Int. J. Dev. Biol. 49: 65-70 (2005)
doi: 10.1387/ijdb.041812nz

Developmental Expression Pattern

Entactin and laminin gamma1-chain gene expression in the early chick embryo

NIKOLAS ZAGRIS*,[1], ALBERT E. CHUNG[2] and VASSILIS STAVRIDIS[1]

[1]Division of Genetics and Cell and Developmental Biology, Department of Biology, University of Patras, Patras, Greece and [2]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, USA

ABSTRACT The expression patterns of entactin and laminin γ1 chain genes were examined by *in situ* hybridization of their mRNAs in the early chick embryo from stage X (morula) to stage HH10-11 (10-somites). The entactin and laminin γ1 transcripts were found in abundance in the embryo at stage X. Entactin polypeptides were detected in embryos at stage X by immunoprecipitation. The expression of the laminin transcripts was intense and of entactin milder in the epiblast and in the hypoblast of embryos at stage XIII (blastula). During gastrulation (stage HH3-4), the laminin γ1 and entactin cRNAs gave strong signals in the cells ingressing through the primitive streak, in the migrating mesenchymal cells and the cells of the lower layer. At the neurula stage (stage HH5-6), punctate groups of cells expressed laminin γ1 strongly in the neural ectoderm, while the signal of expression was milder and more uniform in chordamesoderm. The entactin cRNAs gave a strong punctate pattern of mRNA expression in the neural ectoderm, in mesoderm and in endoderm in embryos at the late gastrula stage (HH4), but mRNA expression was mild in the neural plate and in mesoderm and gave no signal in endoderm and lateral ectoderm in embryos at stage HH6 (neurula). At the 10-somite stage, the laminin γ1 cRNAs gave strong signals in the neural tube and in neural crest cells migrating along the neural tube ventrally and low signals in ectoderm, intense signals in the myotome and milder signals in the dermatome and sclerotome of somites and intense signals in the mesonephric tubules. The punctate pattern of entactin expression was notable in cells at all stages studied. Ubiquitous expression of laminin γ1 and entactin genes during the morula and blastula stages becomes restricted to specific cell populations as the first cell commitments start.

KEY WORDS: *entactin /nidogen, laminin, gene expression, morphogenesis, chick embryo*

Laminin and entactin/nidogen are major glycoproteins of the extracellular matrix, including the basement membranes and of embryonic tissues. The prototype of laminin consists of three distinct polypeptide chains designated as α1 (400 kDa), β1 (210 kDa) and γ1 (210 kDa) (Chung *et al.*, 1979; review Zagris, 2001). Several genetically different isoforms as well as spliced products of the chains have been identified giving rise to various classes of laminin with distinct tissue distributions and co-distributions, unique properties and developmentally regulated expression. Entactin (150 kDa) (Carlin *et al.*, 1981), also referred to as nidogen, is a small rod-shaped molecule with three globular domains (G1, G2, G3) connected by a flexible link and a rod. The entactin carboxyl globular domain G3 binds to laminin on the γ1 chain, the G2 domain binds to the protein core of perlecan, the N-terminal domain of fibronectin and fibrinogen and to collagen IV and the molecule serves as a link between laminin and collagen IV networks (review Miosge *et al.*, 2000). This raised the question whether competition for binding to the entactin G2 domain may provide a mechanism for modifying the extracellular matrix. Entactin may be essential as a bridging molecule for the assembly of the basement membranes and it may modulate the biological activities of the macromolecules to which it binds in the developing embryo. The first extracellular matrix starts to assemble into an organized fibrilar network in the blastocoel at stage XIII (blastula) before the initiation of the first major cellular migrations of gastrulation in the early embryo (Zagris, 2001). Cell interaction with extracellular matrix plays an important role in cell attachment, directional migration, mitogenetic modulation, neurite outgrowth, axon guidance, survival of cells, maintenance of differentiated cell phenotypes and the induction of new

Abbreviations used in this paper: a, dorsal aorta; b, blastoderm; c, extra embryonic coelom; cv, cardinal vein; dm, dermamyotome; e, epiblast; ec, ectoderm; en, endoderm; h, hypoblast; m, mesoderm; mn, mesonephros; mp, splanchnic mesoderm; ms, somatic mesoderm; n, notochord; np, neural plate; nt, neural tube; p, primitive streak; s, sclerotome; v, vitelline membrane.

***Address correspondence to:** Dr. Nikolas Zagris. Department of Biology, University of Patras, Patras, Greece. Fax: +30-26-1099-7650. e-mail: zagris@upatras.gr

0214-6282/2005/$25.00

Printed in Spain
www.intjdevbiol.com

expression patterns. Immunohistochemistry and immunoprecipitation data detected the first expression of laminin β1/γ1 polypeptides in chick embryos at stage XIII (Zagris *et al.*, 2000). Immunohistochemistry studies using antibodies to mouse entactin showed the first presence of entactin in the epiblast and the hypoblast and in the accumulating extracellular matrix in chick embryos at stage XIII (Zagris *et al.*, 1993). In our present work, [^{35}S] methionine labeled embryos at stages X (late morula) and XIII were extracted with EDTA-containing buffer. The EDTA extracts were concentrated and subjected to molecular sieve chromatography on a column of sepharose CL-4B. [^{35}S] methionine labeled homogenates immunoprecipitated with rabbit polyclonal antiserum against mouse entactin (Carlin *et al.*, 1981), recognized a major chick polypeptide at approximately 150 kDa on fluorograms from SDS PAGE electrophoresis in embryos at stages X and XIII (Fig. 1 A,B respectively, arrowhead); this band was absent in precipitations with non-immune rabbit IgG (Fig. 1C) and it is therefore likely to correspond to the mouse entactin. This early localization of entactin to the blastoderm at the late morula stage before the appearance of the laminin polypeptides might be of significance in relation to the organization of the first basement membrane on the ventral surface of the epiblast of the embryo at the blastula stage (stage XIII). The high affinity binding of entactin to laminins mediates the connection of laminins to the collagen IV network and to other proteins such as perlecan and is considered to be an essential step in the stabilization of basement membranes and the pericellular matrix. Massive presence of laminin and entactin coincided with the formation of the first basement membrane on the ventral surface of the epiblast and the epithelialization of the epiblast and was also detected in the extracellular matrix in the blastocoel in embryos at stage XIII (review Zagris, 2001). The presence of entactin and laminin before formation of the primitive streak indicates a primary role of these gene products during the morphogenetic movements of gastrulation. Most studies employing *in situ* hybridization have concentrated on the expression of laminin and entactin genes in the morphogenesis of organs such as the kidney, lung and eye in murine embryos. Our present work employing *in situ* hybridization showed strong signals for laminin γ1 chain and entactin mRNAs in the early embryo from the morula to the early gastrula stages and differential expression of these mRNAs in the forming embryonic tissues and organs in the developing chick embryo. We used specific chain anti-sense RNA probes produced from mouse cDNA for the laminin γ1 chain and entactin to study the tissue specific and temporal patterns of their mRNAs in the early chick embryo. For any given hybridization assay using an anti-sense cRNA probe, a parallel assay was performed using cRNA sense strands to serve as controls. In all cases, only background levels of non-specifically distributed silver grains were detected when the sense cRNA probes were used. Laminin γ1 chain (Fig. 2 A,a) and entactin (Fig. 3 A,a) cRNAs detected strong signals of mRNA expression in the embryo at stage X (blastodisc/late morula). It was notable that the entactin cRNA revealed punctate groups of cells expressing entactin mRNA. At stage XIII (blastula), the laminin γ1 cRNAs gave strong signals of mRNA expression in the epiblast, a sheet of pseudostratified columnar epithelium lying on a basement membrane, which forms all tissues in the embryo and in the hypoblast (Fig. 2 B,b). The *in situ* hybridization results (Fig. 2A) in concert with the immunoprecipitation (Zagris *et al.*, 2000) and immunohistochemistry findings (review Zagris 2001) showed that strong signals of laminin γ1 chain mRNAs were detected at stage X but massive expression of the protein was detected shortly thereafter in the extracellular matrix, including the basement membrane on the ventral surface of the epiblast and in epiblast and hypoblast in embryos at stage XIII. Pertinent to these results are data which showed that also the hypoblast synthesizes non-collagenous glycoproteins which contribute to the extracellular matrix, including the basement membrane, in chick blastula (stage XIII) (review Zagris 2001). The entactin cRNAs (Fig. 3 B,b) detected a weaker signal of mRNA expression in the epiblast and the hypoblast of embryos at stage XIII as compared to the blastoderm embryos at stage X. This may indicate the depletion of stored oogenetic entactin mRNAs before the activation of zygotic transcription of the entactin gene. An interesting question is whether the laminin and entactin mRNA detected at stage X is oogenetic and/or zygotic. It is generally accepted that proteins required for early development are either stored in the egg or synthesized on stored mRNAs. Zygotic gene expression was detectable at stage X but showed marked activation at stage XIII with gradual increase thereafter in the chick embryo (review Zagris *et al.*, 2000). In amphibian embryos at pre-gastrula stages (Darribere *et al.*, 1986), experiments with the transcription inhibitor α-amanitin indicated that laminin-related polypeptides are translation products of a maternal mRNA (Riou *et al.*, 1987). In embryos at the primitive streak stage (HH3-4/gastrula stage), the laminin γ1 chain (Fig. 2 C,c) and entactin (Fig. 3 C,c) cRNAs detected strong signals in the cells ingressing through the primitive streak, in the migrating mesenchymal cells, in the cells of the lower layer, in the ectodermal cells neighboring the embryonic junction while more lateral ectoderm showed mild expression of transcripts. The punctate pattern of entactin expression in the three germ layers at this stage was notable. Pertinent to our results is recent work which localized nidogen/ entactin mRNAs in the three germ layers during gastrulation in the mouse embryo (Miosge *et al.*, 2000). Immunogold detection experiments had shown intense expression of laminin and of entactin, the entactin expression pattern being punctate, in the cells ingressing through the primitive streak and in the extracellular matrix (Zagris *et al.*, 1993; review Zagris 2001); antibody perturbation experiments showed that these molecules were required for the directional

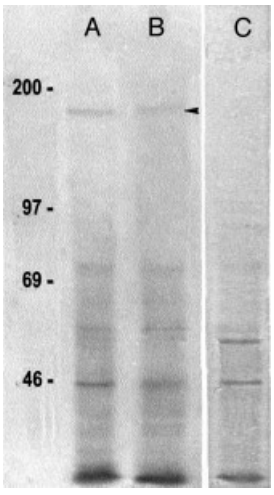

Fig.1. Immunodetection of entactin isolated from early chick embryo. *Embryos at stages X (morula)* **(A)** *and XIII (blastula)* **(B)** *were labeled with ^{35}S-methionine, lysed, precipitated with PYS mouse polyclonal antibodies against entactin; an aliquot of embryos at stage X was precipitated with a non-immune rabbit IgG* **(C)** *The precipitates were analysed in 10% slab SDS-PAGE under reducing conditions and visualized by fluorography. The antiserum against entactin recognized a band at molecular mass approximately 150 kDa (A,B) in embryos at stages XI and XIII (arrowhead) not recognized by the nonimmune rabbit IgG (C) and it is likely to correspond to the mouse entactin. Molecular mass of identified polypeptides was determined according to electrophoretic migration of standards (Rainbow markers, Amersham).*

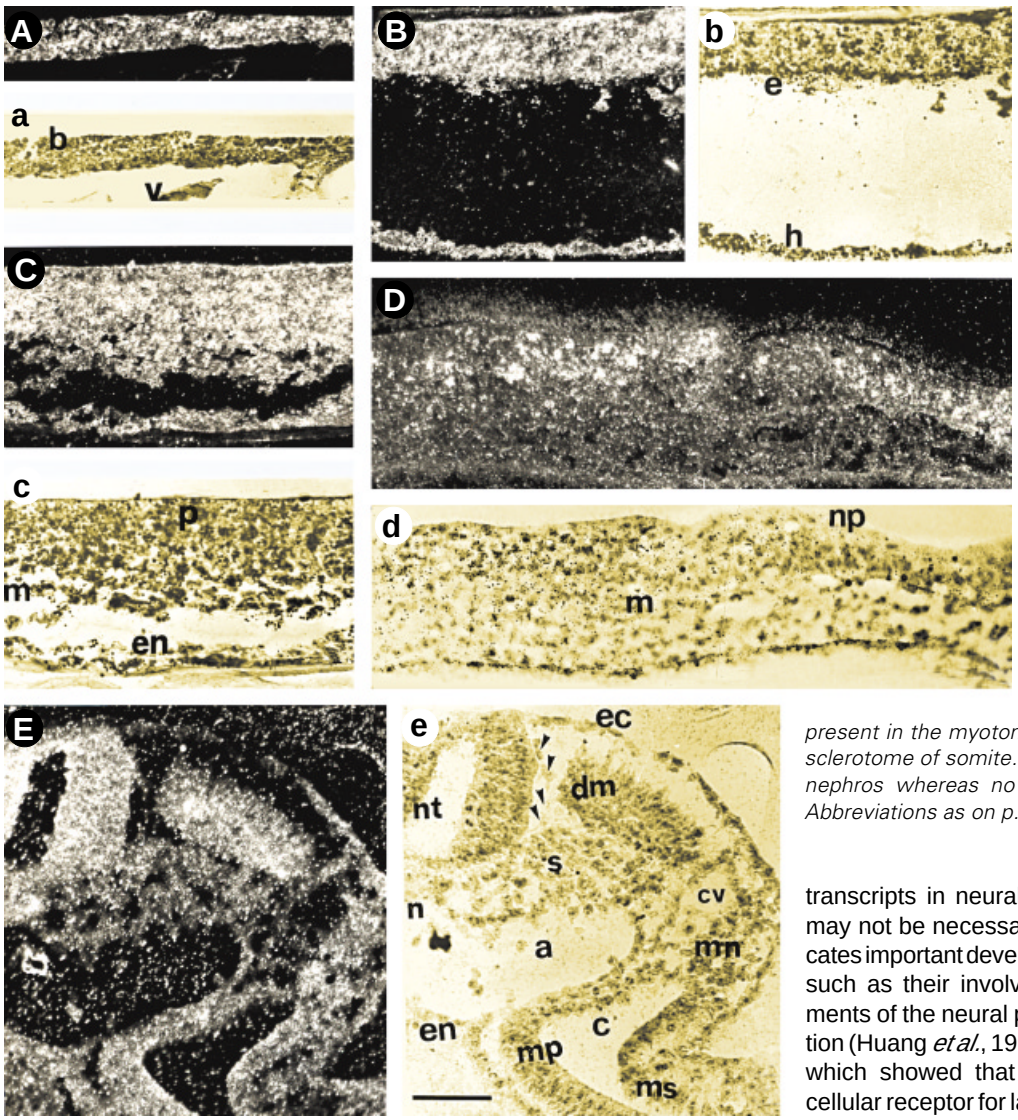

Fig. 2. Localization of transcripts detected by *laminin γ1 chain* cRNA at various stages of chick embryo. *Consecutive transverse sections were processed for* in situ *hybridization as described in* Experimental Procedures. *(A-E) are the dark field pictures of transverse sections from embryos at stages X (morula)* **(A)**, *XIII (blastula)* **(B)**, *HH3-4 (primitive streak)* **(C)**, *HH5-6 (neurula)* **(D)** *and HH10-11 (10 to 13 somites)* **(E)**. **(a-e)** *are the matched bright field pictures. The γ1 cRNA detected strong expression of transcripts in embryos from morula to the early gastrula stages (A-C). Differential expression of laminin transcripts was seen in embryos at early neurula (D) and the 10-somite stage (E). Strong signals were present in the neural plate (D), in the neural tube and notochord (E) and in migrating neural crest cells (indicated by arrowheads E,e). Intense signals were present in the myotome and milder signals in dermatome and sclerotome of somite. Intense signals were observed in mesonephros whereas no signal was detected in ectoderm (E). Abbreviations as on p. 65. Bar, 50 μm.*

migration of cells and, as a consequence, for normal embryonic axis formation (Zagris *et al.*, 1993). At the early neurula stage (HH5-6), the laminin γ1 cRNA (Fig. 2 D,d) revealed punctate groups of cells expressing laminin mRNA strongly in the neural ectoderm while the signal of expression was milder and more uniform in chordamesoderm. This punctate form of laminin expression has been identified in many embryonic tissues such as the embryonic kidney mesenchyme (Ekblom *et al.*, 1980; review Ekblom *et al.*, 1990). The entactin cRNAs gave strong signals which showed a punctate pattern of expression in the neural ectoderm, in mesoderm and in endoderm in embryos at the late gastrula stage (HH4) (Fig. 3 D,d). In embryos at stage HH6 (neurula), the entactin cRNA detected a punctate pattern of mild mRNA expression in the neural plate, in ectoderm neighboring the neural plate and in mesoderm (Fig. 3 E,e) but gave no signal in endoderm and in lateral ectoderm (not shown). After the neural plate folding to produce the neural tube, immunofluorescence had shown strong presence of laminin (Duband and Thiery, 1987; Zagris, 2001) and entactin (Zagris *et al.*, 1993) in the lumen and surrounding the neural tube. The intense expression of laminin transcripts in neural ectoderm and in chordamesoderm may not be necessary for neural determination but implicates important developmental roles for entactin and laminin such as their involvement in the morphogenetic movements of the neural plate bending during primary neurulation (Huang *et al.*, 1990). Pertinent to this is our recent work which showed that the α7 integrin subunit, a specific cellular receptor for laminin, started being expressed in the neural plate and in the adjacent ectoderm during neural plate anchoring to the mesoderm and furrowing to form the neural tube in the chick embryo; inhibition of function of the α7 integrin by blocking antibodies showed that α7 integrin-laminin signaling may play a critical role in tissue organization of the neural plate and neural tube closure (Zagris *et al.*, 2004). At the 10-somite stage (HH10-11), the laminin γ1 cRNA gave strong signals in the neural tube and notochord and low signals in ectoderm (Fig. 2 E,e). In Fig. 2e note the trail of single neural crest cells (pointed by arrowheads) displaying one grain-per cell-label by the γ1 cRNA in Fig. 2E. Neural crest cells migrate extensively along well-characterized pathways through cell-free space rich in laminin/other matrix molecules and laminin is enriched in basement membranes of the neural tube, ectoderm and somite (Duband and Thiery, 1987; Zagris, 2001). It is intriguing neural crest cells express laminin genes strongly (Zagris *et al.*, 2000) and even single cells within a stream of migrating neural crest cells along the neural tube ventrally can be seen labeled (Fig. 2 E,e, arrowheads). The mesonephric tubules which arise from the intermediate mesoderm (nephrotome) lateral to the somites were labeled intensely by the laminin γ1 cRNA (Fig. 2 E,e) as they also were labeled intensely by

the laminin α1 chain (Zagris *et al.*, 2000). Ekblom *et al.* (1980, 1990) showed that laminin participated in the mesenchymal aggregation phase leading to tubule formation and that the expression of laminin α1 chain is transient and locally restricted during kidney organogenesis. Within the somites, the three cell populations displayed variable hybridization signals of the laminin γ1 cRNA. The myotome was labeled intensely, the dermatome and sclerotome were labeled strongly while the migrating fusiform sclerotome cells produced a mild signal by the laminin γ1 cRNA (Fig. 2 E,e). In our previous work, the laminin α1 chain cRNA gave strong signals in the central dermamyotome and hypaxial dermamyotome regions but the epaxial dermamyotome and the sclerotome regions were not labeled (Zagris *et al.*, 2000). Laminin has been detected in basement membranes of the dermamyotome and it is known that also the sclerotome cells synthesize laminin in somites (review Zagris *et al.*, 2000). One can infer from our results transient expression of the laminin genes and/or expression of distinct laminin chain isoforms in the three cell populations in somites. These regional variations are important because they point to variant chains and they imply distinct functions for laminin isoforms during development in the chick embryo. Chick laminin seems to be composed of homologues to several mammalian laminin subunits which are likely to be assembled to various isoforms as indicated by immunodetection (review Brandenberger and Chiquet, 1995) and by amino acid sequence comparison (O'Rear, 1992; Ybot-Gonzalez *et al.*, 1995; Frade *et al.*, 1996) of mouse and chick laminin. The chick homologue of mouse laminin β1 has been identified as B1-1 which has 91% identity over the entire length of the corresponding mouse cDNA (O'Rear, 1992). The amino acid sequence identity of the deduced polypeptide of a chicken laminin chain α-like cDNA fragment shows a very high conservation (88% identity) with mouse α*1* chain (Frade *et al.*, 1996). Variant laminins display distinct distributions and also co-distributions in various tissues and demonstrate overlapping as well as peculiar binding properties in murines and humans (Tiger *et al.*, 1997; Falk *et al.*, 1999). The change in subunit composition of laminin can influence not only their interaction with cellular receptors but also their interaction with other extracellular matrix molecules and could provide specific developmental signals. A comparable pattern of strong signals for laminin mRNA were detected by the laminin α1, β1 and γ1 cRNA probes in the early embryo from the morula stage to early gastrula (Fig. 2 and Zagris *et al.*, 2000). This is an indication that laminin-1 may provide the proper substrate and cues for guiding migrating cells and for folding of epithelial tissues in the early embryo. Among the most puzzling questions concerning the morphogenetic movements have been what triggers the initiation of cellular migrations and how cells reach their appropriate destinations in the early embryo. Integrated movements of cells permit new interactions which transform the simple embryo into a multilayered structure representing the basic body plan of the organism. Whatever the mechanism of control of triggering the initiation of cellular migration, initiation of morphogenetic movements correlates with extracellular matrix deposition.

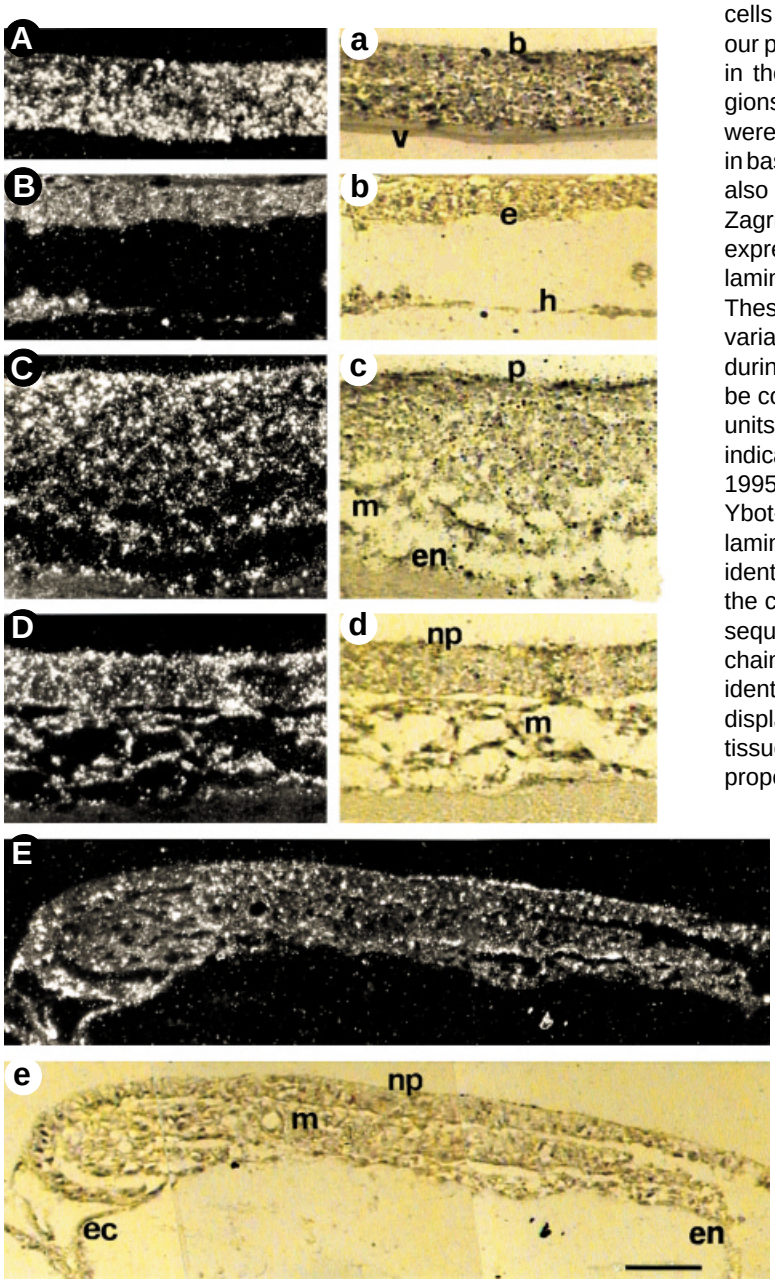

Fig. 3. Localization of transcripts detected by *entactin* cRNA at various stages of chick embryo. *Details and legends are as in Fig. 2 except that the entactin probe was used. (A-E) are the dark field pictures from embryos at stages X* **(A)**, *XIII* **(B)**, *HH3-4 (primitive streak)* **(C)**, *HH4-5 (late primitive streak/early neurula)* **(D)** *and HH5-6 (neurula)* **(E)**; *(a-e) are the matched bright field pictures. The* entactin *cRNA detected strong expression of transcripts in embryos at the morula, mild expression at the blastula and strong expression at the gastrula stages (A-C). Differential expression of entactin transcripts was seen in embryos at early neurula (D,E): strong signals were present in chordamesoderm-ectoderm at the site they interact during the early neurula stage (D) and milder in the neural plate and in mesoderm (E), but no signal in endoderm and in lateral ectoderm (not shown). Note that a punctate pattern of entactin expression is detected in cells at all stages studied. Abbreviations as on p. 65. Bar, 50 μm.*

Experimental Procedures

Embryos

Embryos at stages X (morula), XIII (blastula), HH2-4 (primitive streak/gastrula), HH5-6 (neurula) and HH10-11 (10 to 13 somites) were removed from the eggs, were subsequently fixed in Carnoy fixative (formula B), dehydrated through graded ethanol solutions, embedded in paraffin and sectioned at 5 μm.

Antisera

Antibodies to entactin were prepared against material purified from mouse and were raised in rabbits. Production and specificity of antibodies to entactin were described previously (Carlin *et al.*, 1981).

Radioactive labelling and entactin immunoprecipitation

Embryos at stages X and XIII (20 and 10 embryos, respectively) were removed from the eggs, cleaned and flattened epiblast-side against the surface of vitelline membrane «rafts». Embryos on rafts were placed on Ringer solution containing 335 μCi L-[^{35}S] methionine (1,134 Ci/mmol; New England Nuclear 009T) per milliliter for 5 h at 37ºC. Labeling was stopped at the end of the pulse and blastoderms were detached from the vitelline membrane rafts. Sterile procedures were employed throughout this work. The embryos of each stage X and stage XIII, separately, were solubilized (10 μl/embryo) in lysing buffer [1% (v/v) Triton X-100, 0.02 M NaCl, 0.002 M ethylenediamine tetra-acetic acid (EDTA), 0.004 M ethylene glycol bis (2-aminoethylether)-N,N'-tetraacetic acid (EGTA), 0.04 M tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), pH 7.5] containing 1% aprotinin and 75 μg/ml phenylmethanesulfonyl fluoride (PMSF). Nuclei and insoluble material were pelleted by centrifugation for 5 min at 13,000 rpm. The supernatant (100 μl) was mixed with immunoprecipitation buffer [0.05% (v/v) NP-40, 0.05 M NaCl, 0.0029 M EGTA, 0.01 M Tris-HCl, pH 7.4] (1:6 dilution) and reacted with the preformed antibody-protein A -Sepharose complex by mild agitation. In the embryos at stage X, the homogenate was split into two equal aliquots, each aliquot containing the equivalent homogenate from 10 embryos. The one aliquot reacted with the preformed antibody-protein A-Sepharose complex (experimental) and the other was reacted with the preformed rabbit IgG-protein A-Sepharose complex (control). Protein A-Sepharose CL-4B (6 mg) (Pharmacia) was suspended in Tris-NaCl buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 8.5) by agitation for 10 min and washed five times in the same buffer. The entactin antibody (10 μl) or the rabbit IgG that served as the negative control were diluted with Tris-NaCl buffer (1:10 dilution) and were added to the protein A-Sepharose pellet. Sepharose was suspended by mild agitation and the antibody-protein A-Sepharose complex was allowed to form for at least 1.5 h at room temperature. Sepharose was washed three times with Tris-NaCl buffer and two times with immunoprecipitation buffer. After addition of the supernatant to the preformed antibody-protein A-Sepharose complex or to the preformed rabbit IgG-protein A-Sepharose complex, the immune complex was allowed to form at 4ºC for 3 h with end-over-end mixing. Precipitates were washed six times with immunoprecipitation buffer and the antigen was dissociated from the precipitate by boiling for 2 min in electrophoresis sample buffer containing 10% β-mercaptoethanol (60 μl). Immunoprecipitates were analysed on 10% slab SDS-PAGE and processed for fluorography.

Preparation of probes

The cDNA clones *p54* (nucleotides 4056-5266) and λ*663* (neucleotides 1661-3520) (review Dong and Chung, 1991) for the laminin γ1 chain and entactin, respectively, were used to produce ^{35}S-labeled probes for *in situ* hybridization. The vectors carrying the sequences contain both T3 and T7 RNA polymerase promoters. Both sense and anti-sense cRNAs were transcribed and labeled with ^{35}S-UTP (1000 Ci/mmol; Amersham) by standard methods using T7 and T3 RNA polymerases (Promega). Following transcription of 1μg cDNA, 10 μg yeast tRNA (Sigma) was added as carrier and the cDNA digested by incubation with RQ1 DNase (Promega). Unincorporated nucleotides were removed by gel filtration through a Sephadex G50 (Pharmacia) column and the cRNA probes were precipitated using ethanol. Probes were stored in 10 mM dithiothreitol (DTT) containing 1unit of RNasin for a maximum of three weeks at -70ºC. Before hybridizing to tissue sections, the probes were partially hydrolyzed with alkali to yield fragments of about 100 base pairs long and re-precipitated with ethanol.

In situ *hybridization*

In situ hybridization was carried out as described previously (Dong and Chung, 1991) with slight modifications. The paraffin sections were deparaffinized and rehydrated sequentially in alcohols of decreasing strength down to hydration. The sections were treated with protease K (Sigma) at 10 μg/ml in protease buffer (50 mM TRIS, pH 8.0, 5 mM EDTA) for 7 minutes at room temperature. The digestion was stopped by briefly treating the sections with 0.2% glycine in 1 x PBS. After rinsing with PBS, the sections were fixed in 4% paraformaldehyde in PBS, rinsed and then treated for 10 min in a fresh mix of acetic anhydride diluted in 0.1 M triethanolamine (pH 8.0). Following washing in PBS, the sections were dehydrated and air-dried for 1 h at room temperature. The pre-treated slides were used immediately for hybridization. The probes for *in situ* hybridization were diluted in the hybridization buffer to give a final concentration of 10000 cpm/μl and heated at 80ºC for 30 sec. The hybridization was performed at 50ºC in hybridization buffer containing 50%(v/v) formamide (deionized) (Sigma), 10% dextran sulfate (Sigma), 10 mM DTT, 0.5 mg/ml tRNA (Sigma), 1 x Denhardt's solution (Sigma) and salts (0.3 M NaCl; 20 mM TRIS, pH 8.0; 5 mM EDTA; 10 mM NaH_2PO_4). 30 μl of this solution was applied to each slide, which was then covered with a cover slip and hybridized for 16 h at 60ºC. The hybridization was carried out in a sealed Petri dish containing paper towels saturated with a solution similar to the hybridization buffer. After the period of 16 h, the slides were treated in 5 x standard saline citrate buffered solution (SSC), 10 mM DTT at 50ºC for 30 min to facilitate removal of the coverslips. The slides were washed at high stringency with 50% (v/v) formamide in 2 x SSC plus 10 mM DTT for 20 minutes at 65ºC and were then incubated with 40 μg/ml RNase A (Sigma) in a buffer containing 0.1 M TRIS, pH 7.6, 50 mM EDTA and 0.15 M NaCl for 1h at 37ºC. The first washing step was repeated after RNase A treatment and the sections were quickly dehydrated and air-dried. The labeled slides were dipped in 1:1 diluted NBT-2 emulsion (Kodak, Rochester, USA), air-dried overnight, placed in a light tight box and kept at 4ºC. The emulsion-coated slides were developed after exposure for 22 weeks. The tissue sections were counter stained using Mayer's hematoxylin then examined and photographed under both bright and dark fields. To ascertain the specificity of hybridization, some sections were treated with RNase A and RNase T1 before hybridization. For any given hybridization assay using an anti-sense cRNA probe a parallel assay was performed using cRNA sense strands to serve as controls.

Acknowledgments

This work was supported by grants from the Ministry of Health of Greece, from the General Secretariat of Research and Technology of Greece and grant no. 890752 from NATO.

References

BRANDENBERGER, R. and CHIQUET, M. (1995). Distinct heparin-binding and neurite-promoting properties of laminin isoforms isolated from chick heart. *J. Cell Sci.* 108: 3099-3108..

CARLIN, B., JAFFE, R., BENDER, B. and CHUNG, A.E. (1981). Entactin, a novel basal lamina-associated sulfated glycoprotein. *J. Biol. Chem.* 256: 5209-5214.

CHUNG, A.E., JAFFE, R., FREEMAN, I.L., VERGNES, J-P., BRAGINSKI, J.E. and CARLIN, B. (1979). Properties of a basement membrane-related glycoprotein synthesized in culture by a mouse embryonal carcinoma derived cell line. *Cell* 16: 277-287.

DARRIBERE, T., RIOU, J. F., SHI, D.L., DELARUE, M. and BOUCAUT, J.C. (1986). Synthesis and distribution of laminin-related polypeptides in early amphibian embryos. *Cell Tissue Res.* 246: 45-51.

DONG, L-J. and CHUNG, A.E. (1991). The expression of the genes for entactin, laminin A, laminin B1 and laminin B2 in murine lens morphogenesis and eye development. *Differentiation* 48: 157-172.

DUBAND, J-L. and THIERY, J.P. (1987). Distribution of laminin and collagens during avian neural crest development. *Development* 101: 461-478.

EKBLOM, P., ALITALO, K., VAHERI, A., TIMPL, R. and SAXEN, L. (1980). Induction of a basement membrane glycoprotein in embryonic kidney: Possible role of laminin in morphogenesis. *Proc. Natl. Acad. Sci. USA* 77: 485-489.

EKBLOM, M., KLEIN, G., MUGRAUER, G., FECKER, L., DEUTZMANN, R., TIMPL, R. and EKBLOM, P. (1990). Transient and locally restricted expression of laminin A chain mRNA by developing epithelial cells during kidney organogenesis. *Cell* 60: 337-346.

FALK, M., FERLETTA, M., FORSBERG, E. and EKBLOM, P. (1999). Restricted distribution of laminin α1 chain in normal adult mouse tissues. *Matrix Biology* 18: 557-568.

FRADE, J.M., MARTINEZ-MORALES, J.R. and RODRIGUEZ-TEBAR, A. (1996). Laminin-1 selectively stimulates neuron generation from cultured retinal neuroepithelial cells. *Exp. Cell Res.* 222: 140-149.

HUANG, S., SAINT-JEANNET, J.P., KAN, P. and DUPRAT, A.M. (1990). Extracellular matrix: an immunological and biochemical (CAT and TOH activity) survey of in vitro differentiation of isolated amphibian neuroblasts. *Cell Diff. Dev.* 30: 219-233.

MIOSGE, N., QUONDAMATTEO, F., KLENCZAR, C. and HERKEN, R. (2000). Nidogen-1: Expression and ultrastructural localization during the onset of mesoderm formation in the early mouse embryo. *J. Histochem. Cytochem.* 48:229-238.

O' REAR, J.J. (1992). A novel laminin B1 chain variant in avian eye. *J. Biol. Chem.* 267: 20555-20557.

RIOU, J.F., DARRIBERE, T., SHI, D.L., RICHOUX, V. and BOUCAUT, J.C. (1987). Synthesis of laminin-related polypeptides in oocytes, eggs and early embryos of the amphibian *Pleurodeles waltlii*. *Roux Arch. Dev. Biol.* 196: 328-332.

TIGER, C-F., CHAMPLIAUD, M-F., PEDROSA-DOMELLOF, F., THORNELL, L-E., EKBLOM, P. and GULLBERG, D. (1997). Presence of laminin α5 chain and lack of laminin α1 chain during human muscle development and in muscular dystrophies. *J. Biol. Chem.* 272: 28590-28595.

YBOT-GONZALEZ, P., RUNSWICK, S., SMYTH, N. and EDGAR, D. (1995). Regulated expression of a novel laminin β subunit during the development of the chick embryo. *Differentiation* 59: 215-223.

ZAGRIS, N. (2001). Extracellular matrix in development of the early embryo. *Micron* 32: 427-438.

ZAGRIS, N., CHRISTOPOULOS, M. and GIAKOUMAKI, A. (2004). Developmentally regulated expression and functional role of α7 integrin in the chick embryo. *Develop. Growth Differ.* 46: 299-307.

ZAGRIS, N., CHUNG, A.E. and STAVRIDIS, V. (2000). Differential expression of laminin genes in early chick embryo. *Int. J. Dev. Biol.* 44: 815-818.

ZAGRIS, N., STAVRIDIS, V. and CHUNG, A.E. (1993). Appearance and distribution of entactin in the early chick embryo. *Differentiation* 54: 67-71.

Received: February 2004
Reviewed by Referees: April 2004
Modified by Authors: July 2004
Re-reviewed by Referees and Accepted for Publication: September 2004