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Tick vitellin is dephosphorylated by a protein tyrosine phosphatase during egg development: Effect of dephosphorylation on VT proteolysis

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Abstract

Vitellin (VT) is a phospholipoglycoprotein that is the main component of arthropod egg yolk. Phosphorylation is a recurrent feature of every VT molecule described so far. However, the role played by such post-translational modification during egg development is not yet clear. In the eggs of the hard tick *Boophilus microplus*, VT is a phosphotyrosine-containing protein. VT–phosphotyrosine residues are gradually removed during tick embryogenesis due to the action of a 45 kDa egg tyrosine phosphatase. This enzyme is strongly inhibited by ammonium molybdate, sodium vanadate and cupric ion. The role of phosphotyrosine residues in VT proteolytic degradation was evaluated. Western blots probed with a monoclonal anti-phosphotyrosine antibody demonstrated that the high molecular mass VT subunits (VT 1 and VT 2) are the main targets of dephosphorylation during egg development. Both dephosphorylation and proteolysis of VT 1 and VT 2 are blocked by ammonium molybdate in total egg homogenates. When purified VT was dephosphorylated in vitro with lambda phosphatase and then incubated in the presence of bovine cathepsin D, VT proteolysis increased dramatically. Altogether, these data are the first to show that phosphotyrosine residues are present in a yolk protein, and that such residues might be involved in the regulation of VT breakdown during egg development.

Keywords: *Boophilus microplus*; Yolk degradation; Tyrosine phosphatase; Tick egg development; Proteolysis; Dephosphorylation

1. Introduction

Ixodid ticks are recognized worldwide as vectors of arboviruses and parasitic protozoa (Taege, 2000). *Boophilus microplus* is a tick whose main hosts are bovine cattle. Once attached to its host this tick ingests a huge amount of blood. In the parasitic phase the larvae give rise to fully engorged adults. The females then fall to the ground and during this non-parasitic period egg laying occurs. Egg development takes around 3 weeks, and recently hatched larvae migrate to the upper part of the grass, where they wait for another vertebrate host (Sonenshine, 1991). *B. microplus* is considered the main parasite responsible for damage to the world livestock economy, for several reasons. First, it is the vector of *Babesia bovis*, which causes piroplasmosis, also known as "cattle sadness". This disease can cause a high mortality rate among cattle (Hunfeld and Brade, 2004). Second, *Boophilus* can ingest a very large amount of blood while attached to its host. In severely infested bovines the daily removal of blood is close to 100 ml. Blood loss leads to anemia and, consequently, loss of animal strength. Finally, tick feeding damages the hide and decreases the production of meat and milk.

One strategy to block disease transmission by ticks would be the identification of new molecular targets for the

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interruption of its life cycle. In this respect the non-parasitic phase, when the eggs are laid and embryonic development occurs, is tempting for two reasons. First, this phase occurs away from the host and, therefore, blocking strategies directed to the larvae will not affect the cattle. Second, embryonic development is strictly programmed. During development, an exclusive sequence of cellular and molecular processes occurs, most of which are not present in both larvae and adults.

The main biochemical event of egg development in oviparous organisms is the utilization of yolk components that were stored in the egg during oogenesis. Vitellogenin (VG) is a hemolymphatic phospholipoglycoprotein stored in growing oocytes. Once inside the oocytes, it is conventionally called VT and is the main component of yolk platelets (Sappington and Raikhel, 1998). After oocyte fertilization, several different VT processing mechanisms are triggered, and VT furnishes the building blocks for developing embryos. Several enzymes involved in this process have been characterized in the past few years in different biological models (Fagotto, 1990; Fialho et al., 2005; Hunfeld and Brade, 2004; Sappington and Raikhel, 1998; Sonenshine, 1991; Taege, 2000; Yamamoto and Takahashi, 1993). Such studies have addressed the identification, purification and/or cloning and characterization of VT proteolytic systems in developing eggs of arthropods. However, they do not provide information about the susceptibility of VT itself to proteolytic attack.

VT of most organisms contains covalently bound phosphate (Byrne and Gruber, 1989; Masuda and Oliveira, 1985; Sappington and Raikhel, 1998; Silva-Neto et al., 2002). Despite its wide distribution, the role of phosphorylation during egg development in blood-sucking arthropods is not clear. Usually, maternally derived phosphoproteins found in developing arthropod eggs contain only phosphoserine residues (Sappington and Raikhel, 1998). In the present study, we demonstrate that *B. microplus* VT is a phosphotyrosine-containing protein. We also demonstrate the presence of a protein, tyrosine phosphatase, able to dephosphorylate tick VT. Inhibition of this enzyme blocks VT proteolytic degradation by egg proteases. Furthermore, we show that in vitro dephosphorylation of tick VT increases the rate of its degradation by commercial aspartyl protease.

2. Materials and methods

2.1. Chemicals

Molecular mass standards, glycine, acrylamide, bis-acrylamide, TEMED, *p*-nitrophenylphosphate (pNPP), pCMB, dithiotreithol (DTT), tris, tetramisole, bovine serum albumin (BSA) and sodium acetate were purchased from Sigma Chemical Company (St. Louis, MO, USA). Phenylarsine oxide (PAO), okadaic acid (OKA) and *o*-vanadate were purchased from Calbiochem–Novabiochem Corp. (La Jolla, CA, USA). Mouse monoclonal anti-phosphotyrosine PY-99 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A set of pre-stained molecular mass standards (SeeBlue®) and rabbit polyclonal anti-phosphoserine were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Ammonium molybdate and dimethylsulfoxide (DMSO) were purchased from Merck (Gibbstown, NJ, USA). ECL system was purchased from Amersham. All other chemicals were of analytical grade, from Brazilian suppliers.

2.2. Ticks and eggs

Ticks were obtained from a colony maintained at the Faculdade de Veterinária at the Universidade Federal do Rio Grande do Sul, Brazil. *B. microplus* of the Porto Alegre strain, free of *Babesia* sp., were reared on calves obtained from a tick-free area. Engorged adult females were kept in Petri dishes at 28 °C and 80% relative humidity until completion of oviposition. Eggs were pooled and kept for the desired length of time under the same conditions. To obtain samples during the days after oviposition, a group of eggs was collected every day at the same time and stored at −18 °C for further analysis. A control group of eggs was allowed to complete development in order to determine the exact day of hatching. Eggs were homogenized with a plastic pestle in 0.2 M acetate buffer pH 5.0 and, when indicated, in the presence of a cocktail of protease inhibitors (AEBSF 1.04 mM, aprotinin 0.8 μM, leupeptin 20 μM, bestatin 40 μM, pepstatin A 15 μM and E-64 14 μM) from Sigma Fine Chemicals (St. Louis, MO, USA). This preparation is referred to as egg homogenate. Protein content of egg homogenate was determined by the method described by Lowry et al. (1951) using BSA as standard.

2.3. VT dephosphorylation and proteolytic assays

VT was purified as described previously in the presence of 1 mM ammonium molybdate, using eggs obtained on the first day after oviposition (Logullo et al., 2002). Before use, VT was dialyzed to remove ammonium molybdate. VT dephosphorylation was carried out routinely as follows. Purified VT (80 μg) was incubated in the presence or in the absence of 200 U of recombinant lambda phosphatase from Upstate Biotechnology (Waltham, MA, USA) at 37 °C according to the manufacturer's instructions. Aliquots were removed at times indicated in figure legends. When dephosphorylated VT was used for proteolysis assays, lambda phosphatase was removed after the dephosphorylation reaction by centrifugation in a Centricon-100 device (Millipore, USA). The extent of dephosphorylation was evaluated by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting. In the experiments where VT proteolysis was evaluated, samples were dephosphorylated for only 1.5 h. Lambda phosphatase was

removed as mentioned above, and VT samples were further incubated in the presence of 2.6 mU of commercial cathepsin D (CD) (Sigma–Aldrich, USA) at 37 °C. Reactions were stopped with sample buffer and VT proteolysis was evaluated by SDS-PAGE.

2.4. Tyrosine phosphatase assay

Tyrosine phosphatase was assayed by following pNPP hydrolysis in microplates. This reaction was measured at 37 °C with 1 mM pNPP, 0.2 M sodium acetate pH 5.0 with either total egg homogenate or the partially purified enzyme. Final protein concentration in egg homogenates varied from 0.3–0.6 mg/ml. Reaction was initiated by the addition of pNPP and stopped after 30 min with the addition of 1:8.5 volumes of 2 M NaOH. The amount of *p*-nitrophenol (pNP) formed was determined at 405 nm in an ELISA reader (Thermomax microplate reader, Molecular Devices, Sunnyvale, CA, USA). Specific activity is expressed as nmol of (pNP) released/mg ptn min. Controls lacking enzyme source were used as blanks. Tick VT is a hemeprotein and it commonly absorbs light at 405 nm (Logullo et al., 2002). Thus, absorbance of the blank was also checked in pH-inactivated enzyme samples. The molar extinction coefficient for *p*-nitrophenolate ion utilized for determination of enzyme specific activity was $1.75 \times 10^4\,M^{-1}\,cm^{-1}$ (Sheng and Charbonneau, 1993). Reactions were linear in all experiments. The optimum pH for pNPP hydrolysis was determined with the following mixture of buffers—glycine, sodium acetate, sodium citrate and Tris in (final concentrations, 0.1 M each). The pH was adjusted in each medium from 3.0 to 9.0. In some experiments, pNPP hydrolysis was measured in the presence or absence of different phosphatase modulators, as described in Table 1. In some experiments, we prepared a partially purified preparation of tick tyrosine phosphatase as follows. Eggs from the first day after oviposition were obtained as described above. Eggs (1 g) were homogenized using a Potter–Elvehjem and 10 ml of 100 mM sodium acetate pH 6.0, containing a cocktail of protease inhibitors as described above, at 4 °C. The homogenate was centrifuged at 100,000*g* for 1 h at 4 °C. The pellet and the floating lipids were discarded and the supernatant was applied onto a TSK-Gel Toyopearl SP-650M cation exchange column (25 cm × 1.6 cm), previously equilibrated with 100 mM sodium acetate pH 6.0 (buffer A). The column was next washed with 75 ml of buffer A, followed by 45 ml of buffer B (buffer A + 2 M NaCl) with a flux of 1 ml/min. Protein content of each fraction (1.5 ml) was monitored at 280 nm in an Ultrospec 1000 I spectrophotometer (Amersham Pharmacia Biotech, Sweden). Fractions were then assayed for pNPPase activity as described above. Samples with the highest phosphatase activity were pooled and concentrated in a SVC 100 Savant Speed Vac. This preparation was used for the biochemical characterization of egg tyrosine phosphatase, and was virtually free of VT. In order to obtain the molecular mass of the egg tyrosine phosphatase, the enzyme preparation described above was applied onto a Superose 6 HR gel filtration column coupled to a Shimadzu HPLC system, equilibrated with 100 mM sodium acetate pH 6.0, NaCl 0.15 M. The column was developed with the same buffer at a flow rate of 1 ml/min. Fractions (0.5 ml) were evaluated for their protein content by measuring absorbance at 280 nm, and pNPPase activity was assayed as already described above.

Table 1
Effect of phosphatase inhibitors on pNPP hydrolysis

Effectors	nmol pNP/mg ptn min
Control (without drug)	1.83 ± 0.044
Na^+/K^+ tartrate (1 mM)	1.86 ± 0.251
Na^+/K^+ tartrate (10 mM)	1.48 ± 0.252
Tetramisole (1 mM)	2.14 ± 0.023
Tetramisole (10 mM)	2.37 ± 0.019
NaF (1 mM)	1.28 ± 0.428
NaF (10 mM)	1.16 ± 0.179
Vanadate (100 μM)	0.62 ± 0.507
Vanadate (1 mM)	0.34 ± 0.194
Vanadate (2 mM)	0.13 ± 0.111
Molybdate (10 μM)	0.54 ± 0.246
Molybdate (100 μM)	0.09 ± 0.119
pCMB (100 μM)	0.79 ± 0.004
pCMB (1 mM)	0.64 ± 0.001
K_2HPO_4 (1 mM)	1.89 ± 0.021
K_2HPO_4 (10 mM)	1.73 ± 0.018
Phenylarsine oxide (100 μM)	2.17 ± 0.293
Phenylarsine oxide (1 mM)	1.91 ± 0.055
Okadaic acid (100 nM)	2.04 ± 0.135
Caffeine (500 μM)	2.03 ± 0.011
Caffeine (5 mM)	1.62 ± 0.121

The fractions with high phosphatase activity eluted from TSK-Gel Toyopearl SP-650M column were pooled and assayed against pNPP in the presence of different inhibitors.
Enzyme activity is shown as nmol pNP released/mg ptn min ± S.E. for three independent experiments, performed in triplicate.

2.5. SDS-PAGE and Western blotting

Polyacrylamide gels were run in the presence of 10% SDS (Laemmli, 1970) at a constant current of 13 mA. Gels were stained with Coomassie Brilliant Blue G and destained with a 7.5% acetic acid, 5% methanol solution. SeeBlue® was used as a molecular mass standard. For Western blotting, samples were separated by SDS-PAGE (10%) and transferred at 190 mA for 90 min to a nitrocellulose membrane using 25 mM Tris–HCl, pH 8.3, 192 mM glycine at 4 °C. The membrane was blocked overnight with TBS-Tween 20 (0.1% (v/v))-BSA 5% (w/v) (TTA), and probed with a monoclonal antibody (1:2000) raised against phosphotyrosine (PY-99) or a polyclonal antibody (1:2000) raised against phosphoserine and developed using the ECL system.

3. Results

3.1. Tick VT is a phosphotyrosine-containing phosphoprotein

Every VT molecule described in the literature bears several phosphorylation sites; thus, we began by looking for correlation between proteolysis and the phosphorylation sites in this molecule. VT from *B. microplus* is composed of 11 subunits with a total native molecular mass of 492 kDa (Logullo, et al., 2002). Incubations with anti-phosphotyrosine antibodies provided specific evidence of phosphotyrosine residues in most of its subunits (Fig. 1B). The presence of phosphotyrosine residues was further confirmed by treatment with lambda phosphatase, which readily removed these phosphate groups (Fig. 1B). The rapidly migrating bands in lanes 0.5 and 2 (Fig. 1A) are lambda phosphatase. The use of lambda phosphatase demonstrates the specificity of the primary antibody used towards phosphotyrosine residues present on specific VT subunits such as VT 1 and VT 2. Tick VT also contains phosphoserine residues in several of its subunits (Fig. 1C). However, phosphoserine content of VT 1 and VT 2 is very low and hardly detected. Under our working conditions, lambda phosphatase did not dephosphorylate VT 1 phosphoserine residues and also such treatment did not alter yolk protein solubility (Fig. 1C). Tick VT is the first yolk protein ever described to exhibit phosphorylation of both phosphotyrosine and phosphoserine residues (Fig. 1C).

Tick VT phosphotyrosine residues are dephosphorylated during egg development (Fig. 2). Since VT is composed of eleven subunits, it is hard to establish a correlation between in vitro dephosphorylation carried out with purified VT and in vivo dephosphorylation using total egg homogenates. Fig. 2A shows that VT subunits with higher molecular mass (VT 1 and VT 2) are degraded by egg proteases during development, consistent with a previous report (Logullo et al., 2002) in which the authors followed VT degradation during egg development using purified preparations. In Fig. 2B, Western blot probed with anti-phosphotyrosine antibody revealed a decrease in VT phosphorylation during egg development. Curiously, a 36 kDa polypeptide retained its phosphotyrosine content until day 19 and it was not degraded by proteolysis. VT 1 and VT 2 are prone to dephosphorylation both in vivo and in vitro, undergo proteolysis and are easily resolved in SDS-PAGE. Therefore, in this study, we will focus on VT 1 and VT 2, which can be clearly identified in all figures. Dephosphorylation and proteolysis were particularly evident for VT 1 and VT 2 subunits. This correlation was further confirmed by densitometric scanning of both the membrane and the blot (data not shown). Thus, we infered that proteolysis and dephosphorylation are linked events catalyzed by different molecular entities.

3.2. Tyrosine phosphatase activity in developing eggs

The above set of results suggested the presence of a tyrosine phosphatase activity towards VT during egg

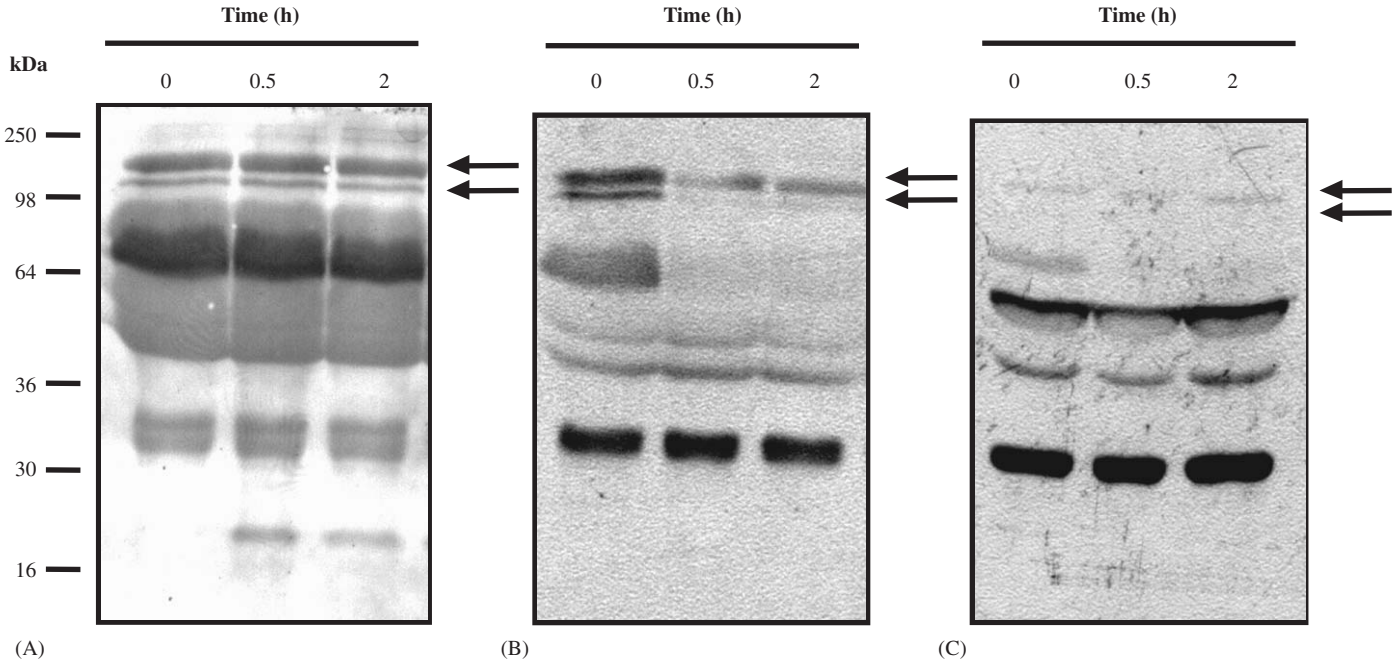

Fig. 1. In vitro VT dephosphorylation. VT (80 μg) was incubated either in the presence (0.5 and 2 h) or in the absence (0 h) of lambda phosphatase for different times at 37 °C. The reactions were stopped with sample buffer. Samples were separated by SDS-PAGE (10%), and dephosphorylation was evaluated by Western blotting using anti-phosphoaminoacid antibodies. Molecular mass standards are indicated at left. (A) Ponceau red-stained nitrocellulose membrane. (B) Western blot with anti-phosphotyrosine. (C) Western blot with anti-phosphoserine. VT 1 and VT 2 subunits are indicated by arrows. Lambda phosphatase corresponds to the rapidly migrating band present in lanes 0.5 and 2 (panel A).

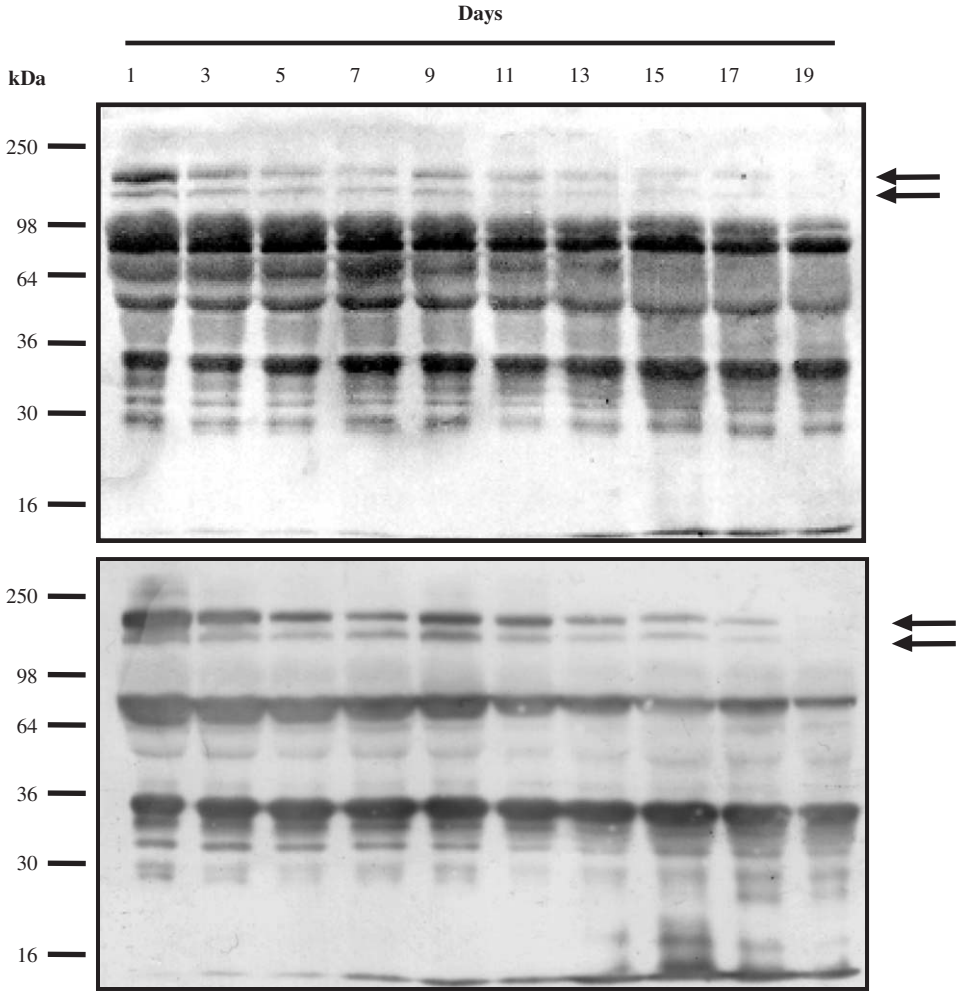

Fig. 2. In vivo VT dephosphorylation during tick egg development. Eggs from different days after oviposition were homogenized in 0.2 M acetate buffer pH 5.0 in the presence of a cocktail of protease inhibitors and molybdate 1 mM. Samples containing 80 μg of protein were separated by SDS-PAGE (10%) and then transferred to a nitrocellulose sheet. The membrane was developed as described in Materials and methods. (Upper panel) Ponceau red-stained nitrocellulose membrane. (Lower panel) Western blot against phosphotyrosine. VT 1 and VT 2 subunits, which undergo extensive dephosphorylation and proteolysis, are indicated by arrows on the right.

development in ticks. A similar enzyme activity targeting VT has never been studied in any biological system. Therefore, we next obtained a partially purified preparation of the tyrosine phosphatase from tick eggs as described in Materials and methods. This enzyme preparation is composed of a main tyrosine phosphatase activity with a molecular mass of 45 kDa (Fig. 3A). Enzyme activity towards pNPP is maximal at pH 5.0 (Fig. 3B) and this reaction is strongly inhibited by sodium vanadate and ammonium molybdate (Table 1), which are classical tyrosine phosphatase inhibitors (Tonks et al., 1988). Moderate to negligible modulation of enzyme activity was observed with drugs commonly used to block lysosomal, alkaline and Ser/Thr phosphatases (Table 1). The pNPPase activity was also inhibited by the SH-specific reagent *p*-chloromercuribenzoate (pCMB) (Belyaeva et al., 2003). Inhibition by pCMB has been reported to occur with other members of the phosphotyrosine phosphatase family (Bose and Taneja, 1998). Km and Vmax for pNPP hydrolysis by partially purified egg tyrosine phosphatase were 0.34 mM and 3.23 nmol pNP/mg ptn min, respectively.

Ammonium molybdate was an effective inhibitor of pNPP dephosphorylation in tick egg homogenates throughout the period of egg development. Fig. 4A shows that pNPPase activity gradually increased, reaching a peak on day 17, and the enzyme remained active until the end of egg development. Inhibition by 100 μM ammonium molybdate throughout this period suggests that the enzyme described in earlier experiments is the main tyrosine phosphatase activity in this system. In the next experiments, we explored the ability of tyrosine phosphatase from developing tick eggs to dephosphorylate VT in vitro. Eggs were homogenized on the first day after oviposition in the presence of a cocktail of protease inhibitors, and incubated either in the presence or in the absence of ammonium molybdate. Following incubation, samples were blotted and probed with anti-phosphotyrosine

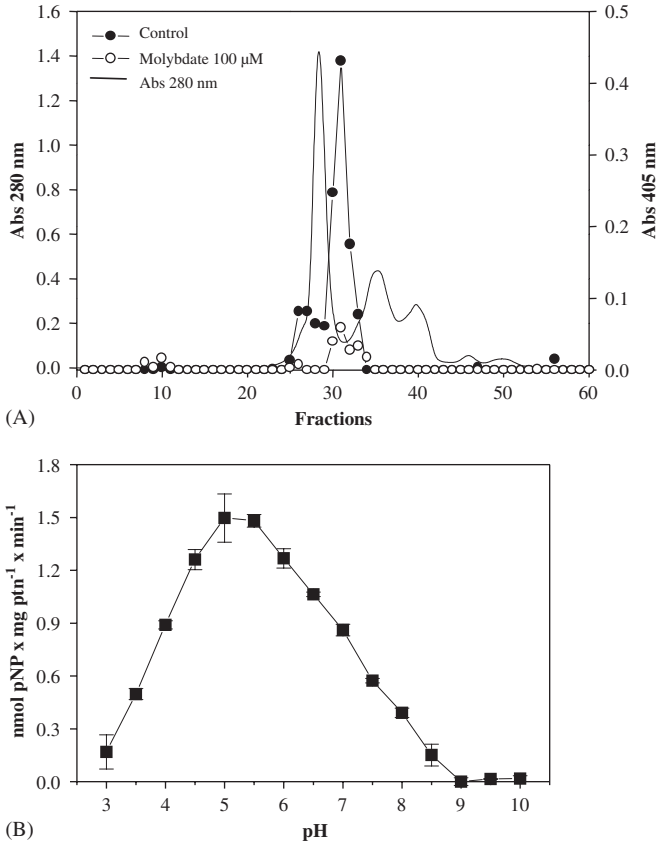

Fig. 3. Biochemical properties of egg tyrosine phosphatase activity. Egg tyrosine phosphatase was partially purified using a TSK-Gel Toyopearl SP-650M column. Fractions with high phosphatase activity were pooled, and the main biochemical properties of tick tyrosine phosphatase evaluated as indicated in Materials and methods. (A) Determination of tyrosine phosphatase molecular mass. (B) pH dependence for pNPP hydrolysis. Enzyme activity is shown as nmol pNP released/mg ptn min. *Error bars* indicate S.E. for three independent experiments.

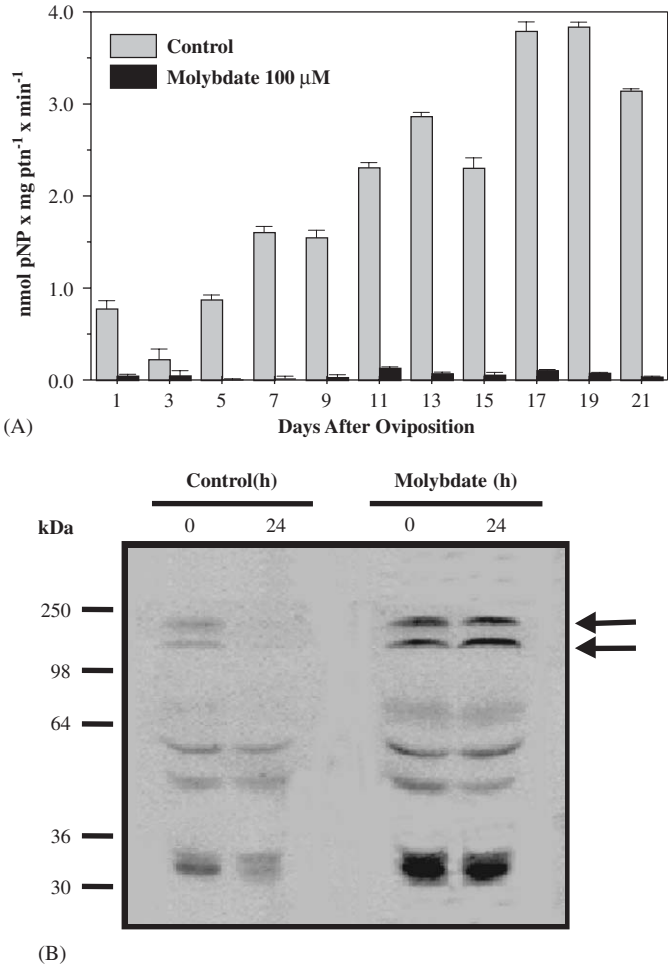

Fig. 4. Tyrosine phosphatase activity in vitro—effect of molybdate on pNPPase and VT dephosphorylation. (A) Eggs from different days after oviposition were homogenized in 0.2 M acetate buffer pH 5.0. Egg homogenates were assayed for pNPPase activity as described under Materials and methods, either in the presence or in the absence of molybdate 100 μM. Enzyme activity is shown as nmol pNP released/mg ptn min. *Error bars* indicate S.E. for three independent experiments. (B) Eggs from the first day after oviposition were homogenized in 0.2 M acetate buffer pH 5.0 in the presence of a cocktail of protease inhibitors, and either in the presence or in the absence of 1 mM molybdate. Samples were incubated at 37 °C for the indicated times, and then an aliquot (90 μg) was separated by SDS-PAGE (10%) and transferred to a nitrocellulose sheet shown in panel B. The membrane was developed as described in Materials and methods using anti-phosphotyrosine antibodies. Arrows on the right show VT 1 and VT 2.

antibody. Fig. 4B shows that VT 1 and VT 2 are readily dephosphorylated in vitro in a reaction blocked by ammonium molybdate. Non-incubated control samples in the absence of ammonium molybdate also show some level of dephosphorylation (Fig. 4B, control). This is due to the time required for processing egg homogenates. Altogether, this second set of results shows that tick VT phosphotyrosine residues are dephosphorylated during egg development in a reaction catalyzed by an egg tyrosine phosphatase.

3.3. Dephosphorylation of VT phosphotyrosine residues enhances its proteolytic degradation by aspartic proteases

The next experiments were performed to test whether dephosphorylation of phosphotyrosine residues enhances the proteolytic attack on VT by egg proteases. Egg homogenates from the first day after oviposition were obtained either in the presence or in the absence of ammonium molybdate, and VT proteolysis was evaluated by SDS-PAGE. Fig. 5 shows that molybdate is able to block VT proteolysis. Tyrosine phosphatases are inhibited by oxidizing agents. Due to the presence of at least one cysteine residue in the catalytic site such enzymes are extremely sensitive to inhibition by Cu^{2+} ion. Cu^{2+}-mediated inhibition of enzyme activity occurs by a reversible oxidative reaction of this ion with cysteine in the active site of tyrosine phosphatases (Kim et al., 2000). In our experiments, pNPPase activity of partially purified egg tyrosine phosphatase was also blocked by Cu^{2+} ion (Fig. 6C). Fig. 6B shows that Cu^{2+} partially blocks VT proteolysis. Therefore, experiments shown in Figs. 5 and 6

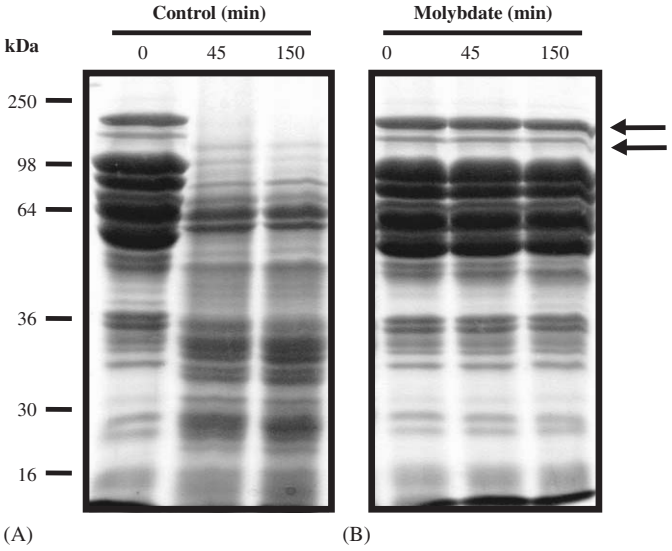

Fig. 5. Effect of molybdate in proteolytic activity of tick eggs. Eggs from first day after oviposition were homogenized in a 20 mM Tris–HCl buffer pH 8.0. Then, the samples were acidified with 200 mM sodium acetate pH 4.0, in the presence or in the absence of 1 mM molybdate. Samples were incubated at 37 °C for the indicated times (min). The reactions were stopped with sample buffer. Samples (80 μg) were separated by SDS-PAGE (10%). (A) Control proteolysis. (B) Proteolysis in the presence of 1 mM molybdate. Arrows on the right show VT 1 and VT 2.

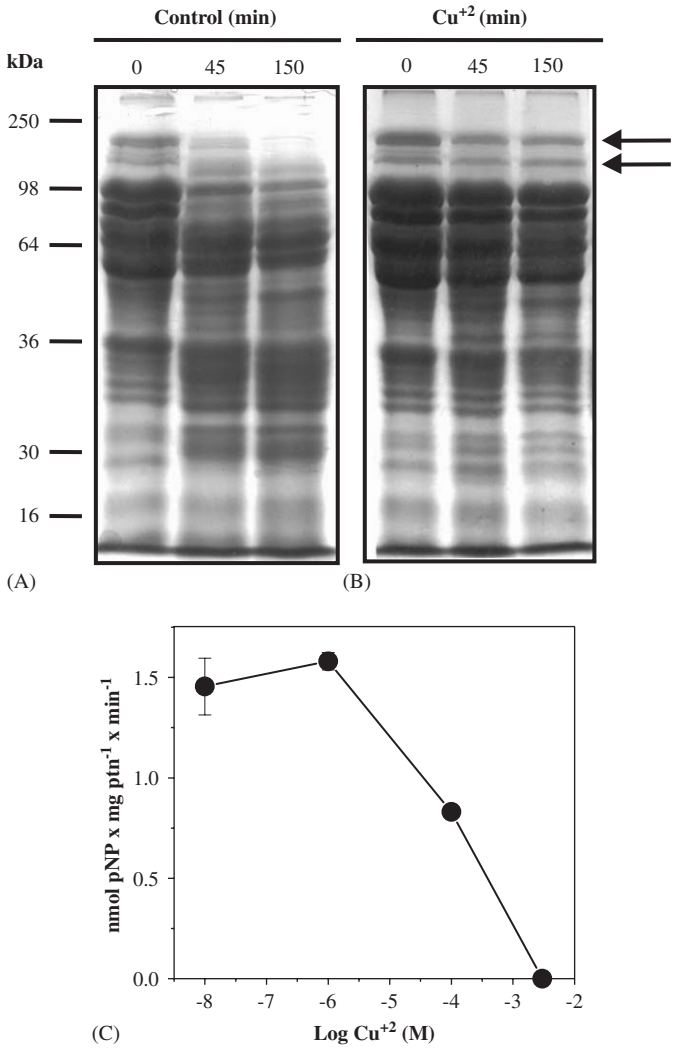

Fig. 6. Effect of Cu^{2+} on proteolytic activity of tick eggs and tyrosine phosphatase activity. Eggs from first day after oviposition were homogenized in 20 mM Tris–HCl buffer pH 8.0. Then, the samples were acidified with 200 mM sodium acetate pH 4.0, in the presence or in the absence of 1 mM Cu^{2+}. Samples were incubated at 37 °C for the indicated times (min). The reactions were stopped with sample buffer. Samples (80 μg) were separated by SDS-PAGE (10%). (A) Control proteolysis. (B) Proteolysis in the presence of 1 mM Cu^{2+}. Arrows on the right show VT 1 and VT 2. (C) shows the effect of different concentrations of Cu^{2+} on tyrosine phosphatase activity against pNPP from the fractions with high phosphatase activity eluted from a TSK-Gel Toyopearl SP-650M.

suggest an involvement of phosphotyrosine dephosphorylation of VT in the rate of its proteolytic degradation. Two aspartic proteases targeting VT, as substrate have already been described in *B. microplus* eggs, *Boophilus* yolk pro-cathepsin D (BYC), and tick heme-binding aspartic proteinase (THAP) (Logullo et al., 1998; Sorgine et al., 2000). Incubation of egg homogenates in the presence of the classical aspartic protease inhibitor pepstatin A completely blocked the degradation of VT 1 and VT 2, as already pointed out (Abreu et al., 2004).

Purified tick VT was incubated in the presence and in the absence of lambda phosphatase. The enzyme was removed by centrifuging samples through a Centricon-100 device, and both VT and dephosphorylated VT (dephosphoVT) were next incubated with commercial cathepsin D (CD). The extent of VT proteolysis was evaluated by SDS-PAGE. Fig. 7 shows that an enhancement of proteolysis occurs when VT is previously dephosphorylated. Even an incubation of VT with CD lasting for 6 h was not able to completely consume VT 1 and VT 2 subunits, as occurs with dephosphoVT after 4 h of incubation. Despite the presence of phosphoserine residues in tick VT, such phosphoaminoacids are not a substrate for lambda phosphatase in our working conditions (Fig. 1C). Altogether, this set of results demonstrates that during tick egg development, VT is gradually dephosphorylated on phosphotyrosine residues by an egg tyrosine phosphatase. Such dephosphorylation could be a mechanism that renders VT prone to proteolytic degradation catalyzed by egg aspartic proteases.

4. Discussion

Protein tyrosine phosphatases are key elements of intracellular signaling (Stoker, 2005). These enzymes can be divided into four classes—classical receptor tyrosine phosphatases, classical non-receptor tyrosine phosphatases, dual-specificity tyrosine phosphatases and the low-molecular-weight tyrosine phosphatases. Such enzymes are involved in several cellular events, including substrate adhesion, cell motility and gene expression (Stoker, 2005). In *Drosophila melanogaster*, tyrosine phosphatases associated with egg development are involved in the

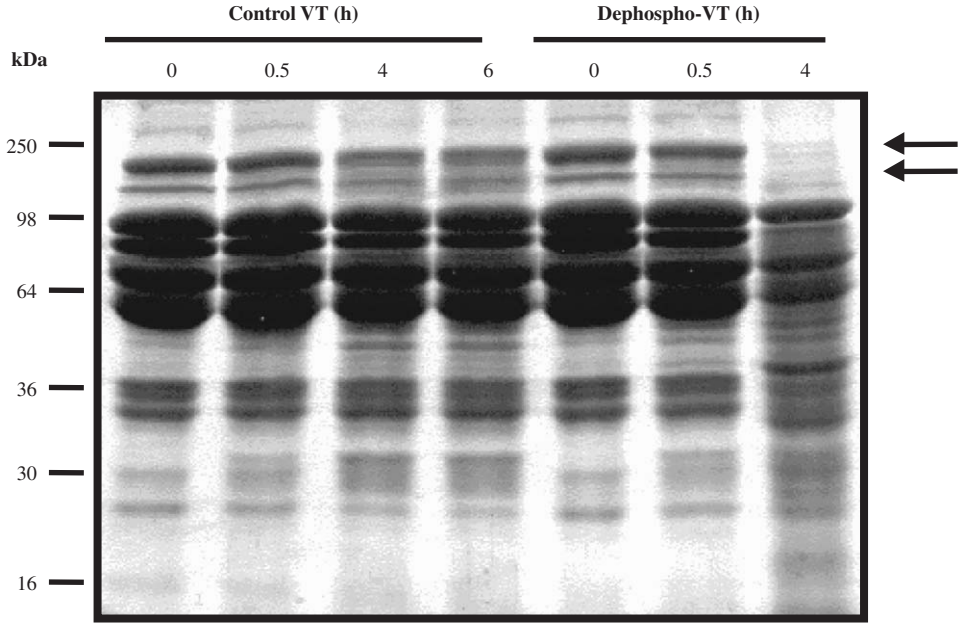

Fig. 7. Effect of dephosphorylation on VT proteolysis by commercial bovine cathepsin D. VT (80 μg) was incubated in the presence (DephosphoVT) or in the absence (Control VT) of lambda phosphatase for 1.5 h at 37 °C. After the reaction, lambda phosphatase was removed by centrifugation on Centricon-100 and samples were incubated in the presence of commercial cathepsin D at 37 °C for the indicated times. The reactions were stopped with sample buffer. Samples were separated by SDS-PAGE (10%) and the gel was stained with Coomassie Blue G. Arrows on the right show VT 1 and VT 2.

transduction of Torso signaling by *corkscrew*, development of central nervous system axons and regulation of the cell cycle by *twine*, a homolog of *cdc25* (Perrimon et al., 1995; Zinn, 1993; White-Cooper et al., 1993). Recently, it was demonstrated in *D. melanogaster* that the evolutionarily conserved transcriptional factor Eyes absent is a non-thiol-based protein tyrosine phosphatase (Tootle et al., 2003). In the flesh fly *Sarcophaga peregrina* an egg-derived tyrosine phosphatase mRNA is stored in growing oocytes, and the expression of the enzyme seems to be modulated during embryo development. However, the function of this enzyme is presently unknown (Yamaguchi et al., 1999).

Fialho et al. (2002) demonstrated that egg fertilization triggers the activity of an acid phosphatase in the eggs of the blood-sucking bug *Rhodnius prolixus*. That study suggested for the first time that dephosphorylation of yolk proteins could be related to their degradation. However, dephosphorylation of *R. prolixus* phosphoaminoacids mediated by acid phosphatase was hard to be demonstrated in vitro and the targets of the acid phosphatase are still unknown. In the present study, we report VT dephosphorylation in the developing eggs of *B. microplus*. The enzyme involved in this process displays the biochemical properties of tyrosine phosphatases. It certainly does not belong to the classical receptor tyrosine phosphatases, since detergent-free 100,000*g* supernatants were used during its isolation. Moreover, it is not a member of the low-molecular-weight tyrosine phosphatases, whose molecular masses are around 20 kDa (Alonso et al., 2004). Finally, immunoblotting conducted with anti-PTP1B antibody, the prototype of classical non-receptor tyrosine phosphatases (Tonks, 2003), failed to recognize the tick enzyme (data not shown). Therefore, the exact classification of this enzyme as well as some aspects of its biology such as its exact distribution in developing eggs remain to be demonstrated.

In most non-mammalian oviparous organisms, one of the early events during embryo development is the initiation of proteolytic processing of VT. The regulation of this process is still unclear, but a growing body of evidence (Abreu et al., 2004; Fagotto 1990; Fagotto and Maxfield, 1994; Izumi et al., 1994; Liu and Nordin, 1998; Logullo et al, 1998; Oliveira et al., 1989; Sappington and Raikhel, 1998; Yamamoto and Takahashi, 1993) indicates that pH may be one of the regulators of yolk degradation. Furthermore, granule restructuring is also linked with VT utilization. Although arthropod VTs contain covalently bound phosphate and carbohydrates, the roles of these constituents in VT utilization have not been ascertained. In fact, several post-fertilization enzyme activities have been demonstrated during arthropod egg development (Chaubey and Bhatt, 1998; Fagotto and Maxfield, 1994; Fialho et al., 1999; Liu and Nordin, 1998; Yamaguchi et al., 1999). Some of them were shown to introduce major modifications in VT structure, including its phosphate moiety. Thus, phosphate removal may be part of a programmed pathway triggered by fertilization. Involvement of phosphatase-mediated VT proteolysis was recently shown by our group in the developing eggs of the blood-sucking bug *R. prolixus* (Fialho et al., 2005). However, in that study, conventional acid phosphatases were involved in the dephosphorylation of an as yet unidentified target. In the present study, we have demonstrated that an enzyme from

a different class of phosphatases, tyrosine phosphatase, targets VT phosphotyrosine sites, its action engenders VT degradation by aspartyl proteases. Inhibition of egg tyrosine phosphatase using two compounds able to block pNPPase activity of partially purified enzyme led to the inhibition of proteolysis (Figs. 5 and 6). Curiously, ammonium molybdate completely blocked proteolysis in the same concentration used to completely block pNPPase activity (Figs. 4A, 5 and Table 1). On the other hand, Cu^{2+} did not completely block proteolysis when used in the same concentration, where it completely blocks pNPPase activity (Fig. 6). This may be due to the chelation of divalent cations by the anionic residues of VT in total egg homogenates (Silva-Neto et al., 1996).

Proteolysis of VT 1 and VT 2 was enhanced upon in vivo and in vitro dephosphorylation of these subunits. These results constitute evidence for a novel mechanism for the regulation of aspartyl proteases and are mainly based on the following data. Inhibition of egg tyrosine phosphatases induced VT proteolysis catalyzed by endogenous egg proteases (Figs. 5 and 6). However, molybdate also blocked the proteolysis of bands which do not exhibit phosphotyrosine residues in the 98–64 kDa range (cf. Figs. 2 and 5). Therefore, there may be some unspecific effect of molybdate on protease activity. In order to evaluate this hypothesis, we used in vitro dephosphorylation assays coupled to in vitro proteolysis. In vitro dephosphorylation induced an increase in VT proteolysis when commercial CD was present in the reaction mixture (Fig. 7). During *B. microplus* egg development, VT 1 and VT 2 are the main proteolytically degraded subunits, as pointed out in Fig. 2, and also demonstrated previously (Logullo et al., 2002). The remaining VT subunits undergo minor modifications in their quantity. Therefore, the proteolytic enzymes associated with tick yolk must be highly selective towards such subunits. The *B. microplus* egg proteolytic system is composed of three different proteases, two aspartic proteases, BYC and THAP (Logullo et al., 1998; Sorgine et al., 2000); and a cysteine endopeptidase named vitellin degrading cysteine endopeptidase (VTDCE) (Seixas et al., 2003). Proteolytic activity towards VT is maximal in the pH range 2.5–4.0. In vivo acidification of yolk granules is achieved due to the presence of a proton pump, and in vitro acidification of egg extracts led to the degradation of VT 1 and VT 2 subunits in a reaction blocked by pepstatin A (Abreu et al., 2004). Such results indicated that at least these two subunits are targeted for degradation by aspartic proteases. It is noteworthy that these are the same subunits extensively degraded during tick egg development. Here, we show that degradation of VT 1 and VT 2 by aspartic proteases is modulated by the phosphorylation level on phosphotyrosine residues of such subunits.

To our knowledge, the present study is the first to establish a link between the major changes in the covalently bound VT phosphate moiety and the rate of its proteolysis in developing eggs of any oviparous organism. The localization of VT domains which are dephosphorylated after fertilization, together with the effects of this phosphate removal on its overall structure, will thus increase our understanding of the role those reactions may play during arthropod embryo development. In conclusion, the present study provides evidence that the mechanisms that underlie the regulation of yolk processing are a potential molecular target for controlling tick life cycle and disease transmission.

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