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Expression of Type IV Collagen-Degrading Activity During Early Embryonal Development in the Sea Urchin and the Arresting Effects of Collagen Synthesis Inhibitors on Embryogenesis

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Abstract Type IV collagen-degrading activity was expressed in homogenates of *Lytechinus pictus* embryos during embryogenesis. Activity was concentrated 1,600-fold by ammonium sulfate fractionation, ion exchange, and gel chromatography and could not be activated further upon trypsin or organomercurial treatment. This enzyme activity could also degrade gelatin but had no affinity for type I, III, and V collagens. Activity was inhibited by addition of excess type IV collagen or gelatin, but was unaffected by addition of excess amounts of non-collagenous proteins of the extracellular matrix. Chelators such as 1,10-phenanthroline or Na_2EDTA reduced activity to control levels. Inhibitors of plasmin and of serine and thiol proteases were without effect. Type IV collagen-degrading activity first became apparent at the stage of early mesenchyme blastula. It then increased by a small increment and remained stable up to the stage of late mesenchyme blastula, coinciding with first detection of collagen synthesis and the appearance of the archenteron. Thereafter, a sharp increase in activity was observed, concurrently with remodelling of the archenteron. Maximum activity was attained at prism stage and was retained throughout to pluteus-larva stage. The specific inhibitors of collagen biosynthesis 8,9-dihydroxy-7-methyl-benzo[b]quinolizinium bromide and tricyclodecane-9-yl xanthate arrested sea urchin embryo development at early blastula, prevented the invagination of the archenteron, and reverted the expression of type IV collagen-degrading activity to non-detectable levels. Removal of the inhibitors allowed embryos to gastrulate and express type IV collagen-degrading activity.

Key words: sea urchin embryogenesis, type IV collagen-degrading activity, collagen synthesis inhibitors

The extracellular matrix (ECM) is important for cell adhesion, differentiation, and proliferation in embryonic tissue [Wessells, 1977; Kleinman et al., 1981; Spiegel et al., 1983; Bernfield et al., 1984]. Wessel et al. [1984] showed that in the sea urchin most of the components of ECM are present in the unfertilized egg and that following fertilization these components redistribute and accumulate in unique patterns associated with specific stages of morphogenesis. Thus, it appears that many spatial and temporal events of embryogenesis are mediated by cell-ECM interactions [data reviewed in McClay and Ettensohn, 1987].

Amongst ECM constituents, collagens appear to play a key role in the developing sea urchin embryo. At least three immunologically distinct collagen types have been identified during embryogenesis by using antibodies to vertebrate fibrillar (types I and III) and basement membrane (type IV) collagens [Wessel et al., 1984; Wessel and McClay, 1987]. Genomic or cDNA clones for a discontinuous collagenous sequence [Venkatesan et al., 1986; Angerer et al., 1988] and for procollagen chains [D'Alessio et al., 1989, 1990; Exposito et al., 1992a,b], as well as the Gly-X-Y encoding sequences to RNA [Saitta et al., 1989], have been isolated, confirming the existence of heterogeneous collagen proteins in the ECM of the developing embryo. Biosynthe-

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sis of collagenous protein, which begins at blastula and increases several fold thereafter, and disposition of cross-linked collagen molecules in the ECM of the embryo are essential for both gastrulation and spiculogenesis [Blankenship and Benson 1984; Wessel and McClay, 1987]. In this context, the use of inhibitors of collagen biosynthesis, such as the lathyritic agent β-aminopropionitrile (BAPN) which reduces the cross-linking of collagen molecules [Kleinman et al., 1981; Butler et al., 1987], has contributed in the elucidation of cell-ECM interactions during embryogenesis. BAPN treatment of sea urchin embryos inhibits deposition of collagen and differentiation of the primary mesenchyme cells and thus results in inhibition of gastrulation and spiculogenesis [Butler et al., 1987; Wessel and McClay, 1987; Wessel et al., 1989].

The activity of enzymes involved in collagen biosynthesis, such as prolyl hydroxylase, has been shown to increase simultaneously with collagen synthesis and deposition in the blastocoel and the invagination of the archenteron at gastrula [Mizoguchi and Yazumasu 1983; Wessel et al., 1984; Wessel and McClay, 1987]. Homeostasis of collagenous proteins, however, depends not only on the biosynthesis of new material, but rather is a dynamic process reflecting the net result of biosynthesis and concomitant degradation. Indeed, a rapid turnover of collagen molecules has been demonstrated during development in the sea urchin, as assessed by Western blots and immunofluorescence [Wessel and McClay, 1987]. This rapid turnover of collagenous protein, together with the fact that mesenchyme cells can overcome barriers of basement membranes (BM) during embryogenesis in the sea urchin, suggests the presence of collagenases and other collagen degrading enzymes. Microinjection studies have demonstrated activities sensitive to collagenases during gastrulation [Spiegel and Burger 1982], but such enzymes have not been detected as yet in the developing embryo.

The simultaneous occurrence of both biosynthesis and degradation of collagenous protein in the developing sea urchin embryo would be consistent with similar findings observed in other biological systems in which ECM remodelling occurs [Bernfield et al., 1984; Folkman, 1985; Maragoudakis, 1989; Missirlis et al., 1990a]. In the present study, we searched for the expression of collagen type IV degrading activity during the early developmental stages of *Lytechinus pictus* embryos. We also tested the effect of two specific inhibitors of collagen biosynthesis, 8,9-dihydroxy-7-methyl-benzo[b]quinolizinium bromide (GPA1734) and tricyclodecane-9-yl xanthate (D609), on the ontogeny of collagenous protein and of type IV collagen-degrading activity during the early stages of embryogenesis in the sea urchin. GPA1734, which prevents the postribosomal hydroxylation of proline and lysine and inhibits deposition of underhydroxylated type IV collagen into pre-existing BM collagen [Maragoudakis et al., 1978a,b] and D609, by an as yet unknown mechanism, have been shown to be specific inhibitors of collagen biosynthesis, to inhibit BM biosynthesis and angiogenesis in the chick chorioallantoic membrane system (CAM), and to retard the growth of Walker 256 carcinoma transplants, presumably via the above mechanism [Maragoudakis et al., 1988a,b; 1990; Missirlis et al., 1990b].

We found that type IV collagen-degrading activity is expressed in the developing sea urchin embryo, first appearing at primary mesenchyme blastula, in coincidence with the appearance and remodelling of the archenteron, and that enzyme activity is expressed only in correlation with biosynthesis of new collagenous protein.

MATERIALS AND METHODS

Fertilization of Sea Urchin Eggs

Gametes were obtained from *Lytechinus pictus* gravid adults (Marinus Inc., Westchester, CA) by intracoelomic injection of 0.5 M KCl. Sperm was collected in sterile petri dishes. Eggs were disseminated in artificial sea water, buffered with 0.18 gr/l of $NaHCO_3$ (ASW) [Misevic and Burger, 1986]. When shedding was completed, eggs were passed through a nylon 80 μm mesh to remove debris of broken spines and fecal pellets. The egg suspension was allowed to settle, washed three times with fresh ASW, and fertilized with a diluted sperm solution, at a ratio of about 50 sperms to one egg. Excess sperm was removed by aspiration of the supernatant. Batches with over 95% fertilization rate and subsequent synchronous development were used. Embryos were diluted with ASW and allowed to grow at room temperature (21°C), in sterile flat glass bowls at a concentration of about 3–5 × 10^3 embryos/ml in ASW also containing 50 U/ml penicillin and 50 μg/ml streptomycin.

Preparation of Enzyme and [^{3}H]-Collagen Substrates

Sea urchin sperm, unfertilized eggs, or embryos obtained at several stages of development (morula, blastula, gastrula, prism, pluteus) were washed several times with ice-cold 50 mM Tris-HCl (pH 7.4) containing 3 mM $CaCl_2$, and suspended in the same buffer at approximately 5×10^4 embryos/ml. All steps were performed at 4°C. The suspension was sonicated in a Bransonic 221 sonicator for five 10 sec bursts, with 2 min intervals between sonications, during which suspensions were kept on ice, and centrifuged at 10,000*g* for 10 min. The 10,000*g* supernatants were adjusted to contain 250 mM sucrose and centrifuged at 100,000*g* for 1 h. Supernatant fractions and pellets resuspended in the same buffer were assayed for protein content by the Folin method [Lowry et al., 1951; Peterson, 1977] using bovine serum albumin as standard, subdivided, and stored at −18°C. Proteins in the 100,000*g* supernatant were fractionated by $(NH_4)_2SO_4$ precipitation at 4°C to 25% saturation and the pellet recovered by centrifugation at 30,000*g* for 30 min. This procedure was repeated to obtain the 25–50% and finally the 50–80% $(NH_4)_2SO_4$ pellet. All supernatant fractions and pellets (resuspended in the extraction buffer) were exhaustively dialyzed against the same buffer and assayed for protein content and type IV collagen-degrading activity. An aliquot (1 ml) of the 25–50% $(NH_4)_2SO_4$ fraction was exhaustively dialyzed against 25 mM Tris-HCl (pH 7.5) Triton X-100 R (Sigma) was added to a final concentration of 0.1% and type IV collagen-degrading activity was further purified by ion exchange chromatography on DEAE-Sephacel and gel chromatography on Sephacryl S-300 as previously described [Karakiulakis et al., 1988]. Void volume (*Vo*) and total volume (*Vt*) were determined using Blue Dextran (average MW: 2×10^6; Sigma) and L-[U-^{14}C]-proline (specific activity 264 mCi/mmol; Amersham International), respectively.

[^{3}H]-type IV collagen was prepared by acetylation of type IV collagen from human placenta (C-7521; Sigma) with [^{3}H]-acetic anhydride (25 mCi, specific activity 9.8 Ci/mmol; Amersham International) as previously described [Karakiulakis et al., 1988, 1989]. Similarly, [^{3}H]-type I, [^{3}H]-type III, and [^{3}H]-type V collagens were prepared by acetylation of human placenta collagens type I (C-7774; Sigma), type III (C-4407; Sigma), and type V (C-3657; Sigma), respectively, with [^{3}H]-acetic anhydride (2 mCi each, specific activity 6.6 Ci/mmol; Amersham International). For each substrate, intact [^{3}H]-collagen molecules were separated from fragmented contaminants, non-incorporated [^{3}H]-acetic anhydride, and [^{3}H]-acetic acid by-product, which would otherwise interfere with the collagenase assay procedure, by gel chromatography on a Sepharose CL-6B column. To determine the [^{3}H] labelling index, 100 μl aliquots of the stock solution of each substrate were hydrolyzed in 6 N HCl for 24 h at 100°C and the amount of [^{3}H] solubilized was taken to reflect the total [^{3}H] present in an aliquot. Protein concentration was determined in an aliquot of the stock solution.

Assay for Type IV Collagen-Degrading Activity

Type IV collagen-degrading activity was measured according to Liotta et al. [1982] as previously described [Karakiulakis et al., 1990; Missirlis et al., 1990a]. Assays were performed with 100 μl (approximately 8 nM, 2 μg protein) of [^{3}H]-acetylated type IV collagen, made up to 0.4 ml with 50 mM Tris-HCl (pH 7.4) also containing 200 mM NaCl, 3 mM $CaCl_2$, and NaN_3 (0.01% final, to prevent contamination with microorganisms during incubations, which might be supplying protease activity). Where indicated, trypsin (EC 3.4.21.4, type III; Sigma) (100 μg/ml), p-aminophenylmercuric acetate (APMA) (1 mM), Na_2EDTA (10 mM), 1,10-phenanthroline monohydrate (2 mM), phenylmethanesulphonyl fruoride (PMSF) (5 mM), N-ethylmaleimide (NEM) (5 mM), benzamidine hydrochloride (5 mM), soybean trypsin inhibitor (type I-S; Sigma) (500 μg/ml), L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone, (tosyl-L-phenylalanine chloromethyl ketone), (TPCK) (2 mM), and L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-hydrochloride, (tosyl-L-lysine chloromethyl ketone), (TLCK) (2 mM) were also included. Activity was also measured using as substrate 2 μg of protein of [^{3}H]-collagens type I, III, or V and [^{3}H]-gelatin (20 μg/ml each of [^{3}H]-type I and [^{3}H]-type IV collagens heated at 60°C for 10 min). Degradation of [^{3}H]-type IV collagen was also measured in the presence of excess amounts (0.5 mg/assay) of type IV collagen, gelatin (0.5 mg/ml each of collagen type I (from calf skin, C-3511; Sigma) and collagen type IV, heated as above), laminin (L-2020; Sigma), and fibronectin (F-4759; Sigma). Finally, enzyme activity was measured in the presence of GPA1734 (up to 200 μM) and D609 formulation A (D609A)

(up to 200 μM) or formulation B (D609B) (up to 100 μM). GPA1734 (MW 305.15 was prepared by Dr. J. Watthey of Ciba-Geigy Corp., Summit, NJ [Watthey et al., 1973]) and D609 (MW 266 was obtained from Dr. Schatton of Merz and Co., Frankfurt, Germany, in two formulations: D609A was pure D609 and D609B was a mixture of D609 and undecanoic acid [1:1 w/w]). The reaction was started by addition of 100 μl of enzyme solution and incubations were carried out at 35°C for 24 h. At the end of the incubation period 200 μl of the mixture was transferred to microcentrifuge tubes containing 100 μl of 6% trichloroacetic acid–0.3% tannic acid and vortexed to stop enzymatic activity. Tubes were transferred to 4°C for 20 min. Undigested substrate was centrifuged out at 6,500*g* for 20 min. An aliquot of the supernatants (200 μl) was transferred to vials containing 3.5 ml of Ultima Gold XR (Canberra-Packard SA, Zurich, Switzerland) scintillation mixture. Radioactivity was measured in a Packard 1900CA liquid scintillation analyzer. All determinations were carried out in triplicate. Results were expressed as % of dpm released per μg of protein. Radioactivity released from [^{3}H]-type IV collagen when incubated with buffer replacing the enzyme preparation was considered as blank. Control values were those obtained with boiled enzyme preparations.

Characterization of the Enzymatic Products by Gel Chromatography

Gel chromatography on Sepharose CL-4B column (1.1 × 40 cm) was performed at room temperature with 0.1 M sodium phosphate buffer saline (PBS) (pH 7.4) containing 0.1% SDS and 0.01% NaN_3 [Karakiulakis et al., 1988]. Fractions of 0.5 ml were collected with a flow rate of 0.075 ml/min and assayed for radioactivity. *Vo* and *Vt* were determined using Blue Dextran and [^{14}C]-proline, respectively. In the experiments designed to study the molecular size of type IV collagen components, aliquots of [^{3}H]-type IV collagen were incubated with enzyme buffer at 35°C for 24 h and then reduced and denatured following suspension in 0.1 M PBS (pH 7.4) containing 5 mM PMSF, 0.1% (w/v) SDS, and 5% (v/v) 2-mercaptoethanol, and heated in sealed tubes at 100°C for 2 min. The mixture was centrifuged at 6,000*g* for 10 min to precipitate non-solubilized substrate and the supernatant applied to the column. In experiments designed to investigate the molecular size of the products of [^{3}H]-type IV collagen after treatment with the Sephacryl S-300 fraction of sea urchin embryos obtained at prism stage of development, or with *Clostridium* collagenase, the substrate was incubated at 35°C for 24 h with the appropriate enzyme solution as described above. Enzyme activity was stopped by addition of 2 mM 1,10-phenanthroline. The mixture was then reduced and denatured, as above, and applied to the column. Fractions eluted were assayed for radioactivity.

Amino Acid Uptake and Incorporation Studies

Embryos, calibrated to 3–5 × 10^3/ml, were cultivated for 28 h, as described, in 2 ml of ASW containing [^{35}S]-methionine (0.4 μCi/ml, specific activity 10 mCi/mmol; Amersham International) in the presence or in the absence of GPA1734 (5–25 μM) or D609A (2–10 μg/ml, 7.5–37.5 μM), or D609B (2–10 μg/ml, 3.75–18.8 μM). Incubation of embryos with radioligands alone at 4°C was used as blank. Aliquots (3 × 100 μl) were removed at different stages of embryo development and centrifuged at 3,000 rpm for 3 min in a Heraeus Biofuge A to precipitate live embryos. Supernatants were aspirated and embryos were resuspended in fresh ASW and centrifuged as above. The procedure was repeated until radioactivity in the supernatant was equal to background. Finally, embryos were hydrolyzed in 250 μl of 0.1 N NaOH overnight. [^{35}S]-methionine uptake was measured in 100 μl aliquots. Results were expressed as dpm per μg of protein.

In a similar set of experiments, the radioligand used was a mixture of [^{3}H]-amino acids (0.1 μCi/ml, specific activities 15–102 Ci/mmol; Amersham International). In this case, the radioligand was added at a specific time of incubation (5, 8, 19, 28, and 41 h after fertilization) to embryos developing in the presence or in the absence of collagen synthesis inhibitors. Embryos incubated with the radioligand alone at 4°C were used as blank. Incubations were terminated after 30 min by adding 1 ml of a cocktail containing 6% trichloroacetic acid, 0.3% tannic acid, 5 mM NEM, and 2 mM 1,10-phenanthroline. Samples were then vortexed, sonicated as described before, and centrifuged at 10,000*g* for 10 min to remove non-protein bound radioactivity. The procedure was repeated until radioactivity in the supernatant was equal to background. Pellets, containing protein bound radioactivity, were resuspended in 250 μl of 0.1 N NaOH overnight. [^{3}H]-amino acid incorporation was

measured in 100 μl aliquots. Results were expressed as dpm per μg of protein.

Assay for Collagen Protein Biosynthesis

Embryos, diluted to 3–5 × 10^3/ml, were cultivated for 28 h, as described, in 5 ml of ASW containing 0.5 μCi of [^{14}C]-proline, in the presence or absence of GPA1734 (5–25 μM) or D609A (2–10 μg/ml, 7.5–37.5 μM) or D609B (2–10 μg/ml, 3.75–18.8 μM). Incubation of embryos with [^{14}C]-proline alone at 4°C was used as blank. In one set of incubations, the collagen synthesis inhibitors were removed after 8 h of incubation by centrifugation at 300*g* for 5 min, aspiration of the supernatant, and resuspension in fresh ASW. This procedure was repeated three times and embryos were resuspended in the same volume (as that aspirated) of ASW containing 0.1 μCi/ml [^{14}C]-proline and incubated for further 20 h. Aliquots (750 μl) were removed from each incubation mixture at several stages of embryo development and assayed for collagen protein biosynthesis as previously described [Maragoudakis et al., 1988a,b, 1989, 1990]. Briefly, hydroxylations and protein synthesis were stopped by addition of cycloheximide and dipyridyl and boiling in a water bath for 10 min. Non-protein bound radioactivity was removed by repeated washings in the presence of 5% trichloroacetic acid and centrifugation. Pellets containing the protein bound radioactivity were dissolved in 0.1 N NaOH. Subsequently, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4) was added and the pH adjusted to neutral with 0.5 N HCl using phenol red indicator. $CaCl_2$ was added to a final concentration of 3 mM and digestion followed by incubating with 10 IU of *Clostridium* collagenase (clostridiopeptidase A, EC 3.4.24.3 from *Clostridium histolyticum,* type VII; Sigma) at 37°C for 4 h. At the end of the incubation period, radiolabelled tripeptides corresponding to collagenous material synthesized by the embryos from [^{14}C]-proline were quantified as mentioned above for the assay for type IV collagen-degrading activity.

RESULTS

Type IV Collagen-Degrading Activity in Sea Urchin Embryos

[^{3}H]-acetylation of type IV collagen from human placenta yielded [^{3}H]-type IV collagen with 2,750 ± 180 dpm/μg of protein. This substrate was used to measure type IV collagen-degrading activity by homogenates of *Lytechinus pictus* embryos obtained at various stages of development. Under the conditions adopted, sea urchins developed to the pluteus larva stage 60 h after fertilization, with intermediate stages attained as follows: 4–5 h for morula stage, 5–14 h for blastula stage, 21–30 h for gastrula stage, and about 40 h for prism stage.

The 10,000*g* supernatant prepared from homogenates of sea urchin embryos obtained at prism stage of development showed considerable activity in [^{3}H] release from type IV collagen. This activity became evident after 6 h of incubation and increased in an almost linear fashion, reaching a maximum between 18 and 24 h of incubation (Fig. 1). Incubation with APMA (1 mM), or with trypsin (100 μg/ml) at 35°C for 4 min followed by addition of soybean trypsin inhibitor (500 μg/ml), did not affect type IV collagen-degradation activity. Addition of Na_2EDTA (10 mM) or 1,10-phenanthroline (2 mM) reduced activity to blank levels (incubation with buffer alone). In contrast, PMSF (5 mM) or NEM (5 mM) or benzamidine (5 mM) or 500 μg/ml of soybean trypsin inhibitor did not inhibit type IV collagen-degrading activity to any significant level. The 10,000*g* pellet from the same developmental stage was devoid of any significant type IV collagen-degrading activity. (Table I). Statistical analysis of the means (Student's t-test) indicated that type IV collagen-degrading activity in the 10,000*g* supernatant of sea urchin embryos at prism differed significantly at the level of $P < 0.01$ when compared to

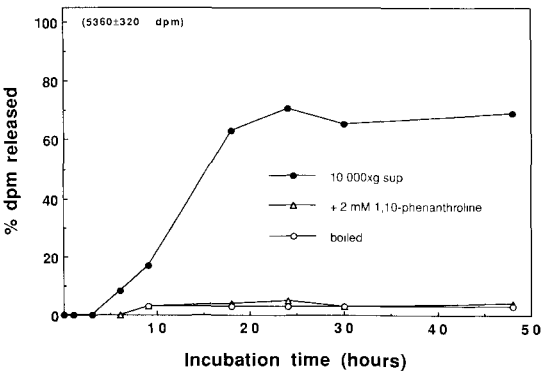

Fig. 1. Time course of radioactivity solubilized from [^{3}H]-type IV collagen by crude homogenates obtained from sea urchin embryos at prism stage of development. Results shown are means of triplicate determinations of radioactivity released, expressed as % of total radioactivity added by the 10,000*g* supernatant (protein content 312 ± 15 mg) (●), by the same fraction in the presence of 2 mM 1,10-phenanthroline (△), and by the corresponding boiled enzyme preparation (○).

TABLE I. Type IV Collagen-Degrading Activity by Various Fractions of Sea Urchin Embryo Homogenates Obtained at Prism Stage

Enzyme fraction[a]	n	Enzyme protein per assay (μg/ml)[b]	Dpm released[b] (%)	Enzyme activity[c]	Relative purification (fold)
10,000*g* supernatant	12	312 ± 15	70.7	0.227	
10,000*g* pellet	12	1,888 ± 110	30.5		
Blank (no enzyme)	12		3.8		
100,000*g* pellet	12	497 ± 30	2.2		
100,000*g* supernatant	12	28 ± 5	68.5	2.5	11
0–25% precipitate of $(NH_4)_2SO_4$ saturation	12	38 ± 4	25.6		
50–80% precipitate of $(NH_4)_2SO_4$ saturation	12	32 ± 2	12.3		
25–50% precipitate of $(NH_4)_2SO_4$ saturation	9	11 ± 2	74.4	6.8	30
boiled	9	11 ± 2	4.6		
+ 2 mM 1,10-phenanthroline	9	11 ± 2	5.3		
+10 mM Na_2EDTA	9	11 ± 2	5.1		
+ 5 mM PMSF	9	11 ± 2	69.1		
+ 5 mM NEM	9	11 ± 2	65.6		
+ 5 mM benzamidine	9	11 ± 2	67.8		
+ 2 mM TLCK	9	11 ± 2	70.9		
+ 2 mM TPCK	9	11 ± 2	66.5		
DEAE-Sephacel	6	1.5	68.7	45.8	200
Sephacryl S-300	6	0.2	72.7	363.5	1,600
+ 2 mM 1,10-phenanthroline	6	0.2	4.5		
+10 mM Na_2EDTA	6	0.2	4.5		
+ 5 mM PMSF	6	0.2	64.4		
+ 5 mM NEM	6	0.2	70.5		
+ 5 mM benzamidine	6	0.2	71.2		
+ 2 mM TLCK	6	0.2	72.7		
+ 2 mM TPCK	6	0.2	71.6		
+ 200 μM GPA1734	3	0.2	66.2		
+ 200 μM D609A	3	0.2	64.7		
+ 100 μM D609B	3	0.2	67.7		

[a][^{3}H]-type IV collagen (5,240 ± 550 dpm, 2 μg) was incubated with various fractions of sea urchin embryo homogenates in the presence or absence of various protease inhibitors and chelators.
[b]Data represent mean values for n determinations from 1–3 embryo fertilization batches.
[c]Enzyme activity is dpm released after 24 h of incubation (expressed as % of total dpm added) per μg of enzyme protein.

blank, to values obtained with the 10,000*g* pellet fraction, or to values obtained in the presence of 2 mM 1,10-phenanthroline or 10 mM Na_2EDTA.

Purification of Type IV Collagen-Degrading Activity from Sea Urchin Embryos

Fractionation of the 10,000*g* supernatant prepared from homogenates of sea urchin embryos obtained at prism stage revealed that type IV collagen-degrading activity was associated mainly with the 100,000*g* supernatant (Table I), while the respective pellet contained only a small fraction of the activity. Ammonium sulfate fractionation indicated that type IV collagen-degrading activity could be precipitated mainly between 25% and 50% saturation, leading to thirtyfold purification. Addition of Triton X-100 to a final concentration of 0.1% in the incubation mixture did not influence type IV collagen-degrading activity significantly.

Further purification of the enzyme isolated in the 25–50% $(NH_4)_2SO_4$ fraction was achieved with ion exchange on DEAE Sephacel and gel chromatography on Sephacryl S-300. The DEAE Sephacel column (1.1 × 15 cm) was equilibrated with 25 mM Tris-HCl (pH 7.5), also containing 0.1% Triton X-100 (to prevent aggregation of the enzyme molecules) [Salo et al., 1983]. The column was eluted in a stepwise fashion with 5 volumes of each of the following: the equilibra-

tion buffer, and 0.05, 0.1, 0.15, 0.2, and 1 M NaCl, all dissolved in the equilibration buffer. Ion exchange chromatography resulted in a further 6.5-fold purification of type IV collagen-degrading activity, which was concentrated in the 0.1 M NaCl fraction, indicating that the enzyme binds with weak ionic forces to DEAE (Fig. 2a). Gel chromatography of this fraction on a Sephacryl S-300 column (Fig. 2b) resulted in a further eightfold purification. Thus, the overall purification of enzyme activity was approximately 1,600-fold (Table I). Incubation with [^{3}H]-type IV collagen showed that type IV collagen-degrading activity purified by gel chromatography was measurable after 6 h of incubation, increased linearly, and reached a maximum after 18 h of incubation (results not shown). Using molecular weight markers we estimated the apparent molecular mass of the purified enzyme(s) to be approximately 70 kDa (Fig. 2b). Following SDS-PAGE [Laemmli, 1970] and electrophoretic transfer to PVDF filters [Sklaviadis et al., 1986] of the proteins present in the 25–50% $(NH_4)_2SO_4$ fraction, a major protein was observed using colloidal gold staining, with an apparent molecular mass of 76 kDa. However, this protein did not cross react with rabbit antibodies raised against peptides of the 72 kDa type IV collagenase isolated from WI-38 human embryonic lung fibroblasts or HT-1080 human fibrosarcoma cell lines (kindly donated by Dr. W.G. Stetler-Stevenson, NIH, Bethesda, MD).

The sensitivity to a variety of protease inhibitors as well as the pH requirements of type IV collagen-degrading activity concentrated either in the 25–50% $(NH_4)_2SO_4$ fraction or by gel chromatography were similar. Maximum activity was present at pH 7, while activity was completely and irreversibly abolished at pH 5, or by boiling for 5 min. Incubation with APMA (1 mM), or with trypsin (100 μg/ml) at 35°C for 5 min followed by soybean trypsin inhibitor (500 μg/ml), did not affect type IV collagen-degrading activity. Na_2EDTA (10 mM) or 1,10-phenanthroline (2 mM) completely inhibited enzyme activity. In contrast, 5 mM of PMSF, NEM, or benzamidine, or 2 mM of TLCK or TPCK, or 500 μg/ml of soybean trypsin inhibitor, or GPA1734 (up to 200 μM), or D609A (up to 200 μM), or D609B (up to 100 μM) did not alter type IV collagen-degrading activity (Table I).

Substrate specificity of the collagen-degrading activity of sea urchin embryos was investigated by using different types of [^{3}H]-collagens and [^{3}H]-gelatin. As shown in Table II, in addition to

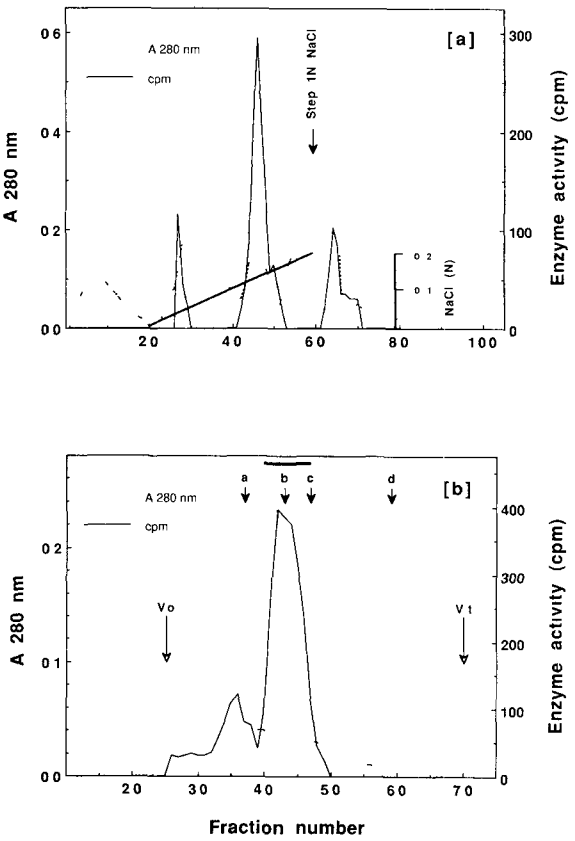

Fig. 2. Purification of type IV collagen-degrading activity in sea urchin embryos obtained at prism, by ion exchange chromatography (**a**) and gel chromatography (**b**) The dialyzed high salt extract was applied to a DEAE-Sephacel column and eluted as described in the text (a) Fractions of 0 5 ml were collected and assayed for protein at 280 nm and type IV collagen-degrading activity (cpm) in the presence of benzamidine (5 mM) and NEM (5 mM) *The thick line indicates NaCl gradient The fraction* with type IV collagen-degrading activity eluted with 0 1 M NaCl from the DEAE-Sephacel column was diluted with the enzyme buffer (50 mM Tris-HCl, 200 mM NaCl, and 10 mM $CaCl_2$, pH 7 4) to about 0 01% Triton X-100 and concentrated to 1 ml in an Amicon cell, YM-10 membrane Triton X-100 was then added to the concentrated sample to a final concentration of 0 1% and the sample was applied to the Sephacryl S-300 gel column (1 1 × 36 0 cm) and eluted with the enzyme buffer containing 0 1% *Triton X-100* (b) *Fractions of* 0 5 ml were collected and assayed for protein at 280 nm and type IV collagen-degrading activity (cpm) in the presence of benzamidine (5 mM) and NEM (5 mM) Lettered arrows indicate *Mr* markers (Sigma reference proteins) a, phosphorylase b (97,400), b, bovine serum albumin (66,000), c, egg albumin (45,000), and d, cytochrome c (14,500) Fractions pooled are indicated by horizontal bar *Vo*, void volume, *Vt*, total volume

type IV collagen, which was degraded by 77%, the enzyme also exhibited gelatinolytic activity, degrading gelatin by 62%; however, no considerable specificity was observed for type I, III, and V collagens, which were degraded by 12, 10, and 22%, respectively. Substrate competition assays showed that type IV collagen-degrading activity

TABLE II. Substrate Specificity of Type IV Collagen-Degrading Activity in Sea Urchin Embryos

Substrate[a]	Dpm added	Dpm released[b]	[^{3}H]-collagen degradation (%)
[^{3}H]-type I collagen (2 μg)	6,600 ± 574	796 ± 209	12
[^{3}H]-type III collagen (2 μg)	1,215 ± 165	125 ± 48	10
[^{3}H]-type IV collagen (2 μg)	6,749 ± 1,119	5,220 ± 480	77
[^{3}H]-type V collagen (2 μg)	5,227 ± 280	1,117 ± 162	22
[^{3}H]-gelatin (2 μg)	6,195 ± 749	3,815 ± 408	62
			Inhibition of [^{3}H]-type IV collagen degradation[c] (%)
[^{3}H]-type IV collagen (2 μg)	6,749 ± 1,119	5,220 ± 480 (C)	0
[^{3}H]-type IV collagen (2 μg) + type IV collagen (0.5 mg)	6,749 ± 1,119	1,084 ± 170	79
[^{3}H]-type IV collagen (2 μg) + gelatin (0.5 mg)	6,749 ± 1,119	2,192 ± 211	58
[^{3}H]-type IV collagen (2 μg) + laminin (0.5 mg)	6,749 ± 1,119	5,065 ± 384	3
[^{3}H]-type IV collagen (2 μg) + fibronectin (0.5 mg)	6,749 ± 1,119	4,904 ± 523	6

[a][^{3}H]-collagens and non-collagenous proteins of the extracellular matrix were incubated as described in Materials and Methods with the Sephacryl S-300 fraction of the sea urchin enzyme (0.1 μg protein per assay) in the presence of NEM and benzamidine (5 mM each).

[b]Results are expressed as dpm (mean of 3–6 determinations ± S.D) released after 24 h of incubation.

[c]Inhibition of [^{3}H]-type IV collagen degradation is expressed as % of control (C).

could be inhibited by excess amounts (0.5 mg/assay) of type IV collagen and gelatin by 79 and 58%, respectively, but was unaffected by excess amounts (0.5 mg/assay) of laminin or fibronectin (Table II), confirming the specificity of the sea urchin enzyme for type IV collagen and gelatin.

Gel Chromatography of Type IV Collagen-Degradation Products

Chromatography of [^{3}H]-type IV collagen on Sepharose 4B, after reduction and denaturation, produced 3 main peaks: A, B, and D (Fig. 3). Peak A was eluted at *Vo*, indicating a component of high *Mr*. Peaks B and D were eluted with a distribution coefficient (*Kd*) of 0.4 and 0.77, respectively, indicating components of smaller *Mr*. Treatment of [^{3}H]-type IV collagen with the Sephacryl S-300 fraction of sea urchin embryos obtained at prism, in the presence of PMSF (5 mM) or NEM and benzamidine (5 mM each), degraded peaks A and B but not peak D. Type IV collagen was degraded to products which were eluted in two major peaks (Fig. 3): the first peak (peak C) was eluted with *Kd* 0.67; the second peak (peak D) was co-eluted with the smaller endogenous components of the reduced and denatured type IV collagen, as indicated by the increased AUC of peak D, with *Kd* 0.79. Treatment of [^{3}H]-type IV collagen with *Clostridium* collagenase also degraded peaks A and B and partially peak D (Fig. 3). The major products were eluted in a peak (E) at *Vt* indicating digestion products of low *Mr*, probably oligopeptides and tripeptides. These products (peak E) were also formed in small quantities by the sea urchin

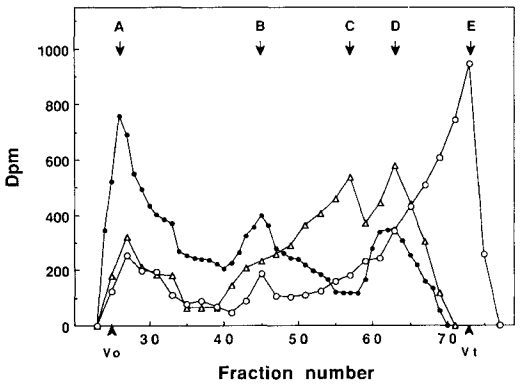

Fig. 3. Characterization of the degradation products of [^{3}H]-type IV collagen by gel chromatography on Sepharose CL-4B. [^{3}H]-type IV collagen (approximately 25 nM) was incubated with enzyme buffer (●), with the Sephacryl S-300 fraction of sea urchin embryos obtained at prism stage of development (0.2 μg/ml protein content) in the presence of benzamidine (5 mM) and NEM (5 mM) (△), and with 50 IU of *Clostridium* collagenase (○). Enzyme activity was stopped in all cases by adding 2 mM of 1,10-phenanthroline. Gel chromatography was performed after reduction and denaturation, as described under Materials and Methods. Results are indicated as radioactivity (dpm) per 0.5 ml fractions collected. Arrows (A–E) indicate peaks. *Vo*, void volume; *Vt*, total volume.

enzyme in the absence of serine and thiol protease inhibitors (results not shown).

Expression of Type IV Collagen-Degrading Activity During Embryonic Development

Type IV collagen-degrading activity could not be detected in either sperm or eggs. Developing sea urchin embryos, however, showed type IV collagen-degrading activity. The rate of activity in the developing embryos was monitored using the 25–50% $(NH_4)_2SO_4$ fraction obtained at different stages of development (Fig. 4). Activity was first detected when embryos reached the stage of early blastula (about 6 h postfertilization) and remained constant throughout to late blastula (about 14 h). Thereafter, there was a sharp increase in type IV collagen-degrading activity, which coincided with the invagination of the archenteron. Following completion of gastrulation (about 30 h) activity continued to rise in an almost linear way, reaching maximum levels when embryos attained the prism stage of development (about 40 h) and retained this level of activity up to the pluteus larva stage of development (about 60 h).

Type IV collagen-degrading activity was also monitored when embryos were cultured in the presence of GPA1734 (5–25 μM), D609A (2–10 μg/ml, 7.5–37.5 μM), or D609B (2–10 μg/ml, 3.75–18.8 μM) and also following removal of the inhibitors after 8 h of treatment. Activity was measured in treated embryos obtained at corresponding postfertilization periods as with the untreated controls. These agents at concentrations up to 200 μM did not inhibit directly the activity of the enzyme (Table I). However, in their presence the embryos could not express any type IV collagen-degrading activity, up to 70 h postfertilization. Upon their removal, though, activity was expressed again (Fig. 4 for 5 and 25 μM GPA1734; Table III).

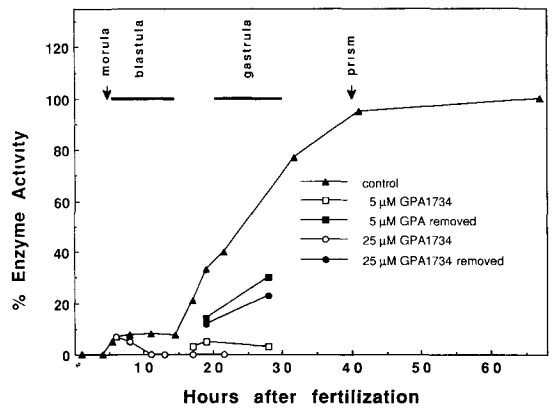

Fig. 4. [^{3}H]-type IV collagen-degrading activity during development of sea urchin embryos The rate of activity shown is that exhibited by the 25–50% $(NH_4)_2SO_4$ fraction (10 μg/ml protein content) obtained at different stages of embryo development (control) (▲) and in the same fraction of embryos cultivated in the presence of 5 μM (□) and 25 μM (○) GPA1734, which were obtained at corresponding postfertilization periods as with untreated controls ■ and ● indicate type IV collagen-degrading activity upon removal of GPA1734 (5 and 25 μM, respectively), as described under Materials and Methods Enzyme activity is expressed as % of that obtained at prism stage of development (about 40 h postfertilization). Results indicated are means of triplicate determinations Arrows and bars denote onset of stage of development

Developmental Arrest by Collagen Synthesis Inhibitors

The development of *Lytechinus pictus* embryos cultivated in the presence of GPA1734 (5–25 μM), D609A (2–10 μg/ml, 7.5–37.5 μM), or D609B (2–10 μg/ml, 3.75–18.8 μM) was morphologically indistinguishable from control embryos up to the stage of early blastula (Fig. 5a for 25 μM of GPA 1734 and data not shown). Subsequent development, however, was arrested and treated embryos remained at early mesenchyme blastula (Fig. 5b). Inhibited embryos continued to swim but did not gastrulate even when control embryos attained the stage of late gastrula (Fig. 5c) or pluteus larva stage (data not shown). In the presence of the agents used, there were a few embryos (less than 5%) which were arrested at early substages of gastrulation. Over 95% of the embryos, even at the lower concentration of the collagen synthesis inhibitors used, underwent primary invagination (the first appearance of the archenteron) but not secondary invagination (elongation of the archenteron). On the other hand, blastulation and mesenchyme cell ingression were relatively insensitive to even the higher concentration of the inhibitors used.

The arrest in development caused by the collagen synthesis inhibitors did not appear to be due to a non-specific embryotoxic effect, for three reasons. 1) Treated embryos remained viable despite their developmental arrest. 2) Embryos gastrulated following the removal of the inhibitors after 8 h of incubation (Fig. 5d), though with a time lag of 4–10 h as compared with controls. Also, about 10% of the embryos either did not gastrulate or were dead. This ratio of mortality was twice as high as compared with untreated controls under the conditions of cultivation adopted. 3) Statistical analysis of the means indicated that the uptake of [^{35}S]-methio-

TABLE III. Correlation Between Inhibition of Collagen Synthesis Induced by D609 and GPA1734 and Expression of [^{3}H]-Type IV Collagen-Degrading Activity*

	Concentration	Dpm collagenous protein/μg total protein[a]	Enzyme activity[a,b] (%)
Control		1,200 ± 124	63
D609A	7.5 μM	320 ± 29	5 ± 2
D609A	37.5 μM	205 ± 22	0
D609A-removed	7.5 μM	835 ± 124	32 ± 6
D609A-removed	37.5 μM	770 ± 63	26 ± 4
GPA1734	5.0 μM	320 ± 25	3 ± 2
GPA1734	25.0 μM	240 ± 26	0
GPA1734-removed	5.0 μM	895 ± 155	30 ± 4
GPA1734-removed	25.0 μM	830 ± 77	23 ± 2.5

*[^{3}H]-type IV collagen-degrading activity (exhibited by the 25–50% $(NH_4)_2SO_4$ fraction) and synthesis of collagenous proteins (in 3–5 × 10^3/ml embryos) were measured, as described under Materials and Methods, 28 h postfertilization (gastrula stage for control embryos) (a) in the absence (controls), (b) in the presence of collagen synthesis inhibitors, and (c) following removal of the inhibitors after 8 h of incubation.

[a]Data represent mean values for triplicate determinations.

[b]Enzyme activity is expressed as % of type IV collagen-degrading activity achieved by control at prism stage (Fig. 4).

nine or the incorporation of a mixture of [^{3}H]-amino acids (per μg of protein) in the developing untreated embryos did not differ significantly when compared to embryos treated with GPA1734 (up to 25 μM), D609A (up to 10 μg/ml, 37.5 μM), or D609B (up to 10 μg/ml, 18.8 μM). In untreated control embryos cultivated in the presence of [^{35}S]-methionine, the rate of uptake increased almost linearly from 300 ± 185 dpm/μg protein (mean ± SD of triplicate determinations) at 1 h of incubation to 10,562 ± 1,487 dpm/μg protein 28 h postfertilization. The respective values for 1 and 28 h of incubation were for 25 μM GPA1734, 100 ± 52 and 9,577 ± 670 dpm/μg protein; for 37.5 μM D609A, 280 ± 104 and 9,400 ± 1,100 dpm/μg protein; and for 18.8 μM D609B, 250 ± 53 and 8,949 ± 2,150 dpm/μg protein. Incorporation of [^{3}H]-amino acids in untreated control embryos, exposed for 30 min periods to a mixture of [^{3}H]-amino acids, was substantially the same for different stages of development and varied from 5,633 ± 1,250 dpm/μg protein 5 h postfertilization to 4,714 ± 160 dpm/μg protein 41 h postfertilization. The respective values obtained in the presence of the above concentration range of inhibitors did not differ significantly from untreated controls (results not shown). The collagen synthesis inhibitors, though, at higher concentrations (≥ 50 μM for GPA1734, ≥ 94 μM for D609A, and ≥ 47 μM for D609B) were embryotoxic. At these higher concentrations, there were over 75% dead embryos at blastula stage, while both the uptake of [^{35}S]-methionine or the incorporation of [^{3}H]-amino acids was significantly inhibited ($P < 0.001$). The uptake of [^{35}S]-methionine 28 h postfertilization was, for 50 μM GPA1734, 850 ± 415 dpm/μg protein; for 94

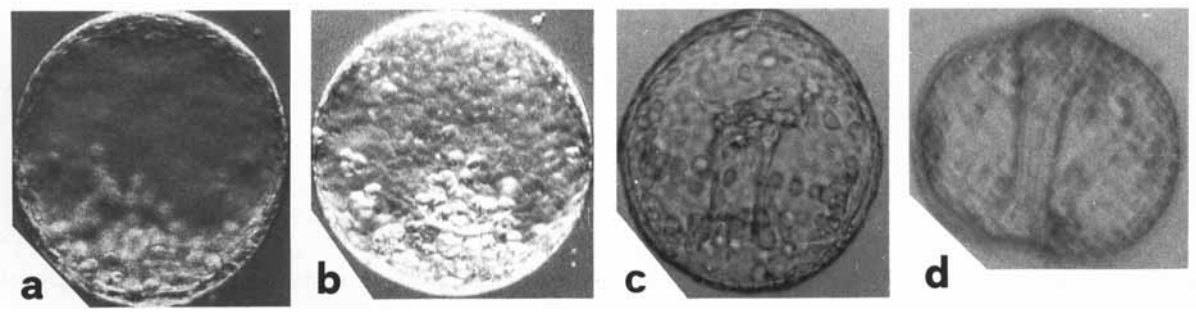

Fig. 5. Developmental arrest of *Lytechinus pictus* embryos cultivated in the presence of GPA1734 (25 μM). Phase-contrast micrographs: Embryos cultivated in the presence or in the absence of the collagen synthesis inhibitor reached early mesenchyme blastula 6 h postfertilization. **a**: Treated embryo at early mesenchyme blastula 6 h postfertilization. **b**: Treated embryo arrested at early mesenchyme blastula 30 h postfertilization. **c**: Control embryos gastrulated 28 h postfertilization. **d**: Removal of GPA1734 (25 μM), 8 h postfertilization, allowed embryos to gastrulate about 35 h postfertilization.

μM D609A, 782 ± 385 dpm/μg protein; and for 47 μM D609B, 640 ± 238 dpm/μg protein; these values did not differ significantly from blank values (335 ± 214 dpm/μg protein) which were obtained with embryos incubated in the absence of the inhibitors at 4°C. Similarly, the incorporation of [^{3}H]-amino acids in embryos treated with embryotoxic concentrations of the inhibitors did not differ significantly from their respective blank values, which were 316 ± 270 dpm/μg protein (results not shown).

Effect of GPA1734 and D609 on Collagen Synthesis in the Developing Sea Urchin Embryo

Collagen synthesis in the untreated embryos was first detected when embryos reached early mesenchyme blastula, about 5 h after fertilization. Thereafter collagen synthesis increased in an almost linear fashion up to pluteus larva stage (Fig. 6, for 0–28 h postfertilization). Treatment of embryos with GPA1734 (5–25 μM) did not significantly affect collagen biosynthesis up to 6 h postfertilization. Thereafter, however, there was a dose dependent inhibition of collagen biosynthesis. Treatment with D609A (2–10 μg/ml, 7.5–37.5 μM) or D609B (2–10 μg/ml, 3.75–18.8 μM) also caused a dose dependent inhibition of collagen biosynthesis, even at 6 h postfertilization. Upon removal of the inhibitors used, 8 h postfertilization, collagen biosynthesis recovered and attained a similar rate of increase as with controls (Fig. 6 for the higher concentrations of the agents used).

DISCUSSION

In the present study we confirm that collagen biosynthesis is a prerequisite for gastrulation of *Lytechinus pictus* and further report that developing embryos express type IV collagen-degrading activity in relation to synthesis of collagenous proteins.

Developing Sea Urchin Embryo Expresses Type IV Collagen-Degrading Activity

Type IV collagen-degrading activity, at the prism stage of development, was purified about 1,600-fold after $(NH_4)_2SO_4$ fractionation and ion exchange and gel chromatography. The apparent molecular mass of the purified enzyme was estimated to be about 70 *kDa*. Metal chelating agents completely inhibited type IV collagen-degrading activity, while plasmin inhibitors as well as thiol and serine protease inhibitors were without effect, indicating that enzyme activity

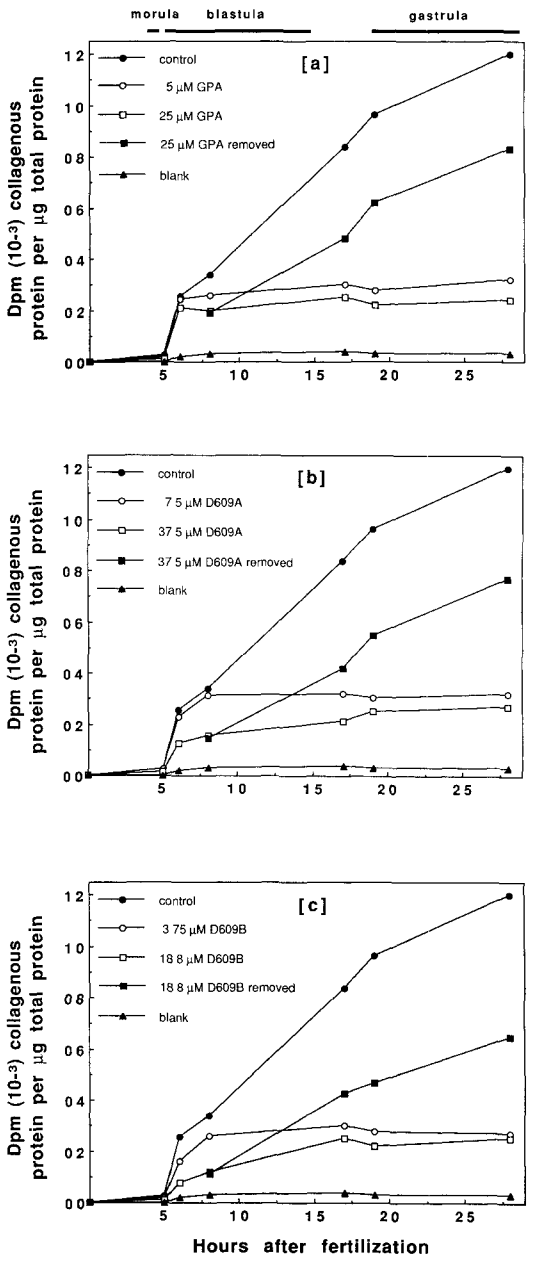

Fig. 6. Effect of GPA1734 and D609 on collagen protein biosynthesis by developing *Lytechinus pictus* embryos Embryos (3–5 × 10^3/ml) were cultivated in the absence of any inhibitor in ASW containing 0 5 μCi of [^{14}C]-proline at room temperature (21°C) (●) (control) or at 4°C (▲) (blank) Incubation of embryos at 21°C in the presence of (**a**) 5 and 25 μM GPA1734, (**b**) D609A (1–10 μg/ml, 7 5 and 37 5 μM) and (**c**) D609B (1–10 μg/ml, 3 75 and 18.8 μM) Embryos were cultivated at 21°C following removal of the collagen synthesis inhibitors after 8 h of incubation, as described under Materials and Methods (■) (only data for the highest dose of the inhibitors removed are presented) Results shown are means of triplicate determinations, expressed as dpm of collagenous protein formed per μg of total protein Horizontal bars denote onset of stage of development

may be attributed to a member of the matrix metalloprotease family (MMP). Type IV collagen-degrading activity was optimal at physiological pH and was irreversibly abolished at pH 5, thus excluding a cathepsin-like protease. Activity could not be enhanced further upon trypsin or organomercurial treatment, indicating that it was isolated in its active state, unlike other MMPs which have been isolated as latent inactive proenzymes [reviewed in Overall, 1991].

Substrate degrading versatility revealed specificity for collagen type IV and gelatin, with no affinity for collagens type I, III, or V or for non-collagenous matrix proteins. Resolution of the enzymatic degradation products of type IV collagen by gel chromatography showed that the enzyme, in the presence of PMSF or NEM and benzamidine, cleaved this substrate to give rise to products which were eluted in two peaks with *Kd* 0.67 and 0.79, respectively. In the absence of serine and thiol protease inhibitors, type IV collagen was also degraded to products of *Mr* size corresponding to oligopeptides or tripeptides, which were eluted at *Vt*, as was the case following treatment with collagenase from *Clostridium histolyticum*. This may be due to neutral extracellular proteases or intercellular lysosomal enzymes, co-purified with type IV collagen-degrading activity, which are masked by the protease inhibitors. However, there are several questions remaining to be answered as to substrate specificity, since not all collagen types were tested, as well as to the precise *Mr* size of the degradation products, which were not identified.

The investigation on the ontogenesis of type IV collagen-degrading activity in the sea urchin revealed that expression of this activity is closely correlated with the rate of biosynthesis of collagenous proteins. Gametes from gravid adults and embryos up to the stage of morula were devoid of any activity. Enzyme activity first became apparent at early blastula stage through to late blastula stage, thereafter increasing sharply during gastrulation, attaining maximum levels at prism stage.

The enzyme activity reported here is different from a collagenase-like hatching enzyme that has been shown to be expressed during early development in the sea urchin [Lepage and Gache, 1990; Roe and Lennarz, 1990]. This hatching enzyme is a 33 kDa metalloprotease and is only transiently synthesized by the sea urchin embryo at late morula stage and early blastula stage. The collagen degrading activity that we observed may be due to a protein similar to those encoded by the SpAN gene in *Paracentrotus lividus* [Reynolds et al., 1992] or the BP10 gene in *Strongylocentrotus purpuratus* [Lepage et al., 1992] with a predicted molecular mass of 64 kDa. These proteins have structural homologies to morphogenetic enzymes of the human bone metalloprotease 1 (BMP-1) and the *tolloid* gene product of the *Drosophila,* while their substrate specificity has yet to be described. The peak activity of these proteins is achieved at prehatching and swimming blastula stages and then decreases and remains constant throughout to prism stage. Further characterization of the protein and identification of the mRNA of type IV collagen-degrading activity will clarify if these molecules are of the same origin or serve similar functions to the SpAN and BP10 gene products. Several properties of the collagen degrading activity that we report indicate similarities with MMP-2 [reviewed in Overall, 1991]. However, we found no cross-reactivity with antibodies raised against peptides of human MMPs-2 isolated from WI-38 fibroblasts or H-1080 fibrosarcoma. Thus, the current study only justifies this enzyme activity as a gelatin/type IV collagen-degrading activity, the precise nature of which is pending further clarification. Also, the cells responsible for expressing this activity as well as the intracellular location of this enzyme remain to be elucidated. The physiological role of this type IV collagen-degrading activity, at present, is not clear. It may be involved in the homeostasis of collagenous proteins, which is reflected in the rapid turnover of collagen molecules [Wessel and McClay, 1987], as well as in the orderly tissue restructure, such as remodelling of the archenteron during gastrulation [reviewed in Wilt, 1987]. This enzyme activity may also enable mesenchyme cells to overcome barriers of BM, while migrating during these same early stages of embryogenesis [Wessel and McClay, 1987].

Collagen Biosynthesis Inhibitors Arrest Collagen Synthesis and Development of the Sea Urchin Embryo at Gastrula Stage

In agreement with previous reports [Blankenship and Benson, 1984; Wessel et al., 1984; Wessel and McClay, 1987], monitoring of collagen biosynthesis by the method of Maragoudakis et al. [1988a,b, 1989, 1990] confirmed the pattern with which collagen has been reported to accumulate in the ECM of the developing em-

bryo of the sea urchin. The fact that the collagen biosynthesis was first detected and sharply increased during the mesenchyme blastula stage may be correlated to type IV collagen of BM detected by transmission electron microscopy studies to form in the blastocoel during this stage of development [Katow and Solursh, 1980]. The above pattern of collagen biosynthesis is also in agreement with reports regarding prolyl hydroxylase activity [Mizoguchi and Yazumasu, 1983], and ultrastructural detection of collagen fibers [Crise-Benson and Benson, 1979], which have been shown to increase at gastrula stage and during subsequent differentiation, but not during developmental processes leading to the mesenchyme blastula stage.

Treatment of *Lytechinus pictus* with the specific inhibitors of collagen biosynthesis GPA1734 or D609 [Maragoudakis et al., 1978a,b, 1990; Missirlis et al., 1990b] during embryogenesis resulted in a dose dependent inhibition of collagen biosynthesis. Morphological observations indicated that early developmental events prior to gastrulation, such as determination of cell polarity and blastulation, were not affected by collagen perturbation. In the presence of these agents at concentrations which inhibit collagen biosynthesis, embryos underwent primary invagination of the vegetal pole (the first appearance of the archenteron) but did not proceed to elongation of the archenteron, suggesting that gastrulation does not solely depend on an initial stimulus but also on a progression of cell-ECM interactions. The inhibitory effects of GPA1734 or D609 on embryogenesis and collagen accumulation in the sea urchin were reversible. Removal of the agents permitted the reaccumulation of collagen within the blastocoel and the restoration of normal development. These observations are in agreement with those reported for BAPN, which has been shown to inhibit lysyl oxidase activity and thus reduce the crosslinking of collagen molecules which then become prone to proteolytic digestion and rapid removal from the matrix [Kleinman et al., 1981; Butler et al., 1987]. As with GPA1734 and D609, BAPN treatment has been shown not to alter the developmental events prior to gastrulation in the sea urchin and to produce a reversibly inhibitory effect on gastrulation [Butler et al., 1987; Wessel and McClay, 1987; Wessel et al., 1989]. The above studies involving inhibitors of collagen biosynthesis, such as GPA1734, D609, or BAPN, suggest that early developmental processes leading to gastrulation do not require synthesis of a collagenous matrix. However, this is in conflict with reports regarding the presence of BM in the blastocoel at mesenchyme blastula stage [Katow and Solursh, 1980]. It is conceivable that in the presence of these agents, embryos do not form normal BM at blastula stage, despite the observation that they are morphologically indistinguishable from controls. This remains to be elucidated by immunohistochemical techniques.

At least three distinct types of collagen have been identified in sea urchin embryos: vertebrate fibrillar (types I and III) and BM collagens [Blankenship and Benson, 1984; Venkatesan et al., 1986; Wessel and McClay, 1987; Saitta et al., 1989; D'Alessio et al., 1990; Exposito et al., 1992a,b]. The use of GPA1734 which has been shown to inhibit biosynthesis of collagens, including BM collagen [Maragoudakis et al., 1978b], indicates that amongst other types of collagen, inhibition of type IV collagen and therefore of BM may also be a contributing factor for the developmental arrest at gastrula stage. This is in agreement with reports that inhibition of synthesis of other components of BM, such as sulfated glycosaminoglycans [Karp and Solursh, 1974] and laminin [McCarthy and Burger, 1987], inhibit gastrulation.

The actions of GPA1734 and D609 described above are not due to a general embryotoxic effect. It was previously shown that both GPA1734 and D609, at concentrations used to arrest embryogenesis in the sea urchin, do not act as non-selective toxic agents, but rather as specific inhibitors of collagen biosynthesis [Maragoudakis et al., 1988a, 1990; Missirlis et al., 1990b]. This is reinforced by the current results. Embryos prior to gastrulation appeared to be unaffected by these agents. When embryos were arrested at early mesenchyme blastula they continued to swim and for many hours were indistinguishable morphologically and biochemically from control embryos at blastula stage. In addition, simple removal of the agents by washing rescued normal development. Furthermore, general metabolism, such as amino acid uptake and incorporation, were not affected at concentrations used to arrest embryogenesis, supporting the suggestion that only selective biochemical events are sensitive to the decreased collagen accumulation in the ECM of the sea urchin embryo. This is also in line with the results of Wessel et al. [1989], who demonstrated that

while disruption of the collagenous ECM by BAPN dramatically affects gastrulation, many transcriptional and morphological events proceed normally. This suggests that many of the events required for cell differentiation in embryos of sea urchins can take place independently of normal gastrulation and collagen deposition in the ECM.

Inhibitors of Collagen Biosynthesis Revealed that Expression of Type IV Collagen-Degrading Activity Depends on Collagen Synthesis

Following treatment of embryos with GPA1734 or D609, type IV collagen-degrading activity could not be isolated after the stage of gastrula, that is, precisely at the stage first inhibited by disruption of collagen processing; upon removal, however, of the inhibitors, activity was recovered (Table III). Since it was demonstrated that these agents did not directly inhibit the isolated enzyme activity (Table I), it appears that lack of expression in type IV collagen-degrading activity by embryos treated with GPA1734 or D609 may be correlated with inhibition of collagen accumulation in the ECM or with subsequent arrest in development that these agents produce. Once collagen biosynthesis and development are restored following washing away of the inhibitors, type IV collagen-degrading activity is expressed again. Although we cannot at this stage exclude the possibility that these agents may inhibit directly the gene expression of the enzyme or interfere with its synthesis by other unknown mechanisms, it may be postulated that there is a temporal dependence of the expression of type IV collagen-degrading activity on collagen biosynthesis. This would be consistent with the observations of Biswas and Dayer [1979], who reported that type I, II, and III collagens are potent stimulators of collagenase production by cultured human skin fibroblasts and human and rabbit synovial cells. It is also possible that the presence of collagen may stabilize type IV collagen-degrading activity.

Preliminary results indicated that addition of GPA1734 or D609 to embryos 5 h postfertilization also produced morphological arrest of embryos and inhibition of collagen biosynthesis. However, the precise effect of these agents on embryo development, collagen biosynthesis, and type IV collagen-degrading activity when added at different stages of embryogenesis remains to be elucidated. Similarly, the effects of these agents on several clearly identified parameters of sea urchin embryogenesis, such as spicule formation, primary and secondary mesenchyme cell migration, diameter of the blastocoel, endoderm and ectoderm cell division, filopodial formation, mRNA transcriptional activity, and others, will be of interest to investigate.

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