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Protein tyrosine phosphatases in *Chaetopterus* egg activation

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Changes in protein tyrosine phosphorylation are an essential aspect of egg activation after fertilization. Such changes result from the net contributions of both tyrosine kinases and phosphatases (PTP). This study was conducted to determine what role(s) PTP may have in egg activation. We identified four novel PTP in *Chaetopterus pergamentaceus* oocytes, cpPTPNT6, cpPTPNT7, cpPTPR2B, and cpPTPR2A, that have significant homology to, respectively, human PTPσ, -ρ, -D2 and -BAS. The first two are cytosolic and the latter two are transmembrane. Several PTP inhibitors were tested to see if they would affect *Chaetopterus pergamentaceus* fertilization. Eggs treated with β-bromo-4-hydroxyacetophenone (PTP inhibitor 1) exhibited microvillar elongation, which is a sign of cortical changes resulting from activation. Those treated with Na_3VO_4 underwent full parthenogenetic activation, including polar body formation and pseudocleavage and did so independently of extracellular Ca^{2+}, which is required for the Ca^{2+} oscillations that initiate development after fertilization. Fluorescence microscopy identified phosphotyrosine-containing proteins in the cortex and around the nucleus of vanadate-activated eggs, whereas in fertilized eggs they were concentrated only in the cortex. Immunoblots of vanadate-activated and fertilized eggs showed tyrosine hyperphosphorylation of approximately140 kDa protein. These results suggest that PTP most likely maintain the egg in an inactive state by dephosphorylation of proteins independent of the Ca^{2+} oscillations in the activation process.

Key words: egg activation, fertilization, invertebrate, protein phosphorylation, protein tyrosine phosphatase.

Introduction

Protein phosphorylation is an essential aspect of the signal transduction pathway for egg activation (Ciapa *et al.* 1991). In several species, protein tyrosine kinase (PTK) pathways lead to egg activation (Kinsey 1996). Initially, much of the evidence indicating that PTK has a role in egg activation was obtained using sea urchins (Kinsey 1997). Tyrosine phosphorylation transiently increases for a subset of proteins after fertilization (Ciapa *et al.* 1991), possibly including the egg receptor for sperm (Abassi & Foltz 1994), is crucial for spindle assembly (Wright & Schatten 1995), and is required for gastrulation (Livingston *et al.* 1998). Furthermore, a PTK-activated phospholipase Cγ (PLCγ) apparently plays a key role in the initial Ca^{2+} release in response to fertilization (Giusti *et al.* 2003). In *Xenopus*, PTK activity is essential for the early events in fertilization, such as vitelline envelope (VE) liftoff and normal calcium wave generation (Glahn *et al.* 1999). Moreover, a *Xenopus* oocyte Src-related tyrosine kinase, Xyk, is activated by tyrosine phosphorylation and translocates upon egg activation, independently or upstream of calcium signaling. In addition, *Xenopus* eggs require the upregulation of egg PLCγ that is associated with Xyk for activation (Sato *et al.* 2000). Xyk is thought to have involvement in the sperm receptor and is localized in the low density detergent-insoluble membrane of *Xenopus* eggs where Src family PTK has been identified (Sato *et al.*, 2002; Draberova & Draber 1993; Yamamura *et al.* 1997).

The involvement of PTK in fertilization has also been demonstrated in other organisms. Mipk, a protein kinase in sea stars that is a $p38^{MAPK}$ homologue, is one of the major tyrosine-phosphorylated proteins in immature oocytes arrested at the G2/M transition of meiosis I and is dephosphorylated during oocyte maturation (Morrison *et al.* 2000). Another MAP kinase in pig oocytes initiates calcium release, cortical granule exocytosis, and progression to the first interphase (Kim *et al.* 1999).

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The phosphorylation state of proteins represents the net effects of both kinases and phosphatases. Since egg protein tyrosine phosphorylation increases rapidly in response to sperm binding (Ciapa *et al.* 1991), protein tyrosine phosphatases (PTP) must inhibit the tyrosine phosphorylation before the sperm binds to the egg. The possible roles of PTP at fertilization have been investigated in the sea urchin (Wessel 1995; Wessel *et al.* 1995). The data showed that the eggs and embryos contain coding information for several PTP, supporting the idea that PTP may play an important role in signaling at fertilization.

Protein tyrosine phosphatases have critical functions in development and differentiation. In *Caenorhabditis elegans, PTP-2* plays an important role during oogenesis and vulval development (Gutch *et al.* 1998). It is thought to participate in a cascade that signals the exit from pachytene arrest. A mutation in *PTP-2* causes abnormal oogenesis, with the oocytes growing 2–3-fold the size of the mature wild type. Two PTP patterns and their functions relative to PTK have been characterized in *Ascaris* eggs by following their activities from the four-cell stage to the fully developed larva (Wimmer *et al.* 1998). PTP activity decreases from the four-cell stage to the larva. In addition, PTP relocalizes during development. The enzyme colocalizes with PTK in the VE in unfertilized oocytes, whereas in fertilized eggs (21 days), PTP is distributed mostly near the mitochondria and sporadically in the cytosol (Wimmer *et al.* 1998).

Recently, a specific function has been found for a PTP at fertilization in zebrafish eggs (Wu and Kinsey 2002). Fyn kinase activity is suppressed in eggs pretreated with peroxyvanadate. This enzyme requires a PTP for dephosphorylation and activation (Wu & Kinsey 2002). This PTP activity co-immunoprecipitates with the Fyn SH2 domain. It was characterized as rPTPα.

Eggs of the marine polychaete annelid, *Chaetopterus*, have several advantages that make them a valuable model system for investigating these questions. First, the sperm make contact with morphologically definable receptors that are restricted to the tips of, and then fuse with, the egg microvilli (Anderson & Eckberg 1983; Eckberg & Anderson 1997). Thus, the domain of the egg surface with which sperm interact is at least partially defined, enabling us to focus on the transduction and/or fusion events and mechanisms between the sperm and egg. Second, like those of most animals, *Chaetopterus* eggs undergo repetitive calcium oscillations in response to fertilization or artificial activation (Eckberg & Miller 1995). Third, artificial activation of these eggs leads to a well-characterized process called differentiation without cleavage that closely mimics many aspects of normal development including a unicellular form of gastrulation and ciliation so developmental activation can be studied specifically (Lillie 1902; Eckberg 1981; Eckberg & Anderson 1995). By contrast, sea urchins have a single Ca^{2+} wave and cortical granule exocytosis; this process is parallel to, but not causal, for development

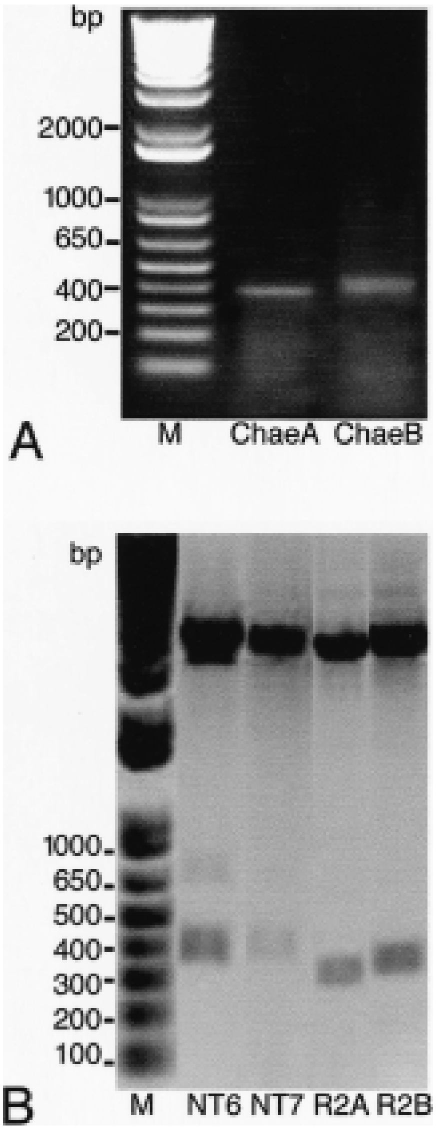

Fig. 1. RT/PCR and cloning of *Chaetopterus* oocyte phosphatase (PTP) mRNAs. (a) Amplification of PTP cDNA. ChaeA and ChaeB represent the products from the different primer sets. Polymerase chain reaction (PCR) products were resolved in a 1% agarose gel (1X TAE). The symbol M represents the marker, a 1 Kb Plus DNA Ladder. PCR yielded products about 360 bp. (b) Ligated PCRII clones were electrophoresed through a 1% agarose gel (1X TAE). The symbol M represents the DNA marker. The clones were verified by restriction digestion with EcoR1 to release the approximately 360 bp inserts. The clones with inserts were sequenced and found to represent four families of PTP, NT6, NT7, R2A, and R2B.

and is the usual event taken as evidence of egg activation. Fourth, elevated Ca^{2+} is apparently the only signal for egg activation, as cytoplasmic pH changes have no role in egg activation (Eckberg & Dubé 1996). The purpose of this study was to identify steps in the activation process that may be dependent on tyrosine phosphorylation. Therefore, we tested whether PTP are present in *Chaetopterus* eggs and studied their role in egg activation.

Materials and Methods

Gamete collection

Oocytes were collected from adult *Chaetopterus pergamentaceus* (Cape Fear Biological, Southport, NC, USA; or The Marine Biological Laboratory, Woods Hole, MA, USA). Gametes were collected, handled and washed as described (Eckberg & Hill 1996). Meiotic maturation to metaphase I was elicited by suspending the oocytes in natural seawater (NSW).

Isolation and sequence of Chaetopterus *cDNA*

Total oocyte RNA isolated by the Trizol (GibcoBRL, Grand Island, NY, USA) method was pelleted and dissolved in 50 µL double deionized water. RNA (2 µg) was reverse-transcribed to cDNA by using the Superscript II RNase H^- Reverse Transcriptase method (GibcoBRL). The cDNA was amplified using degenerate oligonucleotide primers corresponding to the consensus sequences FWRM(I/V)W and HCSAG(V/T/A) (referred to as CHAEA) and XDFWRM and AGVGRT (referred to as CHAEB), the sequences used to isolate PTP from hagfish (Ono-Koyanagi *et al.* 2000). These primers were designed at motifs 5 and 9 of the PTP catalytic domain. They were expected to yield products of ~360 bp. Primers (final concentration of 7 ng/µL) were added to 100 µL polymerase chain reaction (PCR) product. Thirty cycles were performed on a PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA, USA). Each cycle involved an incubation at 94°C for 1 min, 46°C for 1 min, and 72°C for 2 min. An additional incubation at 72°C for 10 min was added at the end of the program. The PCR product was analyzed on a 1% agarose PCR grade gel (Fisher Scientific, Pittsburgh, PA, USA) and the expected fragments of ~360 bp were observed (Fig. 1a). Control reactions using antisense primers yielded no product (data not shown). The PCR products were immediately excised from the gel following the Qiagen (Valencia, CA, USA) PCR purification protocol, and ligated into a pCR II vector using the TA Cloning Kit for molecular studies (InVitrogen, Carlsbad, CA, USA).

Sequence analysis

Twelve cDNA clones that contained the expected band size were sequenced. The vector sequence was removed, and the sequences were analyzed using the basic local alignment search tool (BLAST 2.2.1) (Altschul *et al.* 1997) using the BLATX and BLASTP programs at the National Center of Biotechnology Information (NCBI, Bethesda, MD, USA). To determine the open reading frame and the peptide sequence, we used the European Bioinformatics Institute (EBI, Hinxton, UK) tools for Transeq. The longest reading frame was selected. The amino acid sequence for this reading frame was blasted with the NCBI BLASTP program to ensure that the peptide was a PTP.

Sequence alignment and phylogenetic tree

Alignment of the PTP amino acid sequences was carried out by the multiple alignment program, Clustal

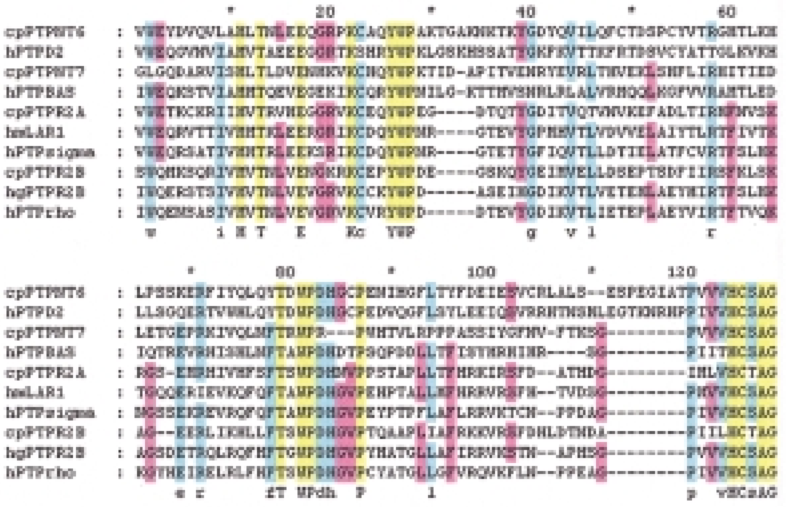

Fig. 2. Sequence analysis of cpPTPs. The deduced amino acid sequences of *Chaetopterus* PTP, cpPTPNT6, cpPTPNT7, cpPTPR2B and cpPTPR2A, and their closest human PTP relatives were determined and compared. A few invertebrate or early vertebrate homologues were included in the alignment. The number above each column is the position of the amino acid. The consensus amino acids are indicated below the sequences. Conserved amino acid sequences in these motifs are shaded. Yellow, 100% identity; blue, 90% identity; pink, 60% identity.

W (Thompson *et al.* 1994). The human PTP data were obtained from a web page for PTP (http://ptp.cshl.edu). The phylogenetic tree of the PTP family on the basis of the alignment was inferred by the NJ method (Saitou & Nei 1987) and viewed using the Tree View program.

Fertilization and imaging of early development

Treatments were carried out in 24-well culture dishes in NSW or calcium-free artificial sea water (CaFASW). After the oocytes underwent germinal vesicle breakdown (GVBD), they were inseminated or treated with the $NaMoO_4$, Na_3V0_4, PTP inhibitor 1, bis N,N-dimethylhydroxamide hydroxooxovandate (DMHV), or 3,4, dephostatin at various concentrations. Then the oocytes were evaluated for 1st and 2nd polar body formation, polar lobe formation and cleavage or pseudocleavage (characteristic of parthenogenetic eggs). In some experiments, the oocytes were allowed to incubate overnight to determine whether or not differentiation proceeded to the formation of pseudolarvae. Development was observed by phase contrast microscopy (Nikon Diaphot).

Immunoblotting

Aliquots of the egg suspension were collected at various times and lysed in 1% SDS sample buffer containing 10mM Na_3VO_4 to prevent protein dephosphorylation occurring in the lysis buffer. The lysates were boiled for 5 min and resolved on a 10% SDS polyacrylamide gel (Laemmli 1970). Prestained molecular weight markers (Kaleidoscope; Bio-Rad, Hercules, CA, USA) and phosphotyrosine markers (Cat, No. 525324; Calbiochem, La Jolla, CA, USA) were resolved in parallel with the samples. The gels were equilibrated with transfer buffer and the proteins transferred electrophoretically to polyvinylidene fluoride (PVDF). Blots were probed with a monoclonal antiphosphotyrosine^{2+}and horseradish peroxidase-conjugated secondary antibody (Calbiochem, San Diego, CA, USA). The bands were visualized by enhanced chemiluminescence.

Fluorescence microscopy

We used immunofluorescence to determine the intracellular location of protein phosphotyrosine. Fertilized

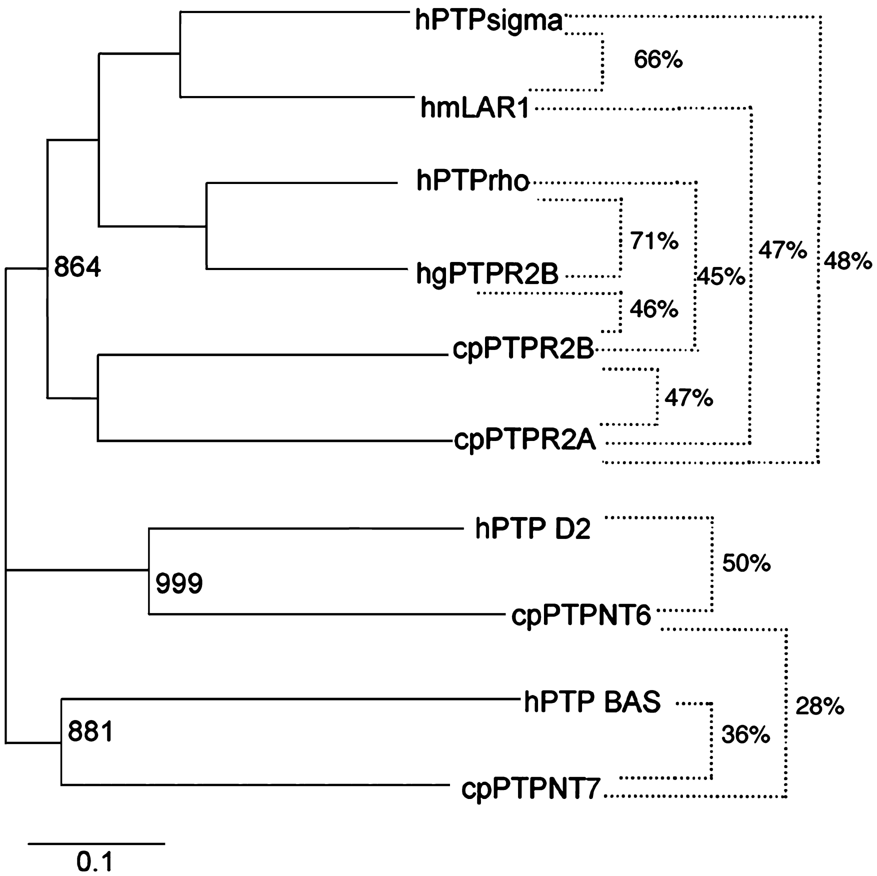

Fig. 3. Unrooted phylogenetic tree of cloned *Chaetopterus* PTP and their closest relatives. Each group represents the cloned cpPTP and its closest relative. The horizontal distance indicates the degree of divergence. The scale below the tree represents the decimal fraction of amino acid substitution events. Four PTP subtypes were identified: two receptor-type and two nontransmembrane. The bootstrap values at the dendrogram nodes give the significance of the sequence similarity within the subtypes. The subdivisions were assigned based on a maximal bootstrap value of 1000.

and Na_3VO_4-activated eggs were fixed in 4.0% paraformaldehyde. The samples were frozen and sectioned at 20 µm. The sections were washed three times with phosphate-buffered saline (PBS), 10 min each, blocked with 1% bovine serum albumin for 2 h and incubated with anti-phosphotyrosine (Calbiochem) at 4°C overnight. The sections were washed three times at 10 min intervals with PBS. The antibodies were detected with Texas red goat anti-mouse IgG antibody (Oncogene Science, Cambridge, MA, USA) for 1 h and washed three times with PBS. The eggs were mounted on a slide with 50% glycerol to prevent evaporation and antibody fading. The sections were analyzed by epifluorescence.

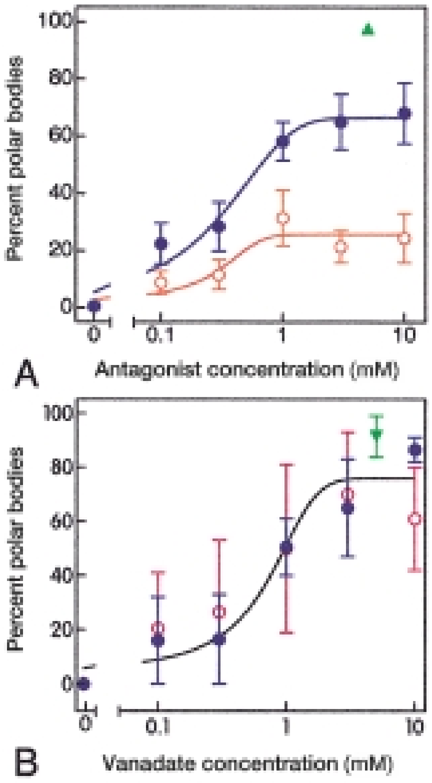

Fig. 4. Egg activation by PTP antagonists. (a) Oocytes that had completed germinal vesicle breakdown (GVBD) were fertilized (triangles) or incubated in the indicated concentrations of Na_2MoO_4 (open circles) or Na_3VO_4 (filled circles). The percentage of eggs with 1st polar bodies was determined 60 min later. Both PTP inhibitors activated the eggs in a concentration-dependent manner. Na_3VO_4, was more effective than Na_2MoO_4. However, the half-maximal stimulatory concentrations for the two compounds were similar. (a) Eggs were incubated in natural seawater (NSW) (filled circles) or calcium-free artificial seawater (CaFASW) (open circles) with indicated concentrations of vanadate. The inhibitor activated eggs in both NSW and CaFASW. Since the data for the two conditions were so similar, the regression line was computed from both data sets pooled together.

Results

cDNAs encoding PTP

We used the PCR approach to determine if PTP were expressed in *Chaetopterus* oocytes. Both sets of degenerate primers produced the expected bands at about 360 bp (Fig. 1b). To determine if the *Chaetopterus* clones were PTP, 12 of them were sequenced. From these we identified four unique PTP-related cDNAs, which we designated cpPTPNT6 (AY174171), cpPTPNT7 (AY174172), cpPTPR2A (AY174173), and cpPTPR2B (AY174174), according to the PTP family to which they belonged (Figs. 1b and 2). These sequences were aligned with the closest human and some invertebrate PTP sequences. The cloned cDNAs contained the PTP signature motifs that identified them as PTP cDNAs. The cloned cDNAs shared 36%–50% amino acid sequence identity as their human counterparts (Fig. 3).

Phylogenetic analysis

To classify the homologous proteins, we derived a phylogenetic tree from our alignment of cpPTP and their closest homologues (Fig. 3). Three PTP domain subtypes were identified: NT7, NT6, and a receptor type. Two of the cDNAs, cpPTPNT6 and cpPTPNT7, represented nontransmembrane PTP subfamilies and were most closely related to the human PTPD2 and PTPBAS (Fig. 3). The other two were receptor-like. Their closest human homologues were PTPσ and PTPρ, which are in the R2A and R2B subfamilies. To determine the significance of the sequence similarity within these subtypes, the bootstrap values at the dendrogram node were calculated. All subtypes were defined based on maximal bootstrap values of 1000.

PTP inhibitors activate Chaetopterus *oocytes*

Our finding of two different rPTP and the fact that the cytoplasmic subfamilies identified contain domains that interact with integrins and cytoskeletal elements allowed us to hypothesize that one or more of these PTP is involved in transducing the activating stimulus at fertilization. Accordingly, we investigated the biological function of PTP in *Chaetopterus* oocytes by testing the effects of various PTP inhibitors on egg activation. We treated oocytes with $NaMoO_4$, Na_3VO_4, PTP inhibitor 1, DMHV, or 3,4, dephostatin at various concentrations and evaluated them for activation and development. DMHV and dephostatin proved toxic to the eggs and were not further studied.

Contrary to our expectations, treatment of oocytes with Na_3VO_4, Na_2MoO_4, or PTP inhibitor 1 did not prevent activation by sperm. Instead, each activated the eggs directly. PTP inhibitor 1 activated VE wrinkling, microvillar elongation and cortical changes associated with egg activation, but no further development (data not shown). Complete egg activation, as determined by polar body formation and pseudocleavage, was concentration-dependent for Na_3VO_4 and Na_2MoO_4 (Fig. 4a). However, at all concentrations tested more eggs activated in response to Na_2VO_4 than Na_2MoO_4. Most Na_3VO_4-activated eggs formed polar bodies by 35 min and underwent pseudocleavage by 170 min. Since the percent activated was always greater after Na_3VO_4-activation, Na_3VO_4 was used in subsequent experiments.

To determine if extracellular calcium is required for egg activation by Na_3VO_4, oocytes were incubated in CaFASW with the inhibitor. Na_3VO_4 also activated the eggs in CaFASW as effectively as in NSW (Fig. 4b). Therefore, egg activation by PTP inhibition bypasses the extracellular Ca^{2+} requirement for activation by fertilization or excess K^+. Thus, PTP must impact a step in the activation process that is independent of the Ca^{2+} oscillations that provide the primary activating stimulus (Eckberg & Miller 1995).

To determine if vanadate-activated eggs develop as K^+-activated eggs do, eggs were incubated in Na_3VO_4 or excess K^+ and observed for the later signs of parthenogenetic activation. Eggs treated with Na_3VO_4

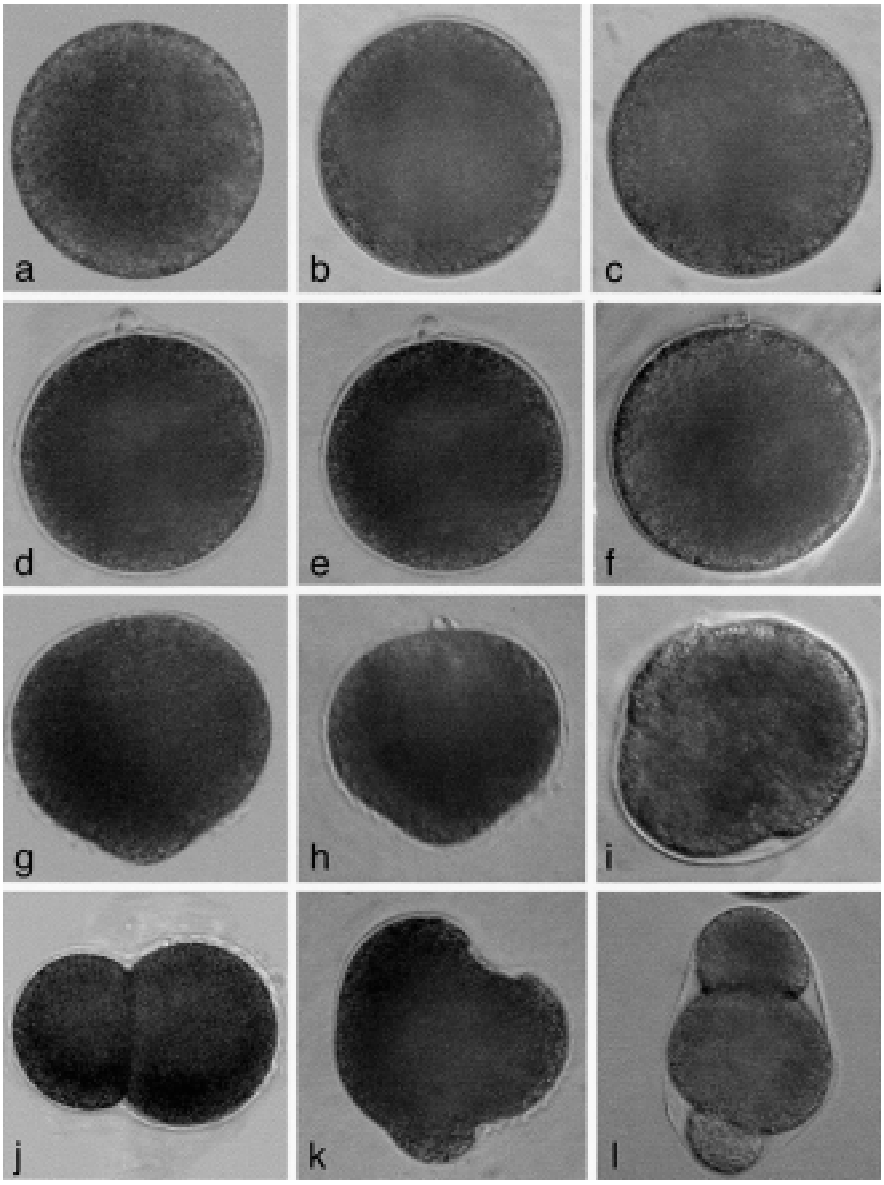

Fig. 5. Morphology of activation by KCl and vanadate. Eggs activated by sperm (a, d, g and j), KCl (b, e, h and k) or Na_3VO_4 (c, f, i and l) were examined microscopically at intervals. Photographs were taken at 0 min (a, b and c) and at the following various developmental stages: polar body (d, e and f), polar lobe (g, h and l), 2-cell stage (j) or beginning of the amoeboid contractions (k and l).

developed similarly to those activated by excess K^+ (Fig. 5). Na_3VO_4 treated oocytes underwent pseudo-cleavage as did KCl-activated eggs when the fertilized eggs cleaved (Fig. 5). Vanadate actually proved to be a more effective agent for activating eggs than excess K^+. K^+-activated eggs generally did not form a 2nd polar body, whereas vanadate-activated eggs formed 2nd polar bodies within 5 min of the time the fertilized eggs did. Embryos and parthenotes were observed swimming after 24 h (data not shown). Since PTP antagonists activated eggs, we investigated the phosphotyrosine-containing proteins in these activated eggs.

Tyrosyl-phosphorylated proteins after fertilization

We performed immunoblots to determine whether tyrosine phosphorylation increased in fertilized or parthenogenetic eggs. Fertilized eggs showed transient hyperphosphorylation of one protein whose molecular mass was ~140 kDa (Fig. 6). pp140 was hyperphosphorylated only in the first 1–3 min after fertilization. This corresponds to the time period during which egg activation becomes irreversible in protostomes (Tyler & Schultz 1932; Allen 1953). Thus, the timing of hyperphosphorylation of pp140 corresponds exactly to the time during which the fertilized egg becomes committed to develop. Exposure of oocytes to excess K^+ did not stimulate tyrosyl phosphorylation of this protein (Fig. 6). Rather, tyrosyl phosphorylation of pp140 gradually decreased, becoming almost undetectable within 60 min of the oocytes first being exposed to KCl. Activation by Na_3VO_4 stimulated phosphorylation of pp140, but pp140 did not become dephosphorylated in vanadate-activated eggs during the period of study (Fig. 6).

Localization of phosphotyrosine in eggs

The identification of a tyrosyl phosphoprotein possibly associated with commitment to egg activation and development led us to identify the sites of tyrosine phosphorylation in eggs during *Chaetopterus* fertilization and vanadate-activation by antiphosphotyrosine immunofluorescence. The time intervals chosen included those of polar body formation (10 and 30 min) and cleavage (60 min). The phosphotyrosyl proteins were primarily restricted to the cortex in fertilized eggs (Fig. 7f). In addition to the cortex, the perinuclear area stained in vanadate-activated eggs (Fig. 7v).

Discussion

Four PTP-encoding mRNA were identified in *Chaetopterus* oocytes. These PTP are among the classical family of PTP and include both cytosolic and receptor-like forms. Each is closely related to a human PTP. Moreover, each appears to be catalytically active, except possibly cpPTPNT7. An aspartic acid

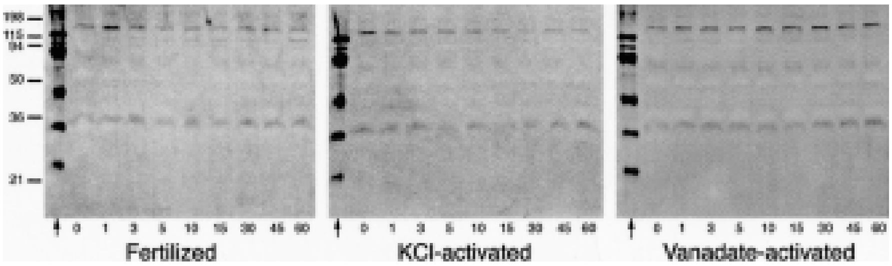

Fig. 6. Tyrosine phosphorylated proteins after fertilization. Immunological detection of protein phosphotyrosine in fertilized, KCl-activated, and vanadate-activated *Chaetopterus* eggs during development. Eggs were sampled at the indicated times (min) after exposure to the stimulus. Eggs were lysed, subjected to SDS-polyacrylamide gel electrophoresis in 10% T gels and blotted to PVDF. Blots were probed with antibody specific for phosphotyrosine. The arrows indicate phosphotyrosine protein standards.

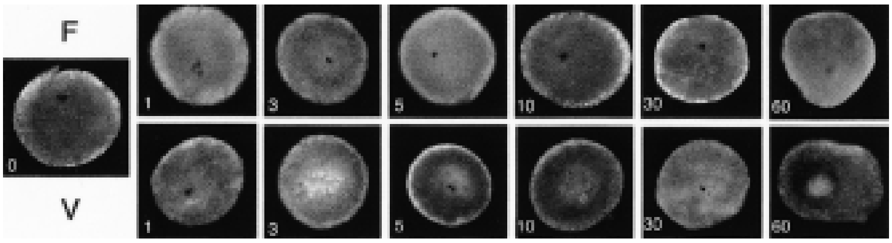

Fig. 7. Localization of tyrosine phosphoproteins in eggs. *Chaetopterus* egg tyrosine phosphoproteins were detected by immunofluorescence. The numbers indicate the time in min after exposure to sperm (F) or vanadate (V).

that is conserved throughout evolution in the WPD loop or catalytic site surface loop (Fig. 2) functions in the general acid-general base catalysis of PTP (Zhang *et al.* 1994); this amino acid is missing from cpPTPNT7. However, each cpPTP contains the PTP phosphate-binding loop HCX$_5$R(S/T) (Charbonneau *et al.* 1989). These homologies indicate that PTP had undergone substantial radiation very early in metazoan evolution. By analogy with their closest human homologues, the cpPTPs may have role(s) in cell growth and development.

The human homologue of cpPTPR2A is hPTPσ. This PTP was cloned and identified as a member of the type IIA family of rPTP, which includes LAR and PTPδ (Pulido *et al.* 1995). PTPR2A are surface glycoproteins characterized by the existence of an extracellular domain composed of three tandemly-arranged Ig-like domains and 5–8 FNIII-like repeats that give them characteristics of adhesion molecules, and two intracellular PTP domains (Edelman & Crossin 1991). PTPR2A exist in multiple isoforms. PTP of this family play a major role in regulating axon growth in embryos and in mediating the autophosphorylation of the epidermal growth factor (EGF) receptor (Graness *et al.* 2000; Johnson *et al.* 2001). A PTP in this family from *Caenorhabditis elegans*, CLR-1, is a negative regulator of the fibroblast growth factor (FGF) -receptor (Kokel *et al.* 1998). Moreover, the related LAR1 is required for patterned outgrowth in medicinal leech (Baker & Macagno 2000). The closest mammalian relative, PTPσ, down-regulates EGF-receptor autophosphorylation and has been demonstrated to be linked to type II diabetes in rats (Graness *et al.* 2000; Ostenson *et al.* 2002).

The other rPTP identified in these studies is in the PTPR2B family. It differs from the PTP2A family in that the extracellular domain is composed of a mepin, A5, µ domain (MAM), an Ig-like domain and four FNIII repeats. The intracellular domain has a cadherin-like domain in addition to the two PTP domains (Andersen *et al.* 2001). Thus, the PTPR2B family is likely to be involved in cell to cell contact and development. However, the function of the PTP in this family, including PTPμ, PTPκ, PTPρ, and PTPλ, are not as defined as those of the PTPR2A family. The closest vertebrate homologue to cpPTPR2B is PTPρ. This homologue is expressed in the developing visual system of *Xenopus* (Johnson & Holt 2000).

In addition to the rPTP, we identified two nontransmembrane PTP, cpPTPNT6 and cpPTPNT7. These also belong to different subfamilies, PTPNT6 and PTPNT7, respectively. These subfamilies differ in that PDZ domains are found in the NT7 family. Both families have a FERM (4.1, ezrin, radixin, moesin) domain and a PTP domain (Moller *et al.* 1994). The FERM domain suggests that they are involved in the regulation of the cytoskeleton and cell adhesion. FERM superfamily proteins interact with integrins through the N-terminus and with the cytoskeleton through the C-terminus, forming a bridge between the plasma membrane and cytoskeleton (Tsukita & Yonemura 1997). *Chaetopterus* cpPTPNT6 was identified as a member of the NT6 subfamily. Its closest human homologue is PTPD2, which, when overexpressed, affects the cytoskeleton, cell adhesion and cell growth (Ogata *et al.* 1999). In addition, Pez, an isoform of PTPD2, when isolated from normal breast cancer cells translocates to the nucleus when cells are induced to proliferate (Wadham *et al.* 2000). Thus, cpPTPNT6 may be involved in regulating the cytoskeleton, cell adhesion, and cell growth. Since the microvillus elongation and VE wrinkling characteristic of pseudocleavage are cytoskeleton-dependent processes, this PTP may regulate them.

The NT7 family, which includes PTPBAS, contains the FERM domain found in the NT6 family in addition to five PDZ domains (Maekawa *et al.* 1994). These are thought to cluster membranes and/or link signaling molecules to a multiprotein complex at a specialized membrane site (Tsunoda *et al.* 1997). PTPBAS was cloned from a basophilic leukemia cell line (Maekawa *et al.* 1994). Domains 2 and 4 of PTPBAS are associated with the FAS receptor (Sato *et al.* 1995). In addition, the PTPBAS PDZ 1 domain associates with IκBα, which activates NF-κB. The close homology of IκB and NF-κB with cactus and dorsal in *Drosophila* indicates that the presence of proteins in *Chaetopterus* that interact with this pathway should not be surprising, even though their functions remain to be determined.

The finding of rPTP and especially the identification of cpPTPR2A led us to hypothesize that one or more of the rPTP may be involved in transducing the activating stimulus by analogy with the roles of its closest hPTP relative. Therefore, we performed studies with PTP antagonists to see if they would affect egg activation. Antagonists selective for the *Chaetopterus* PTP we identified are not available. Therefore, these results give us information concerning the roles of PTP as a group rather than a specific PTP.

In our study, two PTP inhibitors, Na_2MoO_4 and Na_3VO_4, elicited increased protein tyrosine phosphorylation and activated *Chaetopterus* oocytes in NSW. In addition, Na_3VO_4 activated oocytes in CaFASW, so this activation, unlike activation by sperm or excess K^+, is independent of extracellular Ca^{2+}. This suggests that vanadate acts independently from the Ca^{2+} oscillations that provide a primary activating stimulus (Eckberg & Miller 1995).

The tyrosyl hyperphosphorylation of pp140 in response to vanadate indicates that vanadate activates eggs by inhibiting PTP activity. Nevertheless, it also inhibits ATPases, including the Ca^{2+} pumps that maintain a low free Ca^{2+} concentration in resting cells. However, concentrations of vanadate that activated nearly 100% of the eggs only slightly reduced the rate of Ca^{2+} pumping by *Chaetopterus* egg homogenates *in vitro*, and vanadate did not activate sea urchin eggs (K.P. Howell *et al.*, unpubl. data, 2002). These findings rule out the notion that vanadate activates the eggs by eliciting a Ca^{2+} release.

Activation of eggs by PTP inhibition suggests that a PTP maintains the egg in an inactive state by dephosphorylating PTK substrates involved in egg activation. The fact that Na_3VO_4 and PTP inhibitor 1 elicited different aspects of the egg activation process strongly suggests that different aspects of egg activation in this organism, cortical cytoskeletal reorganization and polar body formation, may be regulated independently by protein tyrosine phosphorylation.

This is opposite to the findings in the zebrafish where PTP inhibition prevents the initiation of some of the events involved in egg activation (Wu & Kinsey 2002). Specifically, Fyn kinase requires a PTP for dephosphorylation and activation at fertilization.

The phosphotyrosine content of proteins represents the net effects of both phosphorylation and dephosphorylation. Therefore, inhibition of protein tyrosine phosphatase activity increases protein phosphotyrosine content. An increase in specific protein phosphotyrosine was involved in egg activation by sperm and vanadate, but not by KCl. These results are consistent with other data that found a few tyrosine-phosphorylated proteins in eggs and embryos (Wright & Schatten 1995). Data from the immunoblot studies extend those of earlier studies by indicating that phosphorylation of pp140 may be necessary for egg activation and subsequent development after fertilization. K^+-activation apparently does not involve this phosphorylation.

Phosphorylation of pp140 was greatly stimulated and remained constant in the Na_3VO_4-activated oocytes. This same phenomenon of enhanced phosphorylation stimulated by vanadate also occurs in pig oocytes (Kim *et al.* 1999). However, the phosphorylation in pig eggs apparently involves different proteins than in *Chaetopterus*. The fact that pp140 increased to a constant level in Na_3VO_4-activated eggs and was highly regulated in fertilized eggs (transient hyperphosphorylation during activation followed by a dephosphorylation during polar body formation and cleavage) also suggests that PTP keeps this protein in its inactive form.

Since pp140 hyperphosphorylation in response to vanadate varied from that in response to fertilization, we asked whether the localization of tyrosyl phosphoproteins was the same in vanadate-activated and fertilized eggs. In *Chaetopterus* fertilized eggs the tyrosyl phosphoproteins were primarily cortical, but in vanadate-treated eggs they were consistently perinuclear as well as cortical. Previous studies with PTP inhibitors demonstrated that the regulation of phosphotyrosine-containing proteins affects the organization of microtubules in zebrafish (Sun *et al.* 2002). Since the perinuclear area is the domain of the spindle microtubules in the activating egg, this may also be the case in *Chaetopterus*.

These data suggest that *Chaetopterus* rPTP are probably not involved in the early events of egg activation. Rather, they are likely involved in aspects of later differentiation. The data support the hypothesis that at least one PTP (most likely cytosolic) plays a key role in maintaining the egg in an unactivated state before fertilization. Thus, the maintenance of the unactivated state is an energy-requiring process.

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