm head protein, aroylsulfatase A (Asa), was also used as a negative control. All peptides were synthesized, and purity was confirmed by HPLC (SynPep Corp., Dublin, CA). A PPXY (PY) ligand from WBP-1 (WW-binding protein 1) for YAP was also used as a competitive binding inhibitor (23, 26). For the competitive inhibition assay, PPXY peptide, its derivatives PPGY, PPGF, and PY ligand, were preincubated with Western or dot blots loaded with both wild-type and mutant WW domains at 4 °C overnight followed by incubation of recPAWP and immunodetection. In another competitive inhibition assay, YAP and its mutant YAPH129F were preincubated with recPAWP prior to applying the latter onto the blots and immunodetection. In this study, GST tag was removed from all constructs, since it posed steric hindrance. Affinity-purified anti-recPAWP antiserum was used to detect the presence of recPAWP on the blot. Control blots loaded with the equal amount of proteins were used to demonstrate that the anti-serum had no cross reactivity to the GST-fused WW domain proteins.

Porcine Oocyte Collection and in Vitro Fertilization—Ovaries were collected from pre-pubertal gilts at a local slaughterhouse, and oocytes were collected as described previously (27). Oocytes were washed twice and transferred into TCM-199 medium containing 0.1% polyvinyl alcohol, 10 ng/ml epidermal growth factor, 0.5 μg/ml FSH, 0.5 μg/ml LH, and 0.57 mm aspartame (mTCM-199) at 39 °C in atmosphere of 5% CO2 in humidified air. After 22–24 h, the oocyte-cumulus cell complexes were transferred to mTCM-199 without FSH and LH. They were cultured for additional 20 h under the same condition. Freshly ejaculated sperm-rich fraction was collected from a fertile boar and frozen as described previously (27). A sperm pellet was thawed in 2 ml of Dulbecco’s PBS (calcium- and magnesium-free) supplemented with 0.1% polyvinyl alcohol at 39 °C and separated on a two layer (80 and 60%) Percoll gradient (Amersham Biosciences AB, Uppsala, Sweden). The sperm pellet was re-suspended in modified Tris-buffered medium. The final sperm concentration for IVF was 1 × 106 cells/ml. Fertilized oocytes were cultured at 39 °C in North Carolina State University (NCSU)-23 medium with the addition of 0.4% BSA in atmosphere of 5% CO2 in humidified air.

Intracytoplasmic Sperm Injection (ICSI) and Microinjection of recPAWP—For the manipulation, microdrops of 6 μl of 4% PVP and 20 μl of HEPES-buffered NCSU23 with adjusted osmolarity (HEPES-NCSU23) were placed on a Petri dish and covered with mineral oil. Affinity-purified anti-recPAWP (1 μl) or its preimmune serum (1 μl, diluted 1:200 first) were diluted into 1 μl of sperm suspension (4 × 106/ml) in modified Tris-buffered medium (1:1) as well as the PPXY peptides and AsA control peptide (1 μl each, both with initial concentration of 1 mg/ml in modified KHM injection buffer (78 mM KCl, 0.5 mM MgCl2, 50 μM HEPES, pH 7.0)). This suspension (2 μl) was thoroughly mixed in a 6 μl drop of 4% PVP (final dilution of anti-serum and peptides, 1:8). ICSI was performed using an Eppendorf Cell Tram microinjection system equipped with Prime Tech piezo drill. After the spermatozoon was immobilized with piezo pulses, it was aspirated into a micropipette with PVP solution, with or without experimental molecules, and injected into the oocyte within a microdrop of HEPES-NCSU23. After ICSI, oocytes were washed twice and cultured in a 10-μl drop of NCSU23 for 8 h at 39 °C in atmosphere of 5% CO2 in humidified air. The microinjection of recPAWP into mammalian oocytes was carried out as described in ICSI experiments, except for the absence of an injected spermatozoon. An estimated 10 pl of solution containing 0.1 to 1.0 pg of soluble
recPAWP, control BSA, or AsA peptide (sham injection) was aspirated into the injection pipette. Pronuclear development was determined after processing of the oocytes for immunofluorescence.

**Mammalian Oocyte Processing and Immunofluorescence**—
Sperm-injected and in vitro fertilized oocytes were stripped of zona pellucida by a brief incubation in TL-HEPES containing 0.5% w/v protease (Sigma), fixed for 40 min in 2% formaldehyde in 0.1 M PBS, and permeabilized for 1 h in 0.1% Triton X-100 in 0.1 M PBS before immunofluorescence staining procedures (28). Oocytes were sequentially incubated with the affinity-purified anti-recPAWP (1:50; 40-min incubation) and goat anti-rabbit IgG-TRITC (1:80; 40 min; Zymed Laboratories Inc., South San Francisco, CA). To examine the formation of nuclear envelope and nuclear pore complexes, oocytes were incubated with a nucleoporin-specific mouse monoclonal antibody mAb 414 (1:200, Babco, Berkeley, CA) followed by a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Zymed Laboratories Inc.). Anti-c-Yes serum (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) followed by anti-rabbit IgG (1:80) was used to identify the assembly of c-Yes foci in the ooplasm of activated oocytes (Zymed Laboratories Inc.). The DNA stain DAPI (1:80) was mixed with secondary antibodies. The un-injected and sham-injected oocytes served as controls.

Bull, rhesus monkey, and boar spermatozoa were fixed and processed for immunofluorescence and DAPI staining as described previously (29). The primary antibodies used were anti-PAWP_N and anti-PAWP_C (1:200). Images were acquired with a Nikon Eclipse 1000 microscope with high numerical aperture objectives, and an RTE/CCD 1217 camera (Princeton Instruments, Inc., Trenton, NJ), operated by MetaMorph software. Digital images were edited by using Adobe Photoshop 6.0 software (Adobe Systems Inc., Mountain View, CA).

**Microinjection of recPAWP into Xenopus Oocytes**—
The isolation and manipulation of oocytes were performed as described previously (30). Sexually mature *Xenopus laevis* females were primed with gonadotropin (pregnant mare serum gonadotropin, 50 IU/frog, Sigma) 3 days before operations. Ovarian fragments were removed surgically under hypothermia and subjected to collagenase A digestion (2 mg/ml, Roche Applied Science, Mississauga, Ontario, Canada) in calcium-free OR2 medium (83 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1 mM Na2HPO4, 5 mM HEPES, pH 7.8), with 1 mg/ml soybean trypsin inhibitor (Sigma) for 3 h to obtain follicle-cell free oocytes. Stage VI oocytes were then individually selected and grouped. Collagenase-treated oocytes were stimulated with progesterone (5 μM) and germinal vesicle breakdown was assessed by the appearance of a white spot (maturation spot) on the pigmented animal pole after 3–6 h. Three hours after germinal vesicle breakdown, the mature eggs were arrested at metaphase II with the first polar body present (PB). Injection of alkaline PT extract or recPAWP (0.02 ng/ml) in a total volume of 27 nl with or without PPXY peptides (0.04 ng/ml) was conducted in calcium-free OR2 medium. Artificial activation was achieved by incubating metaphase II-arrested oocytes in OR2 medium with 0.5 μg/ml calcium ionophore A23187 (Sigma) for 60 s. Twenty minutes after injection (or ionophore treatment), oocytes were fix ed in 100% methanol for 30 min, rehydrated in 50% (v/v) methanol, and then transferred to Tris-buffered saline containing SYTOX Green (1:10,000; Molecular Probes) for detection of polar bodies as viewed under an Olympus IMT2-RFL inverted fluorescence microscope.

**RESULTS**

**Characteristics of Anti-PT Serum**—
The anti-PT serum used to immunoscreen the bull testicular cDNA expression library was raised in rabbits against the SDS non-extractable but alkaline extractable bull PT polypeptides (Fig. 1). The full or partial sequence identities of most of the prominent PT polypeptides (asterisks in Fig. 1) of this alkaline extractable group have been described previously (2, 3, 20). PAWP (arrowheads) is a less prominent member of this group, although it appears to be the most antigenic (lanes 3 and 4 in Fig. 1). Our cDNA immunoscreening took advantage of this heightened antigenicity and of the fact that the anti-PT serum from the first boost only recognized PAWP, and a previously cloned and characterized 15-kDa PT protein, SubH2Bv (1).

**Isolation of PAWP cDNA Clone and Sequence Analysis**—Positive cDNA clones were obtained from immunoscreening of a bull testicular cDNA library by using anti-PT serum (first boost), and after appropriate processing the clones were sequenced and found to be identical. The isolated cDNA clone was a transcript of 1.4 kb (see supplemental Fig. 1; GenBank™ accession number AF322215) and the longest open reading frame was a transcript of 1.4 kb (see supplemental Fig. 1; GenBank™ accession number AF322215).
frame was found to code for a 313 amino acid polypeptide (Fig. 2). Its calculated pI was 5.60 and molecular mass 31,966 Da. The BLAST search results of PAWP showed that in its N-terminal half it shares a 49% sequence similarity to WW domain-binding protein 2 (see supplemental Fig. 2; WBP2, accession numbers Q969T9 and P97765, respectively, for human and mouse homologues). In its C-terminal half, PAWP contains an unidentified repeating motif (number of repeats is species variable), which we denote as YGXPPXG (where X represents any residue). In addition, two (the number is species-variable) PY motifs, representing a consensus binding motif for group I WW domain are also found in this region of PAWP. The first PY motifs in bull and human PAWP overlap with the YGXPPXG motif to form YGPPPPGY, while in mouse PAWP, it is the third and fourth PY motifs that overlap with the YGXPPXG motif (Fig. 2).

Cloning of Human and Mouse PAWP—We were able to identify both the human (GenBank™ accession number AK129656) and mouse (GenBank™ accession number AK015863) homologues of bovine PAWP in GenBank™ and amplify them by reverse transcription-PCR of total testicular RNA using appropriate primers. Sequence similarities and unique features of PAWP found in bull, human, and mouse are shown by multiple amino acid sequence alignment in Fig. 2. In all three species, the N-terminal region is highly similar while the C-terminal region is more variable. However, in the C-terminal region, the hallmark YGXPPXG repeats of PAWP appear in all three sequences examined, 12 in bull, 9 in human, and 5 in mouse. Furthermore, all three sequences contain PY motifs (shaded sequences in Fig. 2): 2 in bull, 1 in human, and 6 in mouse. Interestingly, at least one PY motif is always found overlapping with the YGXPPXG motif.

Verification of the Identity of PAWP—Recombinant PAWP (recPAWP) was created in a bacterial system. Its authenticity was verified by immunoprobing with anti-PAWP_N and anti-PAWP_C antibodies. RecPAWP was then used for the production of a polyclonal antiserum (anti-recPAWP) and for affinity purification of all anti-sera raised against PAWP. After affinity purification, all anti-sera, including the serum used to immunoscreen the PAWP clone from the cDNA expression library, labeled a single 32-kD band in Western blots of bull PT extracts and sperm, confirming the identity of the cloned and deduced sequence (see supplemental Fig. 3).

Expression Profile of PAWP—Northern blots of total RNA isolated from various bull tissues were probed with DIG-labeled antisense RNA transcribed from PAWP cDNA and a 1.6-kb transcript was detected in testicular tissue (Fig. 3a). This tissue specificity was also verified by PCR on selected tissues (data not shown). Species-comparative Northern blot of total testicular RNA from bull, human, and mouse (Fig. 3a, lower panel) showed the presence of two transcripts in bull and human and an additional transcript in the mouse. A 1.6-kb band was most prominent in the bull and human, while a 2.6-kb band dominated in the mouse. Tissue-comparative Western blots (Fig. 3b) immunolabeled with anti-recPAWP antibody confirmed the preferential expression of PAWP in the testis. This antibody
was able to detect PAWP homologues in the sperm of different species (Fig. 3c).

Sperm Equivalent Concentration of PAWP—Our estimation of the average amount of PAWP present in a single spermatid from the bull is 0.08 pg by using known concentrations of recPAWP as standards in sperm immunoblot densitometric analysis (data not shown). PAWP is only partially extractable from sperm by DTT or freeze-thaw treatments with the larger portion residing in the insoluble fraction (Fig. 3d).

Localization of PAWP by Immunocytchemistry—Immunoperoxidase staining of the paraffin-embedded bull testicular tissue sections with anti-recPAWP antibody was restricted to the elongated spermatid population, and no immunostaining was evident in the round spermatids by this method. PAWP became prominent in the cytoplasmic lobe of step 11 spermatids, although it first became detectable in step 9 (Fig. 4a). Immunofluorescence labeling of mature spermatids of different species including the bull, rhesus monkey, and pig revealed its localization to the PAS-PT. In the bull, the labeling demonstrated an anterior-posterior gradation in the PAS-PT (Fig. 4b), while in the primate spermatid, it appeared as if PAWP delineated the anterior half of the PAS (Fig. 4c). In rabbit and porcine spermatids, PAWP also appeared as a distinct band in the PAS-PT region (Fig. 4d and e). It is important to note that this immunoreactive region is posterior to the crescent-shaped equatorial segment region of the sperm acrosome (arrow, Fig. 4e). At the ultrastructural level of cauda epididymal sperm sections, immunogold labeling with anti-recPAWP was found predominantly over the post-acrosomal sheath of the mature bull sperm PT (Fig. 5, a and b). No immunoreactivity was found in any other sperm structures (Fig. 5c). A fortuitous oblique section through the caudal face of the sperm head (Fig. 5d) indicated that PAWP is distributed over the surface of the PAS-PT.

Distribution of PAWP during Natural Fertilization and ICSI—The localization of PAWP during fertilization was examined by using both ICSI and IVF techniques in porcine oocytes. Both experiments revealed similar PAWP distribution inside of the ooplasm. Oocytes were fixed and processed 6–8 h post-insemination/IVF (Fig. 6, a–d) and 2–4 h post-ICSI (Fig. 6, e–h) to analyze the dynamics of PAWP at the stage of sperm nuclear decondensation and pronuclear development. Initially, PAWP appeared as a distinct band covering the PAS region below the equatorial region of the acrosome in the intact sperm (Fig. 6b). Migration of PAWP to an anterior pole of the sperm nucleus was observed during the initial swelling thus marking the onset of sperm nucleus decondensation and male PN development (Fig. 6, c and e). As the PAS-PT region of the nucleus dissolved, PAWP moved toward the apical pole of the nucleus (Fig. 6f). At this stage, the apical pole of the sperm nucleus was still intact, due to the characteristic persistence of a complex of subacrosomal PT layer and inner acrosomal membrane (28). When the sperm nucleus swelled, PAWP was seen around and throughout the early male pronucleus (Fig. 6g). In the nascent large male...
pronucleus after ICSI, only trace amounts of PAWP were found in the nucleoplasm (Fig. 6d). In more advanced zygotes with two large, apposed pronuclei surrounded by the nuclear envelope, little PAWP was detected in the nucleoplasm (Fig. 6h).

PAWP Specifically Binds to Group I WW Domain through Its PY Motifs—Examination of PAWP binding to group I WW domain (WWI) was carried out by far-Western analysis, using the recPAWP as a probe. Anti-recPAWP antibody was used to detect the affinity of recPAWP to the immobilized GST-fused WWI domains of YAP65 and Nedd4. Anti-recPAWP demonstrated no cross-reactivity to the GST-fused WWI domains we used in this study (Fig. 7a, lane 2). Both wild-type GST-fused WWI domains of YAP and Nedd4 demonstrated positive binding to recPAWP compared with their respective mutants YAP^{P202A} and YAP^{H129F} (these WWI domain mutants were created by single amino acid residue substitution) (Fig. 7a, lane 3). The specificity of the interaction between recPAWP and the
The WWI domain from YAP was further demonstrated in competitive inhibition studies (Fig. 7b). When YAP and YAPF202A WWI domains were preincubated with either a PPXF-containing construct obtained from WBP-1 or a synthetic PPXY-containing peptide from PAWP (see “Experimental Procedures”), recPAWP was unable to bind to YAP (Fig. 7b, lanes 3 and 4). This observation further strengthened the notion that the interaction between WW-YAP and recPAWP is via the PY motifs of recPAWP. Finally, co-incubation of recPAWP with PPXY mutant derivatives (i.e., PPXYF and PPGF) had no effect on binding of recPAWP to YAP as opposed to the inhibition caused by PPXY (Fig. 7c).

Pronuclear Formation in Porcine and Xenopus Oocytes Microinjected with recPAWP and Alkaline PT Extract—Microinjection of recPAWP consistently induced formation of a single pronucleus in MII-arrested porcine oocytes (Figs. 8, a and b, and 9) as well as in bovine (Fig. 9) and non-human primate (Fig. 9) oocytes and released Xenopus oocytes from metaphase II arrest, as indicated by the second polar body extrusion and

FIGURE 7. In vitro binding (far-Western) analysis of PAWP to type I WW domains via the PY motif of PAWP. a, lane 1 shows that approximately equal amounts (5 μg) of GST-fused WW domain constructs were transferred to nitrocellulose membranes and stained with Ponceau Rouge. The blot in lane 2 was probed with affinity-purified anti-recPAWP showing that there is no cross-reactivity of the anti-serum with the GST fusion proteins. Lane 3 demonstrates positive binding of recPAWP (0.2 μg) to the wild type WWI domain of YAP (YAPwt) and Nedd4 (Nedd4wt). The mutated WW domains of YAP, YAPF202A, and YAPH129F exhibited no binding activity with recPAWP. b, the binding specificity was further analyzed by competitive inhibition studies. Co-incubation of recPAWP and YAPF202A mutant did not show any inhibiting effect on recPAWP binding to YAPwt (lane 1), while co-incubation of recPAWP and YAPwt did (lane 2). When the blots were pretreated with PY construct from WBP-1 and PY-peptide, recPAWP was no longer able to bind to YAPwt (lanes 3 and 4, respectively). c, dot blots of the WW domain of YAP and its mutants showing the binding results after incubating with recPAWP (lane 2), recPAWP plus the PPXY containing peptide of PAWP (lane 3), recPAWP plus the PPXYF peptide (lane 4), and recPAWP plus the PPXF peptide (lane 5). Lane 1 shows that the anti-recPAWP antibody does not react with YAP or its mutants.

FIGURE 8. Parthenogenetic pronuclear development in porcine (Sus scrofa) and frog (X. laevis) oocytes upon microinjection of recPAWP. A large female pronucleus (b) with a nuclear envelope that contains nuclear pore complexes (f) and an up-regulation of c-Yes tyrosine foci (d) were observed in the recPAWP-injected porcine oocytes. Sham-injected oocytes on the contrary remained at MII-arrested stage (a, c, e). Similarly, sham or sham + BSA-injected Xenopus oocytes remained in the MII-arrested state with one polar body present (g). Injection of either alkaline PT extract or recPAWP induced a high degree of oocyte activation evident by the extrusion of the second polar body (h). The forming pronucleus of the activated oocyte, often difficult to observe due to the vast size and the non-transparent outer coat of the Xenopus egg, was caught sinking into the cytoplasm (PN and asterisk in inset) after activation with recPAWP. When the alkaline PT extract or recPAWP was co-injected with the PPXY peptide, the oocytes remained in the MII-arrested state as in the sham and BSA injected eggs (g), while co-injection of the mutated PPXF peptide was ineffective in blocking oocyte activation (see supplemental Fig. 4). DNA was labeled using DAPI in porcine oocytes and SYTOX Green in Xenopus oocytes. Bars = 10 μm.
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pronuclear formation (Figs. 8, g and h, and 10). Importantly, resumption of metaphase II induced by recPAWP could be prevented by the co-injection of a competitive PPXY motif containing peptide derived from PAWP but not by co-injection of the mutated PPXF peptide (Fig. 10), indicating that the interacting oocyte molecule was a group I WW domain containing protein. Also of note was that meiotic resumption induced by the sperm alkaline PT extract was also blocked by co-injection of PPXY peptide (Fig. 10). This inhibitory effect by the PPXY peptide indicates that egg activation elicited by the PT extract is PAWP-mediated, fortifying the PT egg-activating hypothesis put forward by Kimura et al. (18) and Sutovsky et al. (2). Recombinant PAWP-injected porcine oocytes displayed distinct cytoplasmic foci of tyrosine kinase c-Yes (Fig. 8, c and d), a proposed target for YAP and WBP-2, and a fully developed female pronucleus with a presumably functional nuclear envelope containing nuclear pore complexes (Fig. 8, e and f). Sham-injected control oocytes that were examined under differential image contrast microscopy and appeared to form a female pronucleus (counted as positive) had neither c-Yes foci in their cytoplasm nor nuclear pore complexes around their nuclei when examined by immunofluorescence.

**Pronuclear Formation during Pig Fertilization Is a PY-WWI Domain-mediated, Non-redundant Signaling Event**—To test the hypothesis that PAWP plays a distinct and independent role in the control of PN development during mammalian oocyte activation, porcine ICSI interference trials were carried out using anti-recPAWP serum and competitive and mutant PY peptides. When compared with the sperm plus preimmune serum-injected control oocytes, anti-recPAWP plus sperm-injected oocytes demonstrated a high incidence of fertilization/developmental arrest. In the latter group, the ooplasm-exposed sperm nuclei either remained intact or were arrested in the early stages of decondensation (Figs. 11, a and e, and 12a). Oocyte chromosomes also failed to form a pronucleus (Fig. 12a). The PPXY peptide was used in place of the anti-recPAWP anti-serum to examine whether PY-WWI domain interaction was essential for the function(s) of PAWP during fertilization. The ICSI-fertilized oocytes again showed high occurrence of arrested PN development (Figs. 11c and 12a). In contrast, the control oocytes co-injected with sperm and the AsA-peptide demonstrated normal fertilization and PN development (Figs. 11d and 12a). Because ligand interaction with WWI domain modules of signaling proteins is dependent on an intact and unphosphorylated tyrosine (Tyr) residue in PPXY containing ligands (31, 32), we next either substituted the Tyr residue in the synthetic PPXY peptide to phenylalanine (Phe) or phosphorylated the Tyr residue to test the specificity of the competitive PY peptide. The substitution significantly abrogated the competitive inhibitory effect of the PPXY synthetic peptide on ICSI induced male and female pronuclear formation (Fig. 12b). However, phosphorylation of the Thr residue had a lesser neutralizing effect, perhaps because of the susceptibility to dephosphorylation in the oocyte cytoplasm.

**DISCUSSION**

As an ongoing process of identifying and characterizing the proteins of the mammalian sperm PT, we report another member of this group that exclusively resides in the PAS, along with four somatic core histones (4). PAWP was found as a PAS component in the sperm of bull, mouse, rat, man, monkey, pig, and rabbit. The functional PY and signature YGXXPPXG motifs in PAWP, its sequence resemblance to WBP-2, and its ability to promote meiotic resumption and pronuclear formation are indicative of the important role of PAWP during fertilization.

PY motifs are identified in molecules mediating protein-protein interactions, which bind WWI domain protein modules found in a variety of cellular signaling pathways (32, 33). One of the important goals of this study was to examine whether the PY motifs of PAWP are functional. Our *in vitro* data showed that PAWP is capable of binding to various WWI domains including YAP, Nedd4, and dystrophin through its PY motifs but not to groups II and III WW domains. PAWP-WWI inter-

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**FIGURE 9.** Parthenogenetic activation of bovine, monkey, and porcine oocytes by microinjection of recPAWP (rPAWP). The activation was evaluated by the percentage of oocytes that formed a female pronucleus. The alkaline PT extract (PT), containing the majority of PT proteins, was also effective in stimulating meiotic resumption. Statistical analysis was performed by $\chi^2$ test. The difference between columns a and b are significant ($p < 0.01$).

**FIGURE 10.** Microinjection of recPAWP (rPAWP) or alkaline PT extract (PT) is effective in releasing *Xenopus* oocytes from metaphase II arrest. Single trial (all treatments done at the same time with the same group of isolated eggs) showing stimulatory effect of PAWP and PT extract blocked effectively by PPXY and the ineffectiveness of this peptide when the Y is replaced by F (PPXF). Statistical analysis was performed by $\chi^2$ test. The difference between columns a and b are significant ($p < 0.01$). For cumulative data of multiple separate trials with negative and positive controls see supplemental data.
action was highly specific as it was abolished by various point mutations in the WWI domains or PY motifs.

WW domains, named after its two highly conserved tryptophan (Trp) residues, are small functional domains, found in signaling proteins, which mediate protein-protein interactions in a similar fashion to Src homology 3 domains (33). WW domain-containing proteins and their cognate ligands have been demonstrated to be involved in a wide spectrum of cellular events including cell cycle control (Pin1/Ess1), ubiquitin ligation (Nedd4/Rsp5 and smurfl), and transcripational activation (YAP65) (33, 34). Relevant to this study, group I WW domain-containing proteins (i.e. YAP65, Nedd4, and dystrophin) recognize proline-rich effector molecules with a minimal consensus sequence of PY motif (24). Mutations or deletion of this PY motif lead to pathological states such as Duchenne’s muscular dystrophy (35) and Liddle’s syndrome (36, 37).

It is uncertain at this point which of the two PY motifs present in bovine PAWP is responsible for the WWI interaction. However, it has been suggested in the case of dystrophin-β-dystroglycan complex, where β-dystroglycan (ligand) contains two PY motifs, that the non-binding PY motif provides the stability of the complex (38). In the case of PAWP, the first PY motif that overlaps with the YGXXPPXGX motif of PAWP, is more likely to be participating in mediating the PAWP-WW I

**FIGURE 11.** Porcine ICSI with the co-injection of PAWP antagonists, including affinity-purified anti-recPAWP serum and a competitive PY peptide. Porcine oocytes were fixed at 8 h after ICSI and sequentially processed with anti-recPAWP antibody and anti-rabbit IgG-TRITC (a), the PY-peptide (b), or control synthetic peptide (c; AsA). To confirm that the co-injected anti-recPAWP serum indeed bound to its target in the sperm PAS-PT, the eggs injected with anti-recPAWP were fixed 8 h after being injected with a single spermatozoon in medium containing anti-recPAWP anti-serum (d; anti-recPAWP), pre-immune serum to anti-recPAWP (e; the shaded region of the coverslip are significant (p < 0.01)) normally arrested the development of both pronuclei. The asterisks indicate that the latter treatments were significantly different (p < 0.01) than ICSI without co-injection (ICSI), ICSI with preimmune serum (Pre-Im) and ICSI with AsA peptide. Panel b, mutation or phosphorylation of the tyrosine in the PPXY containing synthetic peptide effectively abrogated the competitive inhibitory effect of the peptide on ICSI-induced male (FPN) and female pronuclei (FPN). Each treatment consisted of three trials except two for the KHM control buffer. Statistical analysis was performed by χ² test. The difference between columns marked a and b are significant (p < 0.01).
domain interactions since its synthetic peptide can successfully compete for this interaction.

Given the involvement YAP65 in transcriptional control and its obligatory co-activation by PY containing proteins, PEBP2 (39) and WBP-2 (40) there is a possibility that sperm-contributed PAWP could modulate some aspect of early zygotic transcription. A sequence comparison of WBP-2 and PAWP (see supplemental Fig 2) reveals similar N-terminal halves suggesting conservation of a function that remains to be investigated. The only commonality in the C-terminal halves of these proteins is the shared PY motifs implying the involvement in WWI domain protein interactions. Unique to PAWP in this region is the presence of YGXXPPXG repeating motifs, occasionally incorporating the PY motif, which may provide protein recognition specificity. In fact the repeating YGXXPPXG motif along with the PY motif(s) appear to be the only conserved features of the C-terminal half of PAWP between species. The variability in the C-terminal region of PAWP may stem from interspecies differences in sperm head morphology and the molecular composition of the perinuclear theca. We suggest that it is the conserved N-terminal half of PAWP that dictates its function, while the C-terminal half ensures the binding specificity of PAWP through the YGXXPPPGY motif, which we have shown blocks sperm induced egg activation.

It has been established in mouse that the bulk of early zygotic transcription occurs within the male pronucleus, but the reason for this gender bias is not known (41). One possible explanation is that transcriptional factors bind preferentially to paternal chromatin due to its differential histone modification (42). It is plausible that a transcriptional factor or co-activator could be carried in the sperm head PT and migrates inside the nascent male pronucleus at an early stage of pronuclear development. The signaling properties and timing of PAWP migration from PT to male pronucleus in ICSI and IVF zygotes (this study) fit this pattern. An unrelated transcription factor, Stat4, has also been localized to sperm PT (10) and could be released into ooplasm at fertilization. The combination of a functionally versatile PY motif in PAWP and its strategic localization in the PAS region of sperm, where oocyte-activating factors presumably reside (18), prompted an examination of the involvement of PAWP in fertilization.

In addition to the cortical reaction (data not shown) and second polar body formation, a high rate of parthenogenetic pronuclear development was achieved after recPAWP microinjection into MII-arrested oocytes of different species and taxa. Oocytes injected with recPAWP exhibited a fully developed female pronucleus surrounded by a nuclear envelope equipped with nuclear pore complexes, and c-Yes tyrosine foci were observed throughout the ooplasm. These signs of activation were comparable with eggs fertilized in vitro indicating a resemblance to normal fertilization. In cases of spontaneous activation none of these indicators of activation were found. Interestingly, in ionomycin-activated rat eggs, it was demonstrated that resumption of meiosis and PN development were significantly hindered by Src family tyrosine kinase inhibitors, while cortical granule exocytosis was not (43). Another study in mouse demonstrated that during sequential fractionation of freeze-thaw sperm extracts, the ability to support pronuclear formation was lost before Ca$^{2+}$-releasing ability (44). Drawing from these studies, PAWP may function through its interaction with a WW-domain protein-Src family tyrosine kinase-protein complex (a precedent of interaction set by YAP65).

To confirm and examine the role of PAWP in pronuclear formation, ICSI was chosen for our inhibition experiments over IVF for several reasons. First, the co-injection of PAWP antagonists with the spermatozoa assures their sufficient concentration near the sperm head at the time of PAS-PT solubilization. Second, we avoided polyspermy, which occurs at high frequency during porcine IVF (27). Third, both the full compliment of paternal and maternal influences were taken into account thus enabling us to test the redundancy of any particular signaling molecule. As demonstrated, the anti-recPAWP antibody inhibited the formation of both male and female pronuclei, supporting the role of PAWP in PN development. Since our far-Western experiments showed that the PY ligands of PAWP specifically interact with WWI domain containing proteins, we hypothesized that this interaction, presumably with an oocyte WWI domain containing protein, is essential for egg activation. To test this hypothesis in ICSI, we substituted the antibody with our PAWP-PY peptide. Our hypothesis was confirmed by the prevention of pronuclear development and further validated by the fact that phosphorylation of the tyrosine residue or its substitution with phenylalanine rendered the PY peptide ineffective, as was predicted from previous PY-WWI domain interactions (31), from our far-Western analysis and from our parthenogenic trials.

The precise molecular mechanism by which PAWP contributes to meiotic resumption and pronuclear formation at present is not clear. Although there could be several alternative explanations, our data indicate that no indigenous sperm borne oocyte-activating factor introduced into the egg with the sperm was able to compensate for the PY peptide or anti-PAWP antibody-imposed blockade of fertilization after ICSI. Not ignoring the evidence for sperm-borne phospholipase involvement, a possibility remains that the PAWP-mediated signaling pathway acts upstream of calcium signaling. Most research points to a calcium increase as the sole requirement for resumption of the oocyte cell cycle (45, 46). However, we do not know yet if the PY peptide-induced block of oocyte activation affects sperm-induced calcium oscillations. Alternatively, the PAWP-mediated signaling pathway may act downstream of calcium-induced signaling during fertilization. In any case, the disruption of PAWP signaling prevents metaphase-anaphase transition of oocyte chromosomes and male pronuclear formation, leading to developmental arrest. Our data suggest that PAWP most likely imposes its effects on pronuclear development by interacting with an oocyte-derived WWI domain-containing protein. It is possible that the PAWP-WWI pathway targets tyrosine kinase regulation of the oocyte meiotic spindle and/or male chromatin remodelling factors. Future endeavors will focus on resolving how the PAWP-mediated pathway fits in with calcium oscillations during mammalian egg activation and on the identification of the interacting partner of PAWP in the oocyte.

In summary, we have uncovered a novel sperm-specific WWI domain-binding protein that exclusively resides in the
PAWP promotes meiotic resumption and pronuclear development by specifically interacting through its PY motif(s) with the WWI domain of an unidentified oocyte protein.

Acknowledgments—We thank Melissa Samuel, Miriam Sutovsky, and Jing Gong for excellent technical assistance and Dr. Don Li for help in statistical analysis. We thank Dr. Marius Sudol for the PY and WW domain constructs and Ashley Fraser for her PT31 evaluation. We are indebted to the following colleagues who have supported our work on PAS-PT and PAWP: Tanja Dominko, Diana Takashi, Gerald Schatten, Richard Stouffer, Richard Schultz, Carmen Williams, Jason Knott, and Rafael Fissore.

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