Extracellular matrix modifications at fertilization: regulation of dityrosine crosslinking by transamidation

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Fertilization is accompanied by the construction of an extracellular matrix that protects the new zygote. In sea urchins, this structure is built from glycoproteins residing at the egg surface and in secretory vesicles at the egg cortex. Four enzymatic activities are required for the transformation of these proteins into the mechanically and chemically resilient fertilization envelope: proteolysis, transamidation, NADPH-dependent oxidation and peroxidation. Here, we identify the Strongylocentrotus purpuratus enzymes responsible for the formation of ε(γ-glutamyl)lysine crosslinks (transamidation). We find that these two transglutaminases are activated by local acidification and act on specific substrates within the fertilization envelope (including ovoperoxidase, rendezvin and SFE9). Surprisingly, these enzymes also regulate dityrosine crosslinking both by direct conjugation of ovoperoxidase and by modulating hydrogen peroxide production. Together, these results emphasize how transglutaminases can coordinate the activities of other enzymes during extracellular matrix transmogrifications.

KEY WORDS: Extracellular matrix, Fertilization, Transamidation, Transglutaminase, Sea urchin

INTRODUCTION

Transamidation occurs in all organisms during development and in adult homeostasis (Griffith et al., 2002; Lorand and Graham, 2003), with individual transglutaminases (TGs) acting on both intra- and extracellular substrates (Aeschlimann and Thomazy, 2000). These enzymes are involved in G-protein-mediated cell signaling (Mhaouty-Kodja, 2004), keratinization of cornified epithilia (Candi et al., 2005; Lorand and Graham, 2003), insolubilization of the cell wall in the alga Chlamydomonas reinhardtii (Waffenschmidt et al., 1999), hardening of the chitinous cuticle of arthropods (Iijima et al., 2005) and the stabilization of proteins required for clotting (Chen et al., 2005; Karlsson et al., 2004; Lorand and Graham, 2003; Piacentini et al., 2000; Verderio et al., 2004). During mammalian blood clotting, for example, the transglutaminase factor XIII crosslinks serum fibrin to other extracellular matrix proteins at injury sites (Esposito and Caputo, 2005). Factor XIII proenzymes are stored as homodimers of A subunits (XIIIa) in platelets or as insolubilization of the cell wall (Esposito and Caputo, 2005) and the stabilization of proteins required for clotting (Chen et al., 2005; Karlsson et al., 2004; Lorand and Graham, 2003; Piacentini et al., 2000; Verderio et al., 2004). During mammalian blood clotting, for example, the transglutaminase factor XIII crosslinks serum fibrin to other extracellular matrix proteins at injury sites (Esposito and Caputo, 2005). Factor XIII proenzymes are stored as homodimers of A subunits (XIIIa) in platelets or as circulating heterotetramers of 2A and 2B subunits (XIIIa-XIIIb) in serum (Adany and Bardos, 2003). Platelet activation releases thrombin, the protease that unleashes factor XIII activity by exposing the catalytic site of this transglutaminase, whereas free Ca²⁺ regulates catalysis by controlling substrate access to its active cysteine (Adany and Bardos, 2003; Esposito and Caputo, 2005).

Transglutaminases are also functional at fertilization, participating in egg extracellular matrix modification. ε(γ-glutamyl)lysine crosslinks are the major contributor to teleost chorion hardening (Chang et al., 2002; Ha and Iuchi, 1998; Oppen-Berntsen et al., 1990; Yamagami et al., 1992), and are associated with morphological changes on the surface of the sea urchin fertilization envelope (Battaglia and Shapiro, 1988; Chandler and Kazilek, 1986; Larabell and Chandler, 1991; Mozingo and Chandler, 1991; Veron et al., 1977). The fertilization envelope establishes a physical block to polyspermy that protects the zygote from environmental and microbial agents (Kay and Shapiro, 1985; Wong and Wessel, 2006a). This extracellular matrix is constructed from two populations of glycoproteins, the nascent egg extracellular matrix (the vitelline layer) and the contents of cortical secretory vesicles (cortical granules) hemifused to the egg plasma membrane (Wong et al., 2007).

Four major enzymatic activities are essential for the proper assembly of the fertilization envelope: proteolysis, transamidation, hydrogen peroxide synthesis and peroxidase-dependent dityrosine crosslinking. The serine protease CGSP1 (cortical granule serine protease 1), which is derived from the cortical granules, cleaves linkages between the vitelline layer and the plasma membrane, thereby releasing the fertilization envelope from the cell surface (Carroll and Epel, 1975; Haley and Wessel, 1999). CGSP1 also regulates the activity of another cortical granule enzyme, ovoperoxidase (Haley and Wessel, 2004), which is responsible for establishing dityrosine crosslinks between adjacent proteins (Foerder and Shapiro, 1977; Kay and Shapiro, 1987; LaFleur et al., 1998; Wong and Wessel, 2008). Hydrogen peroxide, an essential substrate for ovoperoxidase activity, is synthesized from free oxygen by the NADPH reductase domain of the dual oxidase Udx1 (urchin dual oxidase 1) (Foerder et al., 1978; Warburg, 1908; Wong et al., 2004). Together, Udx1 and ovoperoxidase crosslink the proteins found throughout the fertilization envelope, thereby establishing the permeability of this zygotic extracellular matrix (Kay and Shapiro, 1985; Veron et al., 1977; Wong and Wessel, 2008). Inter-protein crosslinking occurs in two discrete subdomains of the fertilization envelope: first at the microvillar casts, the segments of the vitelline layer that cover the egg microvilli, followed by the intercast region, the area between the microvillar casts (Chandler and Kazilek, 1986; Larabell and Chandler, 1991; Mozingo and Chandler, 1991). Transamidation of proteins localized at microvillar casts is associated with the morphological change from a rounded (‘igloo’) to a pointed (‘tent’) shape (e.g. the I-to-T transition), and for fertilization envelope thickening (Battaglia and Shapiro, 1988; Chandler and Kazilek, 1986; Larabell and Chandler, 1991; Mozingo and Chandler, 1991; Veron et al., 1977), whereas the intercast region is predominantly crosslinked by ovoperoxidase (Larabell and Chandler, 1991; Mozingo and Chandler, 1991). Unlike the other enzyme activities, the transglutaminases responsible for these morphological changes remains undefined.
Materials and Methods

Animals

Strongylocentrotus purpuratus gametes were obtained and handled as previously described (Wong and Wessel, 2004).

RNA in situ hybridization

Antisense digoxigenin-labeled RNA probes (DIG RNA Labeling Kit (SP6/T7); Roche Diagnostics Corporation, Indianapolis, IN, USA) representing S. purpuratus eTG (nucleotides 1274 to 1802) and nTG (nucleotides 62 to 403) or negative control probe (neomycin phosphotransferase II) were used at 0.1 μg probe/ml to detect native transcript in oocytes according to previously published protocols (Areñas-Mena et al., 2000).

Quantitative PCR

Real-time quantitative PCR was conducted on about 0.1 μg-equivalents of total RNA isolated from hand-collected oocytes or eggs (Song et al., 2006). Primers representing the 3'-end of each transglutaminase open reading frame were used to amplify products for eTG (199 bp using F=5'CAGT-GAGAATTGGATGGTTGG with R=5'GGTTGCCTGTCTTCTTTGGT) and nTG (169 bp using F=5'CCACCCGGAGATGAAGACTGA with R=5'TGTCCTTITGGAAGAACATC) (Epel et al., 1970). The DTT-released and exudate samples were dialyzed extensively against TE, then lyophilized and resuspended in distilled water; (4) soft fertilization envelopes (SFEs) were isolated from the washed zygotes used to collect zygotic seawater, as previously described (Wong and Wessel, 2004).

Polyclonal antibody analysis

One-dimensional polyacrylamide gel electrophoresis (PAGE) was accomplished in pre-cast 4-20% polyacrylamide Tris-Glycine gels (Nu-Sep Incorporated, Lawrenceville, GA, USA). Total protein was transferred onto high-capacity nitrocellulose (Pall Corporation, Pensacola, FL, USA), and then blocked using Blotto [3% non-fat dry milk (w/v)], 10 mM NaCl, 50 mM Tris, 0.05% Tween20 (v/v) (pH 8.0)]. Primary antibodies were diluted in Blotto [eTG sera at 1:100 dil or nTG sera at 1:1000 dil (v/v)], followed by horseradish peroxidase-conjugated secondary antibodies diluted in Blotto [1:5000 dil (v/v); Jackson ImmunoResearch Laboratories Incorporated, West Grove, PA, USA]. Blots were washed extensively in TBS-Tween20 [170 mM NaCl, 50 mM Tris, 0.05% Tween20 (v/v) (pH 8.0)] before development. Specific proteins were detected by chemiluminescence using a luminol-hydrogen peroxide solution [1.25 mM luminol, 68 μM coumaric acid, 0.0093% hydrogen peroxide (v/v), 0.1 M Tris (pH 8)]. Following development, blots were each stripped with two washes in 200 mM glycine-HCl, 0.05% Tween20 (pH 2.5) at 80°C for 20 minutes, and then equilibrated to TBS-Tween20. Blots were then stained with 0.1% Amido Black dissolved in destaining solution [10% acetic acid, 45% methanol (v/v)], followed by extensive destaining in the same solvent.

Immunofluorescence localization was performed on paraffin embedded tissue fixed with 4% paraformaldehyde. Antibody probing was performed essentially as described previously for double-labeling (Wessel and McClay, 1986). Anti-nTG sera [1:2000 dil (v/v)] was detected with anti-rabbit Cy5 Fab [1:25 dil (v/v); Jackson ImmunoResearch Laboratories Incorporated, West Grove, PA, USA], then remaining anti-nTG sites were blocked with unlabeled anti-rabbit Fab [1:3 dil (v/v); Jackson ImmunoResearch Laboratories Incorporated, West Grove, PA, USA] (Negoescu et al., 1994). Hyalin staining was used as a counterstain for cortical granules, and was probed for at the same time as the unlabeled-anti-rabbit Fab incubation. Anti-hyalin monoclonal antibody 2B7 [1:3 dil (v/v)] (Wessel et al., 1998) was detected with anti-mouse Alexa Fluor 564 (Invitrogen Corporation, Carlsbad, CA, USA). Samples were imaged using an LSM510 META laser confocal microscope (Carl Zeiss Corporation, Thornwood, NY, USA). Z-stacks were taken in 0.5 μm steps.

Electron microscopic analysis was performed on paraformaldehyde-fixed whole-mount eggs and zygotes (see above), immunoprobed with preimmune sera [1:200 dil (v/v)], anti-nTG sera [1:500 dil (v/v)], or anti-eTG sera [1:200 dil (v/v)] followed by anti-rabbit IgGs conjugated to 10 nm colloidal gold (Jackson ImmunoResearch Laboratories Incorporated, West Grove, PA, USA). These samples were prepared and imaged as previously described (Wong and Wessel, 2006b). Each colloidal gold particle was scored according to its location along the egg cortex or the fertilization envelope, and normalized over the length of each structure analyzed. Data are reported as the number of particles per millimeter, per egg or per embryo, averaged over three to six cells.

Inhibition studies: drug concentrations

All enzymatic inhibition assays were conducted on about 200 acid-dejellied eggs in a final volume of 200 μl of seawater. Unless otherwise noted, commercial inhibitors were used at a final concentration of: 10 mM cadaverine-HCl (cad; Sigma-Aldrich); 1× mini EDTA-free protease.
inhibitor (p. inh; Roche Diagnostics Corporation, Indianapolis, IN, USA); 10 μM diphenyleneiodonium (DPI; Sigma-Aldrich, St Louis, MO, USA); or 1 mM 3-aminotriazole (3AT; Sigma-Aldrich, St Louis, MO, USA).

**In vivo cadaverine and tyramide labeling ± functional blocking analysis**

Quantification of transglutaminase-dependent or peroxidase-mediated inter-protein crosslinking within the fertilization envelope was measured using the fluorophore conjugates cadaverine-Alexa Fluor 488 or tyramide-Alexa Fluor 594, respectively (Invitrogen Corporation, Carlsbad, CA, USA). About 200-acid-dejellied eggs were preincubated on ice for 30 minutes with various dilutions of purified IgGs (control used ASW only) or chemical inhibitors in 200 μl of seawater. An equal volume of 20 μM cadaverine-Alexa Fluor 488 and 1:4000 dilution of tyramide-Alexa Fluor 594 stock solution (see manufacturer handbook) were added with homospecific sperm (1:10,000 final concentration), diluting the IgGs (125, 250 or 500 pg affinity-purified IgG per egg) or chemical inhibitors to their final concentration (see the section ‘Inhibition studies’). Twenty minutes after insemination, zygotes were washed five times with ice-cold ASW, and then resuspended in a 1% paraformaldehyde solution in ASW. Incorporation of each competitor molecule was imaged on an LSM510 META laser confocal microscope (Carl Zeiss Incorporated, Thornwood, NY, USA) at the equatorial plane of the fertilization envelope. Fluorescence intensity in the fertilization envelope was quantified with Metamorph software (Molecular Devices, Sunnyvale, CA, USA). Mean values from 20 individual zygotes per treatment are reported.

**Scanning electron microscopy**

Morphological alterations of microvillar casts in the fertilization envelopes were observed on zygotes fertilized in the presence of respective inhibitors (250 pg/egg of each affinity purified anti-TG IgG was used), as above without Alexa Fluor conjugates. Zygotes were then fixed and post-processed as previously described ( Battaglia and Shapiro, 1988). Samples were coated with gold-palladium (60:40) using an Emeitch sputter coater (Emitech Limited, Kent, England) immediately before digital imaging on a Hitachi S-2700 scanning electron microscope (Hitachi High Technologies America, Pleasanton, CA, USA) using Quartz PCI software (Quartz Imaging Corporation, Vancouver, BC, Canada).

**Chemical isolation of and analysis of labeled fertilization envelopes**

Fertilization envelopes were chemically isolated from live zygotes inseminated in the presence or absence of 3-aminotriazole (Sigma-Aldrich, St Louis, MO, USA) as documented elsewhere ( Santiago and Carroll, 1987; Veron et al., 1977). Briefly, 30 minutes after insemination in the presence of 100 μM cadaverine analogs [cadaverine HCl (cad-HCl) or cadaverine-Alexa Fluor 488 (cad-AF)], zygotes were washed three times with ASW and then twice with CFSW. Soft fertilization envelope were dissolved by incubating zygotes in 2 M urea, 100 mM DTT, 250 mM EDTA in CFSW for 15 minutes, and repeating once. Supernatants from successive incubations were pooled, and proteins were isolated using methanol chloroform precipitation (Wessel and Flugge, 1984) and resuspended in 2× sample buffer (5 mM Tris, 20% sucrose, 2% SDS (w/v) (pH 6.8)).

Polyacrylamide gel electrophoresis (PAGE) was accomplished in precast 4-20% polyacrylamide Tri-Glycine gels (Nu-Seph Incorporated, Lawrenceville, GA, USA) or using the ZOOM system (Invitrogen Corporation, Carlsbad, CA, USA). For the latter, fertilization envelope proteins were first focused on a linear ZOOM pH 3-10 isoelectric gradient and then subjected to separation on a 4-20% polyacrylamide Tri-Glycine gel (Invitrogen Corporation, Carlsbad, CA, USA). For both types of electrophoresis, gels were imaged for fluorescence using a Typhoon scanner (488 nm excitation, 526 band pass emission filter) driven by proprietary software (Amersham Biosciences, Piscataway, NJ, USA). Gels were then stained for total protein with Colloidal Coomassie (Nu-Sep Incorporitated, Lawrenceville, GA, USA).

**Mass spectrometry**

In-gel samples were digested according to manufacturer instructions using a Pierce In-Gel Tryptic Digestion Kit (ThermoFisher Scientific Incorporated, Rockford, IL, USA), and then samples were cleaned using Pierce PepClean-18 columns (ThermoFisher Scientific Incorporated, Rockford, IL, USA). Resuspended peptides were processed on an LTQ Ion Trap mass spectrometer (ThermoFisher Scientific Incorporated, Rockford, IL, USA), and the spectra were analyzed with Bioworks and SEQUEST software programs (ThermoFisher Scientific Incorporated, Rockford, IL, USA).

**Population-level measurement of hydrogen peroxide and ovoperoxidase activity**

Hydrogen peroxide production or ovoperoxidase activity was measured for about 200 pretreated or control eggs using Amplex Red (Invitrogen Corporation, Carlsbad, CA, USA) on a microtiter plate system, as previously published (Wong et al., 2004). The output from each well was normalized to the kinetics of eggs or sperm alone, and is reported as percentage of control reactions (preimmune or ASW alone).

**In vivo cadaverine incorporation on the egg surface by pH**

pH dependence of transamidation on the egg surface was assessed using the crosslinking of cadaverine-Alexa Fluor 488 (Invitrogen Corporation, Carlsbad, CA, USA) to endogenous substrates in the vitelline layer or on the plasma membrane. About 100 freshly shed eggs were resuspended in seawater buffered with 10 mM citrate at various pH values (pH 5-8) to assess transglutaminase activation. To measure activity at a constant pH, the eggs were resuspended in 200 μl citrate-buffered ASW containing 10 μM cadaverine-Alexa Fluor 488. To measure activity in response to a rapid pH shift, the eggs were first incubated in the appropriate citrate-buffered ASW for 5 minutes, and then the fluid was exchanged for normal ASW (pH 8) containing 10 μM cadaverine-Alexa Fluor 488. Ten minutes after addition of cadaverine-Alexa Fluor 488 solutions, eggs were washed five times with ice-cold ASW, and then resuspended in a 1% paraformaldehyde solution in ASW. Conjugation of fluorophore to the egg surface was imaged on an LSM510 META laser confocal microscope (Carl Zeiss Incorporated, Thornwood, NY, USA), taking 20 μm z-stacks using 2-μm optical slices at 2-μm steps through the equatorial plane of the egg. Stacks were projected onto a single image for quantification using LSM510 software (Carl Zeiss Incorporated, Thornwood, NY, USA). Fluorescence intensity at the egg surface was quantified with Metamorph software (Molecular Devices, Sunnyvale, CA, USA). Mean values from five to eight individual eggs per treatment are reported.

**Statistical analysis**

Comparative analysis of cadaverine or tyramide conjugates within intact fertilization envelopes was carried out using a two-tailed Student’s t-test, comparing experimental sets against control only.

**RESULTS**

Two transglutaminases are differentially localized in the sea urchin oocyte and egg

The genome of the sea urchin Strongylocentrotus purpuratus (Sea Urchin Genome Sequencing Consortium et al., 2006) contains two transglutaminases (TGs), named according to their subcellular location (as described below): an extracellular (eTG) and a nuclear (nTG) isoform. The two isoymes are derived from independent genes (Fig. 1A). Consistent with other family members (Lorand and Graham, 2003), neither S. purpuratus transglutaminase contains a signal peptide. Their open reading frames are 34% identical, with 26% and 29% identity between the N and C sequences outside of the signal peptide. Their open reading frames are 34% identical, with 26% and 29% identity between the N and C sequences outside of the signal peptide.
relative to oocytes). Antibodies generated against each isozyme specifically identify the said proteins (see Fig. S1 in the supplementary material). Anti-nTG antiserum predominantly detects a protein of ~110 kDa (Fig. 2A), near its predicted molecular weight (~83.9 kDa). Anti-eTG serum detects a ~210 kDa protein (Fig. 2A), a mass over twice the predicted size (~82.9 kDa), suggesting eTG may exist as a homodimer that is stable under these analytical conditions. This association may occur during its secretion, as observed with other extracellular transglutaminases (Adany and Bardos, 2003; Griffin et al., 2002), because in vitro synthesis of eTG does not yield dimers (see Fig. S1C in the supplementary material).

Biochemical fractionation and in situ localization also reveal differential distribution of the egg transglutaminases. nTG is highly enriched in the nucleus (Fig. 3B), although a fraction of the nTG population resides in the vitelline layer near the microvilli (Fig. 3L) and is retained in the fertilization envelope (Fig. 2C), primarily at the microvillar casts (Fig. 3L,F). eTG, conversely, is exclusively outside of the egg. It resides in the vitelline layer, enriched near microvilli (Fig. 3D,J,L), but is more evenly distributed throughout the mature fertilization envelope than the egg surface enrichment would predict (Fig. 3F,L). About 20% of the original egg population of eTG is retained with the zygote (Fig. 2A), while the remainder is probably released into the media (Fig. 2C).

Both TGs participate in fertilization envelope transamidation

Native ε(γ-glutamyl)lysine crosslinking is difficult to detect because of the small mass change it adds to the participating proteins and the insolubility of the resultant aggregates (Nemes et al., 2005). To overcome these limitations, we used low concentrations of Alexa Fluor 488-conjugated cadaverine (cad-AF) as a reporter of in vivo transamidation (Fig. 4A). High concentrations of unlabeled cadaverine (cad-HCl) can inhibit protein crosslinking, just as with putrescine or glycine ethyl ester (Battaglia and Shapiro, 1988; Nemes et al., 2005). Thus, we used cadaverine analogs to overcome the two major hurdles to observing transglutaminase function in vivo.

Cadaverine analogs primarily affect the microvillar cast transition of the fertilization envelope in a dose-dependent fashion. When present during normal fertilization, cad-AF reporter accumulates at microvillar casts (Fig. 4C), consistent with the enriched
Transamidation of specific substrates in the fertilization envelope

The covalent incorporation of the cad-AF reporter was used to identify transglutaminase substrates within the fertilization envelope. To enrich for labeled substrates, we dissolved fertilization envelopes with a solution of urea (Inoue and Hardy, 1971), dithiothreitol (Hall, 1978; Veron et al., 1977) and calcium chelators (Bryan, 1970; Carroll et al., 1986). Only softened fertilization envelopes formed in the presence of 3-amino triazole (+3AT) were sensitive to these chemicals, as indicated by their solubility (Fig. 5A) and the migration of their constituents into a polyacrylamide gel (Fig. 5A’). Including low concentrations of cad-AF during fertilization envelope formation allowed us to identify specific proteins modified by transamidation (Fig. 5A’). Resolution of these tagged samples by two-dimensional electrophoresis, followed by mass spectrometry analysis of the resolved spots, identified SFE9, rendezvin and ovoperoxidase as TG substrates within the fertilization envelope (Fig. 5B).

Transglutaminases control dityrosine crosslinking through two paths

In addition to being transamidated (Fig. 5), ovoperoxidase is sensitive to transglutaminase (Fig. 4), CGSP1 (Haley and Wessel, 2004) and Udx1 (Wong et al., 2004) activity. Given this preliminary network of enzyme regulation, we investigated whether transamidation affects other enzymatic activities during fertilization envelope assembly. Reporters used to measure the major activities include (Fig. 6A): conversion of Amplex Red to resorufin by exogenous horseradish peroxidase to measure Udx1 production of hydrogen peroxide on a population level (Wong et al., 2004), or without horseradish peroxidase to measure ovoperoxidase activity (see Fig. S4 in the supplementary material); incorporation of tyramide-Alexa Fluor 594 to measure ovoperoxidase activity per zygote (Wong and Wessel, 2008); and incorporation of cad-AF to measure transamination per zygote (Nemes et al., 2005).

Inhibition of transamidation reduces both hydrogen peroxide production and ovoperoxidase activity. As expected, chemical or antibody inhibition of transamidation blocks cad-AF accumulation in the fertilization envelope (Fig. 6B,C). Excess cad-HCl and anti-eTG IgGs also suppress Udx1-dependent resorufin accumulation and ovoperoxidase activity (Fig. 6E; see Fig. S4 in the supplementary material). The specific knockdown of hydrogen peroxide production by anti-eTG might account for the extra potency of the anti-eTG versus anti-nTG on tyramide-Alexa Fluor 594 incorporation into the fertilization envelope (Fig. 4D; Fig. 6E), suggesting that both TGs control ovoperoxidase activity but eTG also regulates Udx1.

Environmental acidification activates extracellular transglutaminases

Analogous to its sibling factor XIII (Adany and Bardos, 2003; Esposito and Caputo, 2005), we predicted that proteolysis would activate the extracellular transglutaminases. Alternatively, oxidation
by hydrogen peroxide could activate the catalytic cysteine residues in each TG (Griffin et al., 2002; Lorand and Graham, 2003). To distinguish these hypotheses, we assessed how the inhibition of each major enzyme activity affects cad-AF incorporation into the fertilization envelope and microvillar cast transmogrification (Fig. 6A). We used protease inhibitors to block CGSP1 (Haley and Wessel, 1999), diphenyleneiodonium (DPI) to block Udx1 production of hydrogen peroxide (Wong et al., 2004) and 3-aminotriazole (3AT) to block ovoperoxidase (Showman and Foerder, 1979).

Surprisingly, Udx1 and ovoperoxidase, but not CGSP1, regulate fertilization envelope transamidation. Inhibition of either hydrogen peroxide production (DPI) or ovoperoxidase (3AT) blocks incorporation of cad-AF, whereas protease inhibitors have no direct effect (Fig. 6B,C). Protease inhibitors do, however, suppress hydrogen peroxide production by Udx1, and consequently deplete dityrosine crosslinking by ovoperoxidase (see Fig. S4 in the supplementary material). Consistent with previous observations (Carroll et al., 1986; Mozingo and Chandler, 1991), only direct inhibition of transamidation with excess cad-HCl affects the morphological transition of the microvillar casts (Fig. 6D). Thus, transamidation at the microvillar casts occurs before transglutaminase activity is modified by Udx1 or by ovoperoxidase (Fig. 6C).

As CGSP1 does not affect transamidation, and transglutaminase activity occurs in advance of dityrosine crosslinking, we next sought non-enzymatic sources of transglutaminase activation. Many transglutaminases are activated by free Ca\textsuperscript{2+} (Lorand and Graham, 2003), but the 10 mM concentrations of Ca\textsuperscript{2+} in seawater are not sufficient to initiate transamidation. Instead, the activating factor is related to cortical changes during fertilization (Battaglia and Shapiro, 1988). The cell surface is transiently acidified between 30-120 seconds after fertilization (Paul et al., 1976; Smith et al., 2002), timing that is coordinate with exocytosis of cortical granule contents (Chandler and Heuser, 1979; Haley and Wessel, 2004; Kay and Shapiro, 1985; Matrese et al., 1997) and with peak transamidation activity (Battaglia and Shapiro, 1988). MIGHT acidic environments activate the transglutaminases, as observed for the teleost...
transglutaminase that crosslinks the chorion (Ha and Iuchi, 1998)? We tested this by determining whether cad-AF could be covalently linked to the egg surface by simply acidifying the seawater, mimicking the transient pH shift following fertilization (Fig. 7A). Indeed, lowering the seawater to pH to 5 increases transamidation (Fig. 7C), as reported by a punctate incorporation of cad-AF on the cell surface (Fig. 7B) that is consistent with the microvillar enrichment of these enzymes in eggs (Fig. 3). Surprisingly, a rapid shift from pH 5 to pH 8 by seawater exchange significantly abolished surface labeling (Fig. 7A,C). Thus, local acidification at the egg surface is sufficient to activate the transglutaminases, and the gradual return to alkaline pH 8 conditions 3-5 minutes after fertilization (Paul et al., 1976; Smith et al., 2002) probably inactivates them.

**DISCUSSION**
Identification of the transglutaminases (TGs) completes the molecular dissection of the sea urchin fertilization envelope, some 150 years since its first description (Derbès, 1847) and 100 years after its first major enzyme activity was measured (Warburg, 1908).
Ironically, the last enzyme identified is the first one active during fertilization envelope assembly (Battaglia and Shapiro, 1988; Larabell and Chandler, 1991; Veron et al., 1977).

Both eTG and nTG reside on the egg surface until fertilization, when they are transiently activated and locally modify the fertilization envelope (Fig. 8). eTG is present as inactive homodimers, the most effective form for extracellular transglutaminases (Griffin et al., 2002) such as mammalian factor XIIIa (Adany and Bardos, 2003; Esposito and Caputo, 2005); nTG may be stored as a monomer or a heat-sensitive dimer. At fertilization, the acidic lumen of the cortical granule increases pH just prior to exocytosis (Morgan and Galione, 2007); these protons are pumped out of the cell, thereby temporarily acidifying the cell surface (Paul et al., 1976; Smith et al., 2002). This transient acidification reversibly isomerizes and activates the transglutaminases (Lorand and Graham, 2003); when the local environment returns to pH 8 after 3-5 minutes (Paul et al., 1976; Smith et al., 2002), transamidation is repressed (Battaglia and Shapiro, 1988). Transamidation could be further repressed by hydrogen peroxide-mediated oxidation of its catalytic cysteine (Lorand and Graham, 2003) and/or by excess dityrosine crosslinking (Foerder et al., 1978; Wong et al., 2004; Wong and Wessel, 2008) as both Udx1 and ovoperoxidase activities ramp up as transamidation peaks (Fig. 8).

We suggest the following network of extracellular modifying activities among the major enzymes contributing to fertilization envelope assembly (Fig. 8): coordinate with cortical granule exocytosis, local acidification at the egg surface (Paul et al., 1976; Smith et al., 2002) (1) activates both transglutaminases. Alkalimization of the cortical granule lumen (Morgan and Galione, 2007) primes the auto-activation of CGSP1 (Haley and Wessel, 1999) and ovoperoxidase (Deits and Shapiro, 1985) so they are active when secreted. CGSP1 activity (2) allows the egg vitelline layer to separate from the plasma membrane. Proteolysis also directly activates Udx1, perhaps releasing the N-terminal peroxidase domain from its NADPH reductase region (Wong et al., 2004) to lengthen the half-life of hydrogen peroxide. Within the first 2 minutes of fertilization (Larabell and Chandler, 1991), (3) a surface-associated population of eTG activates Udx1 while (4) the remaining transglutaminases are released with the vitelline layer. As cortical granule structural proteins are woven into the vitelline layer, active transglutaminases retained at the microvillar casts mediate the I-to-T transmogrification, crosslinking microvillar cast proteins such as SFE9 (Wessel, 1995) prior to any crosslinking of the intercast regions (Chandler and Heuser, 1980; Mozingo et al., 1994). Intercast ovoperoxidase could
also be permanently tethered to proteoliasin by transamidation, limiting its redistribution to other domains of the fertilization envelope (Mozingo et al., 1994; Weidman et al., 1985). (6) Soluble CGSP1 and active transglutaminases released from the zygote surface and/or not retained in the fertilization envelope both diffuse away from the zygote, while the permeability of the fertilization envelope remains unrestricted. (7) As production of hydrogen peroxide from Udx1 increases, (8) ovoperoxidase-dependent crosslinking increases among the structural proteins. This burst of dityrosine crosslinking may also covalently modify the transglutaminases retained in the fertilization envelope, permanently shutting them off. This leaves only proteoliasin-tethered ovoperoxidase to complete inter-protein crosslinking (Chandler and Heuser, 1980; Larabell and Chandler, 1991; Mozingo et al., 1994), thereby establishing the permeability barrier associated with the mature fertilization envelope (Kay and Shapiro, 1985; Veron et al., 1977; Wong and Wessel, 2008).

This regulatory enzyme of the sea urchin fertilization envelope is parsimonious with decades of observations. First, blocking transamidation impairs microvillar cast transformation (Battaglia and Shapiro, 1988; Chandler and Kazilek, 1986; Mozingo and Chandler, 1991), thereby establishing the permeability barrier associated with the mature fertilization envelope (Kay and Shapiro, 1985; Veron et al., 1977; Wong and Wessel, 2008).

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This regulatory enzyme of the sea urchin fertilization envelope is parsimonious with decades of observations. First, blocking transamidation impairs microvillar cast transformation (Battaglia and Shapiro, 1988; Chandler and Kazilek, 1986; Mozingo and Chandler, 1991), thereby establishing the permeability barrier associated with the mature fertilization envelope (Kay and Shapiro, 1985; Veron et al., 1977; Wong and Wessel, 2008).
Based on the knowledge that cadaverine analogs are conjugated to endogenous glutamine residues (Nemes et al., 2005), we predict the following transamidation pairings within the fertilization envelope: (1) SFE9 and the vitelline layer rendezvous may stabilize the microvillar casts, consistent with the enrichment of SFE9 within this subdomain (Wessel, 1995) and the ability to incorporate cadaverine-Alexa Fluor 488 to the vitelline layer at the egg surface (Fig. 7). (2) Transamidation may tether specific proteins to the fertilization envelope. Ovoperoxidase glutamines, for example, could be covalently bound to proteolaisin lysines, thereby establishing more permanent association between these two proteins (Somers et al., 1989; Weidman et al., 1985); it is still possible to resolve the two proteins after pharmacological inhibition of ovoperoxidase (Deits et al., 1984; Showman and Foerder, 1979) because transamidation is repressed in the intercast region, where the majority of these proteins reside in the expanded fertilization envelope (Mozingo et al., 1994). Alternatively, (3) crosslinking of intra-enzyme glutamine and lysine residues may positively regulate ovoperoxidase, perhaps locking it in an active state following hysteretic modification (Deits and Shapiro, 1985; Deits and Shapiro, 1986). We hypothesize that a glutamine-conjugated analog would produce results consistent with these substrate pairings.

Thus, the two major crosslinking activities required for sea urchin fertilization envelope assembly are co-regulated. The major structural proteins of the S. purpuratus fertilization envelope proteome (Wong and Wessel, 2006b) are crosslinked by these enzymes at either microvillar casts or intercast domains (Wong and Wessel, 2008), establishing a mechanically ‘hardened’ shell for early embryogenesis. The relatively uniform contribution of these two enzyme activities is conserved among sea urchins (Cariello et al., 1994; Veron et al., 1977), even though other animals do not require two enzymes to achieve an analogously ‘hardened’ matrix: Transamidation is sufficient to harden the matrices of mosquito (Iijima et al., 2005) and most teleost eggs (Chang et al., 2002; Ha and Iuchi, 1998; Oppen-Berntsen et al., 1990; Yamagami et al., 1992); peroxidase is required for the teleost Tribulon (Kudo, 1988); and a zinc-dependent protease accomplishes the same task for amphibians (Lindsay and Hedrick, 2004). What, then, do these complementary interactions imply about the assembly of the sea urchin fertilization envelope? Might transamidation activity be carried over from its role during extracellular matrix modifications in the oocyte?

Post-translational modification is essential for the regulation of protein activity and is capable of changing individual molecules and/or cellular morphology. When these modifications are absent or go awry, however, the associated changes can gravely affect tissue integrity such that overall health is at risk. The absence of transamidation negatively impacts the survival of some animal zygotes (Battaglia and Shapiro, 1988; Chandler and Kazilek, 1986; Chang et al., 2002; Ha and Iuchi, 1998; Larabell and Chandler, 1991; Mozingo and Chandler, 1991; Oppen-Berntsen et al., 1990; Veron et al., 1977; Yamagami et al., 1992). Similarly, inhibition of an extracellular transglutaminase blocks normal peroxidase-dependent isodityrosine crosslinking of Chlamydomonas reinhardtii cell walls, which is lethal to the single cell alga (Waffenschmidt et al., 1999; Waffenschmidt et al., 1993). Aberrant transglutaminase activity is also associated with human pathologies, including skin defects (Candi et al., 2005; Lorand and Graham, 2003), blood clotting disorders (Adany and Bardos, 2003; Lorand and Graham, 2003) and neurodegenerative diseases (Aeschlimann and Thomazy, 2000; Cooper et al., 2002). Similarly, excess NADPH oxidase or peroxidase activity, each of which generates reactive oxygen permeability (Kay and Shapiro, 1985; Veron et al., 1977) of the fertilization envelope – characteristics that have been attributed primarily to dityrosine crosslinking. Second, the transglutaminase inhibitor glycine ethyl ester (Nemes et al., 2005) blocks ovoperoxidase activity at high concentrations (Foerder and Shapiro, 1981; Hall, 1978; Turner et al., 1985). Third, despite the activity of transglutaminase-dependent crosslinking, a single peroxidase inhibitor [e.g. diphenyleneiodonium (Wong et al., 2004), 3-aminotriazole (Showman and Foerder, 1979) or para-aminobenzoic acid (Hall, 1978)] is sufficient to abolish crosslinking of the intercast regions of the fertilization envelope (Mozingo et al., 1994), making it sensitive to mechanical shearing, chemical dissolution and gel electrophoresis (Showman and Foerder, 1979; Wessel, 1995; Wong et al., 2004). Finally, inhibition of CGSP1 blocks inter-protein crosslinking (Carroll et al., 1986) associated with thickening of the microvillar casts and intercast regions (Mozingo and Chandler, 1991). The same inhibitor also blocks hydrogen peroxide production (Coburn et al., 1981) and, logically, establishment of the fertilization envelope permeability barrier (Epel et al., 1970; Wong and Wessel, 2008).
radicals, can accelerate oxidative damage of proteins generally (Davies et al., 1987a; Davies et al., 1987b; Fridovich, 1998), expand the severity of tissue damage at sites of bacterial infection (Heinecke, 1999; Winterbourn et al., 2000) or at athleroschlerotic plaques (Jacob et al., 1996; Lambeth, 2007), advance the aging process through disruption of tissue integrity (Levine and Stadtman, 2001), and/or facilitate the proliferation of cancer (Lambeth, 2007; Winterbourn et al., 2000). The intersection between transamidation, dityrosine crosslinking and disease is clearest in the immune system, where misregulation of the orchestrated enzyme network could domino into chronic inflammation owing to improper clotting (Lorand and Graham, 2003) or excessive oxidative damage (Heinecke, 1999; Lambeth, 2007; Winterbourn et al., 2000). Although directed gene expression is a crucial source of regulation on a cellular level, additional levels of control are essential once these enzymes are released into the extracellular matrix. Our current knowledge that sea urchin transglutaminases and ovoperoxidase regulate each other during fertilization envelope assembly may
reveal how other extracellular matrix modifiers are managed throughout development and homeostasis, allowing us to differentiate normal from pathological activities.

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Supplementary material
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References


