Hierarchies of Protein Cross-linking in the Extracellular Matrix: 
Involvement of an Egg Surface Transglutaminase in 
Early Stages of Fertilization Envelope Assembly

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Abstract. The involvement of transglutaminase activity in fertilization envelope (FE) formation was investigated using eggs from the sea urchin, Strongylocentrotus purpuratus. Eggs fertilized in the presence of the transglutaminase inhibitors, putrescine and cadaverine, had disorganized and expanded FEs with inhibition of the characteristic I-T transition. The permeability of the FE was increased by these agents, as revealed by the loss of proteins from the perivitelline space and the appearance of ovoperoxidase activity in supernates from putrescine-treated eggs. [3H]putrescine was incorporated into the FE during fertilization in a reaction catalyzed by an egg surface transglutaminase that could also use dimethylcasein as a substrate in vitelline layer-denuded eggs. Egg secretory products alone had no transglutaminase activity. The cell surface transglutaminase activity was transient and maximal within 4 min of activation. The enzyme was Ca2+ dependent and was inhibited by Zn2+. We conclude that sea urchin egg surface transglutaminase catalyzes an early step in a hierarchy of cross-linking events during FE assembly, one that occurs before ovoperoxidase-mediated dityrosine formation (Foerder, C. A., and B. M. Shapiro. 1977. Proc. Natl. Acad. Sci. USA. 74:4214-4218). Thus it provides a graphic example of the physiological function of a cell surface transglutaminase.

The assembly of the sea urchin fertilization envelope (FE) provides a powerful system for analysis of the modulation of extracellular matrix (ECM) modifications. The process of FE assembly is temporally and spatially well controlled. Assembly is initiated by a Ca2+-dependent wave of exocytosis from egg cortical vesicles that results in the stoichiometric and catalytic conversion of a thin, tightly apposed egg glycocalyx, the vitelline layer (VL), to an elevated, highly cross-linked, and insoluble fertilization envelope (reviewed in Kay and Shapiro, 1985). The sequence of events that participate in this process has been partially defined biochemically and morphologically including: (a) the cleavage of VL attachments to the plasma membrane which permits its elevation from the egg surface (Carroll and Epel, 1975a); (b) structural rearrangement of the fibrous VL network into a trilaminar ECM by the incorporation of molecules released from cortical vesicles into the VL scaffold (Chandler and Heuser, 1980; Chandler and Kazilek, 1986); and (c) the ovoperoxidase-catalyzed covalent cross-linking of proteolipid and other components into the assembled FE (Foerder and Shapiro, 1977; Kay and Shapiro, 1987; Weidman et al., 1985; Weidman and Shapiro, 1987). These events construct the hardened, impermeable structure that protects the embryo well into the blastula stage of development.

This assembly sequence has been clarified by recent molecular insights which have contributed substantially to the classical observations of FE formation. However, certain older observations about FE assembly have remained unexplained by the above paradigm. For example, in the early assembly phase, there is a morphological transition of the microvillar casts in the FE from igloo ("I" form) to tent ("T" form) structures (Veron et al., 1977), the reason for which is unexplained in molecular terms. Additionally, Lallier (1970, 1971) discovered that glycine ethyl ester and other primary amines inhibit the apparent thickening and hardening of the FE. Since primary amines are effective competitive inhibitors of transglutaminases (Lorand et al., 1979), these early studies suggested that transglutaminases might be involved in fertilization envelope assembly. Although the presence of intracellular transglutaminase has been confirmed in the sea urchin egg (Cariello et al., 1984), no evidence directly implicates transglutaminase in a specific step of FE assembly.

Transglutaminases are thought to play several roles in biology, with substantial evidence in only a few examples. The best studied, that of the fibrin clot, involves the transamidative cross-linking of fibrinogen by secreted factor XIII (Lorand, 1972). Similarly, a transglutaminase released from...
damaged amebocytes is responsible for the coagulation of blood plasma in the lobster (Lorand et al., 1963; Fuller and Doolittle, 1971). The formation of the copulation plug in the guinea pig also depends upon transglutaminase activity (Williams-Ashman et al., 1972). Recently, the identification of a cell surface transglutaminase in mammalian hepatocytes (Slife et al., 1985; Tyrrell et al., 1986) and lung matrices (Cocuzzi and Chung, 1986) has led to speculation regarding its potential influence on the pericellular environment of these systems. However, both the overall biological significance and the specific targets for these cellular enzymes remain obscure.

To explore the association of transglutaminase in ECM modification, we have pursued the initial observations of Lallier (1970) and Lorand and Conrad (1984). Here we report that an egg surface transglutaminase is transiently activated at fertilization to catalyze specific structural changes in the nascent FE. As such, it acts as an early cross-linking event that modulates subsequent FE assembly. Thus, a well-coordinated hierarchy of cross-linking events appears to be responsible for the efficient assembly of a specialized ECM, the FE.

Materials and Methods

Gamete Preparation

Gametes from Strongylocentrotus purpuratus were obtained from mature animals collected in the intertidal regions of the Olympic Peninsula, WA. Artificial spawning was induced by intracoelomic injection of 0.5 M KCl; eggs were collected in filtered sea water (FSW) (Millipore/Continental Water Systems, Bedford, MA) and sperm were collected dry. Spawning was allowed to proceed for 1 h after which time the eggs were passed through three layers of cheesecloth and dejellied by lowering the pH of the FSW to 5.5 with gentle stirring for 4 min. The eggs were then washed in several changes of fresh FSW, pH 7.8. Gametes were maintained at 10°C at all times.

Transglutaminase Inhibitors and Fertilization Envelope Formation

The effects of the primary amines, putrescine, cadaverine and glycine ethyl ester (Sigma Chemical Co., St. Louis, MO), on the assembly of the fertilization envelope were examined at the levels of light and electron microscopy. These compounds are potent competitive inhibitors of transglutaminase activity (Lorand et al., 1979). In some experiments the ovoperoxidase inhibitor, aminotriazole (ATA; 2 mM) was also included. Control experiments lacked primary amines or contained the secondary amine, sarcosine ethyl ester (Sigma Chemical Co.).

The ability of the FE to expand from the egg surface was examined by light microscopy. Aliquots of eggs were fertilized in 50 mM Tris-FSW, pH 8.0 containing either putrescine, cadaverine, sarcosine ethyl ester, and/or ATA. At 15-min postinsemination aliquots of these suspensions were transferred to a temperature-controlled microscope stage (10°C) and the diameter of the FE was measured using phase optics and an optical micrometer. At least 50 eggs were examined for each sample; each experiment was carried out in triplicate with eggs from each of four different females. Photographs were obtained using bright field optics and Kodak Plus-X film.

For scanning electron microscopy, aliquots of the inseminated eggs were fixed in 2% glutaraldehyde (EM grade; Polysciences, Inc., Warrington, PA) in FSW containing 50 mM Tris for 1 h at 10°C. They were subsequently washed four times in FSW and postfixed for 1 h at room temperature in 1% OsO4 in FSW containing 50 mM cacodylate, pH 7.4. Fixed eggs were washed three times in distilled H2O and dehydrated in an ascending series of EtOH to 100%. After dehydration, they were transferred to envelopes made of 53 μM Nite cloth (Teklo, Inc., Elmsford, NY) and infiltrated with Freon 112 (E. I. Du Pont de Nemours & Co., Inc., Newtown, CT) and affixed to 200 mesh copper grids. Sections were examined on a Phillips 201 TEM and photographed on Kodak EM film.

Egg Surface Transglutaminase Assay

The incorporation of 1H]putrescine (30 Ci/mmol; Amersham Corp., Arlington Heights, IL) into the FE was measured at 10°C using small volumes of eggs (1-3 ml) inseminated in FSW containing 50 mM Tris and 2 mM ATA. At 15-min postinsemination, 40 mM nonradioactive putrescine was added to all samples and the eggs were subjected to five passes through an homogenizer (Wheaton Industries, Millville, NJ) with a glass piston to remove the FE. No egg lysis occurred under these conditions and 80% of the eggs were denuded of their FE. The eggs settled (1 g) for 5 min, after which the supernate containing the FE was collected. The supernate was centrifuged at 500 g for 1 min and the FE pellets were resuspended in 5 ml of FSW containing 50 mM Tris and 40 mM cold putrescine. A similar wash protocol was repeated 10 times, after which aliquots of the FE were examined for radioactivity in a beta counter (model LS 1801; Beckman Instruments, Inc., Palo Alto, CA).

Since it was impractical to obtain large quantities of radiolabeled FEs, N,N-dimethylcasein (Sigma Chemical Co.) was used as an artificial substrate for measurement of egg surface transglutaminase activity. For these experiments, unfertilized eggs were denuded of their vitelline layers in 10 mM dithiothreitol-FSW for 7 min and washed six times in 100 vol of fresh FSW. A 20% suspension of the denuded eggs was activated with acetic acid (see Weidman and Kay, 1986). Immediately after activation, aliquots of the eggs were resuspended 1:1 in FSW containing 50 mM Tris (pH 7.8), 1.0 mg/ml dimethylcasein, 2 mM ATA, and varying concentrations of [1H]putrescine at 10°C. At intervals after this resuspension, nonradioactive putrescine was added to 40 μM, and egg supernates were collected and precipitated with 20% cold TCA for 30 min on ice. The pellets were resuspended, washed eight times in 10% TCA with centrifugation, one time in 95% cold EtOH, and then solubilized in 0.1 N NaOH, and assayed for radioactivity. Protein concentrations were assayed by the Lowry (Lowry et al., 1951) or BCA procedures (Smith et al., 1985).

Primary Amine-induced Release of FE or Perivitelline Space Proteins

To ascertain whether the insertion of specific proteins into the FE or perivitelline space was altered by primary amines, we assayed for the presence of ovoperoxidase in the supernate of fertilized eggs. Suspensions of unfertilized eggs were inseminated in FSW containing varying quantities of putrescine and allowed to settle for 15 min, after which aliquots of the supernates were collected. Supernates were only collected from suspensions in which 100% fertilization was achieved. To the supernate was added soybean trypsin inhibitor (10 μg/ml Sigma Chemical Co.) and it was centrifuged at 15,000 g for 1 min to remove sperm. Ovoperoxidase activity was assayed using the guaiacol assay for peroxidase (Foerder and Shapiro, 1977) and a spectrophotometer (model DU-7; Beckman Instruments, Inc.). To determine the total quantity of ovoperoxidase that is released during cortical exocytosis, the supernates from activated, VL-denuded eggs were assayed for ovoperoxidase in the same manner. As a control, the effect of up to 40 mM putrescine on ovoperoxidase activity in these supernates was also analyzed. To correlate the quantity of ovoperoxidase released from the VL-denuded and intact egg suspensions, the quantity of eggs in suspension was determined for each experiment.

Results

Morphological Effects of Primary Amines

The fully assembled FE has several characteristics as viewed by light microscopy. It expands to an average diameter of 106

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Figure 1. Bright field micrographs of eggs photographed 15 min after insemination in buffered sea water (a) and in buffered sea water containing 40 mM putrescine (b). The structure of the fertilization envelopes (arrow) differ greatly; the normal FE is highly refractile and appears to be ~5 \( \mu \)m thick, while the putrescine-treated FE is less refractile and has expanded further from the egg surface than normal. Note that the putrescine-treated egg occupies an eccentric location within the perivitelline space, unlike normal eggs which are centrally located. The hyaline layers (arrowhead) are visible on both eggs, with this layer forming a cast over the site formerly occupied by the fertilization cone at the 11 o'clock position in b. Bar, 8 \( \mu \)m.

\[ \text{Figure 2. Measurement of the extensibility of the FE as a function of putrescine concentration. FE diameters were measured at 15 min postinsemination using bright field optics (see Materials and Methods) and the approximate surface areas were calculated. The surface area of the FE increased under the influence of putrescine alone (solid circles) with maximal expansion occurring at 40 mM. FEs formed in the presence of putrescine and 2 mM aminothiazole (open circles) were slightly larger, but also achieved maximal expansion at 40 mM putrescine.} \]
I-form morphology was maintained (Fig. 3 c), even if they were examined up to 20 min after insemination. Moreover, FEs formed in the presence of primary amines had a heterogeneous distribution of I-form casts, with areas of low and high density (compare Figs. 3, c and d). In control eggs, the I- (or T)-form casts were homogeneously distributed. Sarcosine ethyl ester had no effect on FE morphology.

By TEM, several characteristics of FE assembly were observed to be altered in the presence of putrescine, in addition to the inhibition of the I-T transition (Fig. 4). The ordered structure of the normal FE matrix (Fig. 4 a) was disrupted in eggs fertilized in the presence of exogenous primary amine (Fig. 4 b), although FE thickness was approximately the same as that of control eggs. The perivitelline space of control eggs contains a high concentration of flocculant material, probably protein, that can be observed by TEM (Fig. 4 a); this material was almost completely absent in putrescine-treated eggs (Fig. 4 b).
Figure 4. Transmission electron micrographs of a normal FE (a) and an FE assembled in the presence of 40 mM putrescine (b) fixed at 15-min postinsemination. (a) The normal FE (arrow) exhibits T form morphology and its matrix is highly ordered. The perivitelline space (P) has collapsed in size due to preparation for TEM (compare with Fig. 1), but contains a high density of flocculant material, probably proteins from the cortical vesicle secretion. The region beneath the perivitelline space is the hyaline layer (H) where sections of egg microvilli can be seen (arrowhead). (b) The FE assembled in the presence of 40 mM putrescine (arrow) retains I form morphology and its matrix appears to be disorganized, but it is approximately the same thickness as the normal FE. Note that the perivitelline space (P) is practically devoid of the dense, flocculant material. The hyaline layer (H) and sections of microvilli (arrowhead) can be observed beneath the perivitelline space. Bar, 0.1 μm.

Ovoperoxidase Release from Transglutaminase-inhibited FEs

To examine whether specific proteins, normally present in the fertilization envelope or perivitelline space, were released from FEs assembled in the presence of primary amines, we assayed for ovoperoxidase activity in supernates from fertilized eggs. We discovered that, after normal activation, ~15–17% of the total ovoperoxidase that is secreted during the cortical reaction passes through the assembling FE into the surrounding seawater (as compared to its release from VL-denuded eggs). Transglutaminase inhibitors led to an augmented release of ovoperoxidase. For example, an in-
increasing amount of ovoperoxidase was found in the surrounding seawater as a function of increasing concentrations of putrescine, with up to ~40–45% of the total ovoperoxidase from the FE and perivitelline space being released (Fig. 5). This interference with normal ovoperoxidase localization occurred over the same concentration range as the increase in envelope surface area, with an effect first being found at 5 mM putrescine. In addition to ovoperoxidase release, numerous other proteins, probably from the perivitelline space, also appeared in the supernate of putrescine-treated eggs as confirmed by SDS-PAGE (data not shown).

Egg Surface Transglutaminase Activity

Putrescine was incorporated into the FE when eggs were inseminated in the presence of \([\text{H}]\)putrescine (Fig. 6). At the low concentrations shown, which are far below the \(K_m\) for putrescine (see below), the incorporation was linear. This incorporation was diminished by excess cold putrescine, but not by sarcosine ethyl ester. It was impractical to collect large quantities of radiolabeled FEs with which to examine characteristics of the enzyme involved in putrescine incorporation. Hence, we relied upon the use of VL-denuded eggs for this purpose, as described in Materials and Methods.

By activating VL-denuded eggs in FSW containing dimethylcasein we were able to observe the incorporation of \([\text{H}]\)putrescine into the exogenous acceptor protein (Table I). We observed that unactivated eggs possessed low activity which increased up to fourfold after activation. The egg itself, and not its secretory products, was responsible for \([\text{H}]\)putrescine incorporation into casein; no incorporation was seen by incubation of egg cortical vesicle secretion product (see Materials and Methods) in casein-containing FSW. The incorporation of \([\text{H}]\)putrescine was inhibited by excess unlabeled putrescine or cadaverine, low levels of \(\text{Zn}^{2+}\), and \(\text{Ca}^{2+}\) dependent (Table I). Sarcosine ethyl ester was ineffective as an inhibitor of this reaction. The apparent \(K_m\) for putrescine in this assay was 12 \(\mu\)M.

The above data strongly suggested the presence of an egg surface transglutaminase. To explore the kinetics of its activity after egg activation, we artificially activated eggs and measured \([\text{H}]\)putrescine incorporation into casein as a function of time. As shown in Fig. 7, the low transglutaminase activity seen with unactivated eggs increased twofold at ~1 min after activation, and reached a maximum at ~4 min, after which it gradually decreased to the unactivated level. This transient activation of transglutaminase occurred concomitantly with cortical vesicle exocytosis, i.e., at the time the vitelline layer would be detaching from the plasma membrane of eggs under physiological conditions.

**Discussion**

The above data indicate that an egg surface transglutaminase participates in an early event of fertilization envelope assembly.
dimethyl casein as a function of time as promoted by activated, VL-
denuded eggs. Denuded eggs were suspended in dimethylcasein and
tivity was measured in terms of the amount of [3H]putrescine in-
were obtained and assayed for TCA-precipitable radioactivity. Ac-
corporated per gram casein.

Figure 7. Incorporation of [3H]putrescine incorporation into
dimethyl casein as a function of time as promoted by activated, VL-
denuded eggs. Denuded eggs were suspended in dimethylcasein and
[3H]putrescine containing sea water at varying intervals after acti-
vation. After 2 min of incubation, supernates from the suspensions
were obtained and assayed for TCA-precipitable radioactivity. Ac-
tivity was measured in terms of the amount of [3H]putrescine in-
corporated per gram casein.

We have shown that the FE is abnormally expanded when it is assembled in the presence of transglutaminase
inhibitors. These inhibitors block the I-T transition and lead to an asymmetric distribution of microvillar casts on the fer-
itellogen envelope. Transglutaminase-inhibited FEs have an altered laminar appearance and permit leakage of proteins,
including ovoperoxidase, from the perivitelline space. In ad-
dition, the transglutaminase substrate, putrescine, is incor-
porated into elevating fertilization envelopes during fertiliza-
tion. The activity of the responsible enzyme appears to be transiently increased, with maximal levels occurring within
4 min after egg activation.

Transglutaminases are Ca^{2+} dependent (Lorand and Con-
rad, 1984) and inhibited by specific exogenous primary
amines (Lorand et al., 1979) or very low concentrations of
Zn^{2+} (Lorand and Conrad, 1984). The egg surface enzyme
has all of these characteristics. Hence, these data lend strong
support to the hypothesis of Lallier (1970, 1971) that normal
fertilization envelope assembly is dependent upon transglu-
taminase activity. A cytosolic transglutaminase has been
shown to be activated in the sea urchin egg after fertilization
(Cariello et al., 1984), but its potential relationship to FE
formation was not discussed. Indeed, the cytosolic enzyme
may have no relationship to FE assembly, since we have
found an egg cytosolic transglutaminase with properties dis-
tinct from those of the egg surface enzyme (Battaglia, D. E.,
unpublished data). Certain previous data, presented only in
abstract or thesis form (Campbell-Wilkes, 1973), have sug-
gested that transglutaminase might play a role in fertilization
envelope formation, however, these data were presented in
too little detail to compare with the present work.

The most striking conclusion from our studies is that a hi-
ernarchy of cross-linking events appears to be operative dur-
ing the FE assembly process. It was previously established
that the ultimate cross-linking of dityrosine residues by
ovoperoxidase happens 7-10 min after fertilization (Foerder
and Shapiro, 1977), whereas the transglutaminase activation
observed here occurs within the first 4 min. Transglutamin-
ase is therefore active while the cortical reaction initiates
vitelline layer elevation and the modifications of this glyco-
calyx are just beginning. Just after this the VL matrix is dra-
matically altered via the insertion of paracrystalline materials
(Chandler and Heuser, 1980) along with the proteolaisin-

Moreover, the phenomena described above were seen even
in the presence of the ovoperoxidase inhibitor, aminotri-
azole.

The timing of transglutaminase activity probably explains
the hyperextension of the FE and its associated structural ab-
normalties in the presence of primary amines. Elevation and
expansion of the VL matrix from the egg surface is induced
by the osmotic pressure created by the secretion of cortical
vesicle contents into the perivitelline space (Loeb, 1908;
Hiramoto, 1955a, b) concomitant with the cleavage of its at-
tachment sites to the plasma membrane by a cortical protease
(Carroll and Epel, 1975a, b; Alliegro and Schuel, 1988).
The egg then becomes suspended by the colloid-like nature
of the fluids in the perivitelline space (Hiramoto, 1955a, b).
Inhibition of transglutaminase activity could permit VL ex-
pansion to exceed normal limits. This effect, most marked in
certain zones, could cause the asymmetric distribution of
microvillar casts on the FE (e.g., Fig. 1, c and d), abnormal
insertion of secreted proteins into its matrix, its excessive
permeability to perivitelline proteins, and the eccentric loca-
tion of the egg within the perivitelline space.

The $K_m$ for [3H]putrescine incorporation into casein by
VL-denuded eggs is two to three orders of magnitude lower
than that required for the induction of hyperextensibility of
the matrix (Fig. 2) or leakage of ovoperoxidase (Fig. 5). A
reasonable explanation for this difference is that the gly-
cocalyx substrates are intimately positioned near the activated
transglutaminase; thus, exogenous inhibitors could be needed
at extremely high concentrations to match the effective con-
centrations of the endogenous substrate in the VL.

The transient nature of transglutaminase activity suggests
several possibilities for its control. By analogy to blood
coaugulation factor XIII (Lorand, 1972), the activation me-
chanism may be proteolytic in nature, since proteases are in-
cluded in the cortical granule contents (Carroll and Epel,
1975b; Alliegro and Schuel, 1988). Alternatively, since it is
known that the egg surface greatly increases in surface area
during cortical exocytosis, followed by internalization of
significant portions of the plasma membrane within 5 min af-
ter activation (Schroeder, 1979), management of plasma
membrane domains may control its presentation to its sub-
strate. These points will be better addressed with subcellular
systems and isolated enzyme preparations.

Evidence for other cell surface transglutaminases, possi-
bly integral or peripheral membrane proteins, has been
provided in mammalian hepatocytes (Tyrrell et al., 1986)
and pulmonary tissue (Cocuzzi and Chung, 1986), although recent evidence suggests that their surface localization may be an artifact of the cell homogenization procedures that were used to localize the enzymes (Juprelle-Soret et al., 1988). Our studies with intact eggs do not suffer from this difficulty. Investigations on cell surface transglutaminases are directed towards the possibility that they may be involved in intercellular adhesion or modification of fibrous extracellular matrices. Such a role is shown here directly in the sea urchin egg which possesses one of the few cell surface transglutaminases with a known biological function. We believe that egg surface transglutaminase activity represents the initial stage in a hierarchy of cross-linking activity that is integral to the modification of an extracellular matrix, the fertilization envelope.

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