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Temporary Disruption of the Retinal Basal Lamina and Its Effect on Retinal Histogenesis

Willi Halfter,*,[1] Sucai Dong,* Manimalha Balasubramani,* and Mark E. Bier†

**Department of Neurobiology, University of Pittsburgh, 1402 E Biological Science Tower, Pittsburgh, Pennsylvania 15261; and †Department of Chemistry, Mellon Institute, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213-2683*

An experimental paradigm was devised to remove the retinal basal lamina for defined periods of development: the basal lamina was dissolved by injecting collagenase into the vitreous of embryonic chick eyes, and its regeneration was induced by a chase with mouse laminin-1 and α2-macroglobulin. The laminin-1 was essential in reconstituting a new basal lamina and could not be replaced by laminin-2 or collagen IV, whereas the macroglobulin served as a collagenase inhibitor that did not directly contribute to basal lamina regeneration. The regeneration occurred within 6 h after the laminin-1 chase by forming a morphologically complete basal lamina that included all known basal lamina proteins from chick embryos, such as laminin-1, nidogen-1, collagens IV and XVIII, perlecan, and agrin. The temporary absence of the basal lamina had dramatic effects on retinal histogenesis, such as an irreversible retraction of the endfeet of the neuroepithelial cells from the vitreal surface of the retina, the formation of a disorganized ganglion cell layer with an increase in ganglion cells by 30%, and the appearance of multiple retinal ectopias. Finally, basal lamina regeneration was associated with aberrant axons failing to correctly enter the optic nerve. The present data demonstrate that a transient disruption of the basal lamina leads to dramatic and probably irreversible aberrations in the histogenesis in the developing central nervous system.

Key Words: **basal lamina; chick embryo; retina; axonal navigation.**

INTRODUCTION

Basal laminae are extracellular matrix sheets that separate epithelial tissues from the adjacent connective tissue. The constituents of basal laminae comprise members of the laminin protein family, collagen IV, collagen XVIII, nidogen, agrin, and perlecan (for reviews see Timpl, 1996; Erickson and Couchman, 2000). The laminins function as the principal cell adhesion proteins in basal laminae (Colognato and Yurchenko, 2000), whereas collagen IV provides the two-dimensional scaffold that is critical for basal lamina stability (Timpl *et al.,* 1981; Yurchenko and Ruben, 1987). Nidogen, perlecan, and agrin bind to either laminin and/or collagen IV and provide further stability to the basal lamina sheath (Durkin *et al.,* 1988; Mann *et al.,* 1989; Denzer *et al.,* 1995).

The major basal laminae of the developing central nervous system are the pial basal lamina of the brain and the retinal basal lamina, also referred to as the inner limiting membrane. The pial basal lamina covers the brain and spinal cord, whereas the retinal basal lamina separates the retinal neuroepithelium from the adjacent vitreous body in the eye. Recent data have shown that the retinal and pial basal laminae are critically important in the development of the nervous system: enzymatic removal of the retinal or pial basal lamina, for example, leads to aberrant axons and neurons that exit the brain and retina and invade the meninges or the vitreous (Halfter, 1998; Halfter and Schurer, 1998). Further, mutant mice with defects in basal lamina proteins (Costell *et al.,* 1999) and their receptors (Georges-Labouesse, 1998) show cortical and retinal dysplasia. Finally, several hereditary diseases in humans, such as Fukuyama muscular dystrophy, muscle–eye–brain disease, and Walker–Warburg syndrome, summarized as cobblestone lissencephaly (Gleeson and Walsh, 2000), show breaches in the pial basal lamina that are combined with cortical and retinal dysplasias (Nakano *et al.*, 1996; Hiltia *et*

[1] To whom correspondence should be addressed. Fax: (412) 648-1441. E-mail: whalfter@pitt.edu.

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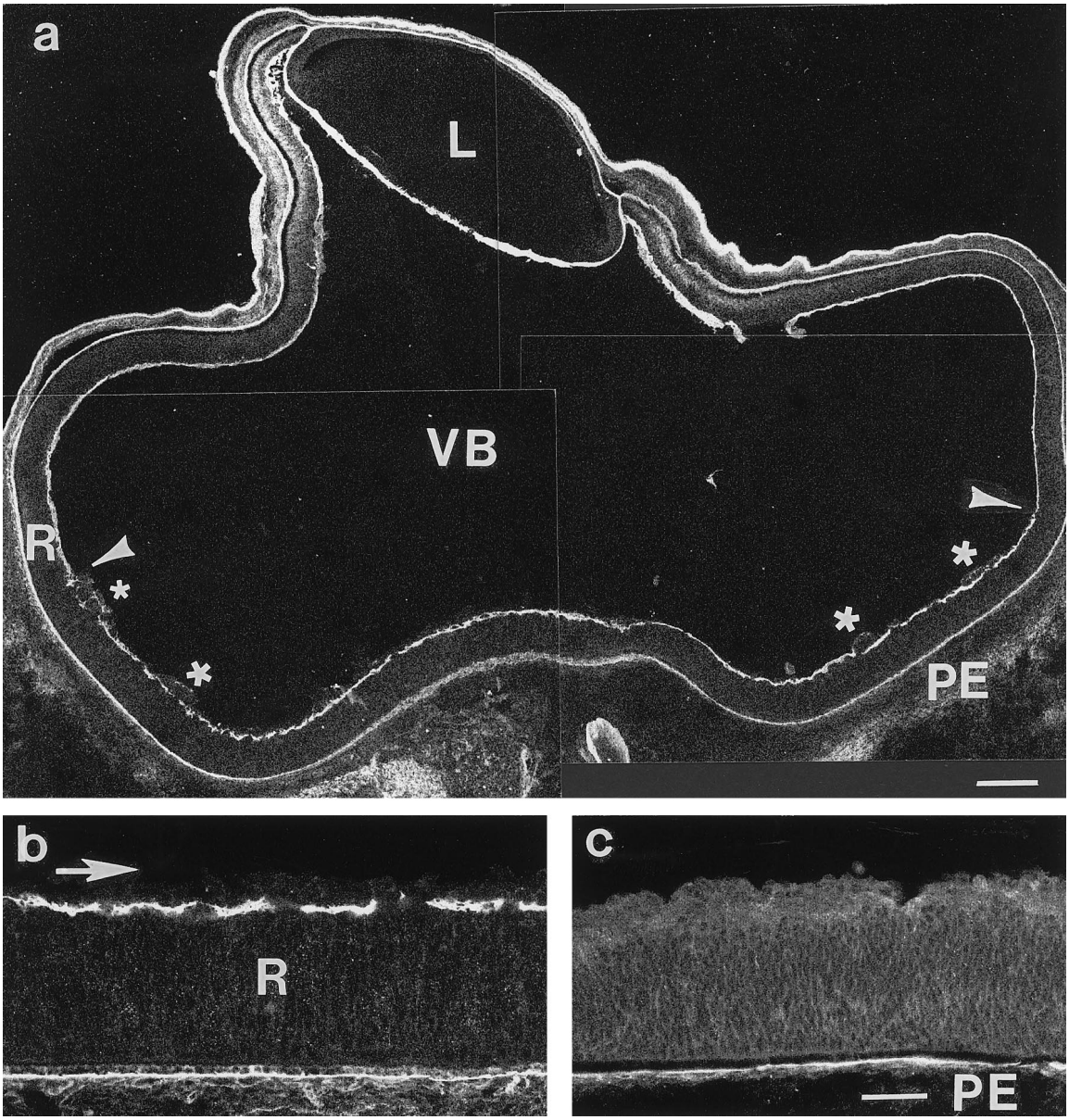

FIG. 1. Fluorescence micrograph showing a cross section through an E5 chick eye after pulse-chase injections of collagenase and laminin-1/α2-macroglobulin. The collagenase had been injected at E4 followed by the laminin-1/macroglobulin chase 10 h later. The embryo was allowed to survive another 14 h. Immunocytochemistry using a MAb to chick laminin-1 shows that the injected mouse laminin-1 caused the reconstitution of the chick retinal basal lamina. The border of the centrally located regenerated basal lamina and the peripherally located *de novo*-formed basal lamina is indicated by arrowheads. Stars indicate ectopias along the retina (R). VB: vitreous body; PE: pigment epithelial basal lamina; L: lens. A high-power view of the retina (b) shows that the regenerated basal lamina is not as perfectly continuous as a control basal lamina and is located deeper in the retina than normal. A high-power view of the retina from an eye chased by an injection of α2-macroglobulin alone is shown in (c): the retinal basal laminin has not regenerated. Bar: a, 200 μm; b and c, 50 μm.

al., 1997; Williams *et al.,* 1984). The similarity of cortical and retinal dysplasias in targeted mouse mutations, human hereditary diseases, and chick embryos after enzymatic basal lamina disruption implicates the retinal and pial basal lamina as an essential player in early brain histogenesis and axonal navigation. How the retinal and pial basal lamina are connected to brain histogenesis is as yet unknown.

The present study introduces a new experimental procedure that allows the disruption of the chick retinal basal lamina for a limited period of development. Histological

studies show that a temporary breach of only a few hours in the retinal basal lamina leads to ectopias in the retina, a scattering of ganglion cells, and to aberrant optic axons at the optic disc, closely resembling retinal dysplasias in humans and mice with mutations of basal lamina proteins. Many of these aberrations are irreparable and of lasting consequence for further brain development. Our study shows that a main function of the retinal basal lamina is to provide a barrier to keep retinal cells and axons from invading the vitreous and to provide an attachment for the neuroepithelial cells.

MATERIALS AND METHODS

Antibodies

The following monoclonal antibodies (MAbs) were used: MAb 3H11 to laminin-1 (Halfter, 1993), MAb 1G12 to nidogen-1 (Halfter *et al.*, 2000), MAb 6C4 to collagen XVIII (Halfter *et al.*, 1998), MAb 6D2 to agrin (Halfter, 1993). MAbs to perlecan (MAb 33-2) (Bayne *et al.*, 1984), to islet-1 as marker for ganglion cell nuclei (Ericson *et al.*, 1992; Austin *et al.*, 1995), and to neurofilament-associated protein (MAb EC/8) (Ciment *et al.*, 1986) were obtained from the Developmental Studies Hybridoma Bank, University of Baltimore. A MAb to β-dystroglycan (MAb 43DAG 1/8D5) and a rabbit antiserum to laminin-1 from the EHS mouse tumor were purchased from Novocastra (Newcastle, UK) and from GIBCO/Life Sciences (Gaithersburg, MD), respectively. A rat polyclonal antiserum to human placenta laminin-2 (merosin) was generated by immunizing a rat with 1 mg/ml human laminin-2 (Gibco/BRL), and polyclonal antisera to mouse nidogen-1 and chick collagen IV were generous gifts from Dr. U. Mayer, School of Biological Sciences, Manchester, UK, and K. von der Mark, University of Erlangen, Germany (Mayne *et al.*, 1982).

Histology

Heads of chick embryos were fixed in 4% paraformaldehyde in 0.1 M potassium phosphate buffer (pH 7.4) for 1 h. After washing in CMF and cryoprotecting with 30% sucrose for 4 h, the specimens were embedded in O.C.T. compound (Miles, Elkhart, IN) and sectioned in a horizontal plane with a cryostat at 25 μm. Sections were mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA). To facilitate islet-1 staining, the slides were dehydrated in absolute methanol and acetone, each for 1 min, and air-dried. Sections to be stained for collagen IV were pretreated with 10 μg/ml pronase as described (Halfter *et al.*, 2000). The sections were permeabilized with 0.05% Triton X-100 and 1% BSA for 10 min and incubated with hybridoma supernatants or primary antiserum for 1 h. After three rinses, the sections were incubated with 1:500 Cy3-labeled goat-anti mouse or goat-anti rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) for another hour. After two final rinses, the specimens were mounted in 90% glycerol and examined with an epifluorescence microscope (Zeiss, Thornwood, NY) or a confocal microscope (Fluoview; Olympus, Lake Success, NY). Islet-1 positive ganglion cells were counted at ×40 for a defined length of the retina at two positions adjacent to the optic disc. To obtain a comparative measure for ganglion cell numbers from different embryos, the number of ganglion cells from each noninjected control eye was set at one and compared to the number of ganglion cells from its corresponding contralateral experimental eye by calculating their ratio. The means of these ratios were calculated for each experimental paradigm.

To visualize individual neuroepithelial cells and the basal lamina at the same time, the retinae were mounted, scleral side down, onto membrane filters that had been coated with a suspension of DiI crystals (Halfter, 1998). Following immunolabeling with an anti-laminin MAb (see above) and a Cy2-labeled secondary antibody, the retinae were removed from the filters, embedded in 1% agarose, and sectioned with a vibratome at 250 μm. Some of the preparations were viewed as whole mounts using a confocal microscope.

Disruption and Regeneration of the Retinal Basal Lamina in Vivo

Retinal basal laminae were enzymatically removed *in vivo* by injecting 0.2–0.5 μl of 100 U/ml (~70 μg/ml) collagenase (Type VII; Sigma, St. Louis, MO) into E3 to E5 chick eyes (Halfter, 1998). For basal lamina regeneration experiments, 0.5–1 μl of 100 U/ml collagenase was injected into E3–E5 eyes, and the collagenase was chased at various time points with 1 μl of laminin-1 (Gibco/BRL) and α2-macroglobulin (M6159; Sigma) at 1 mg/ml each. The embryos were killed 2 h to 3 days after the laminin/macroglobulin chases, and sections through the heads were stained with chick-specific antibodies to laminin-1, nidogen-1, collagen XVIII, collagen IV, perlecan, and agrin to determine the presence of an intact retinal basal lamina. Successful basal lamina regeneration was obtained with four different batches of laminin-1 and two batches of α2-macroglobulin. α2-Macroglobulin alone (1 mg/ml) or α2-macroglobulin (1 mg/ml) in combination with human laminin-2 (1 mg/ml; Gibco/BRL) and 1 mg/ml mouse collagen IV (Gibco/BRL) were injected as well. For antibody-blocking experiments, 25 μl of laminin-1 at 1 mg/ml was mixed with 5 μl of polyclonal antisera to laminin-1, laminin-2, or nidogen-1 and incubated for 1 h prior to injection into the chick eyes.

Isolation of a Laminin Gamma 1 cDNA and in Situ Hybridization

Basal laminae were isolated from E10 chick retina (Halfter and von Boxberg, 1992), and the proteins were separated by SDS–PAGE and visualized by Coomassie blue staining. The band at 200 kD was excised and digested with trypsin, and the peptides were analyzed by capillary-LC nano-ESI MS/MS. The column used was 100 μm i.d. fused silica capillary packed to 10 cm with C-18 POROS R10 particles (PerSeptive Biosystems, Framingham, MA). Solvent A was 0.1% (v/v) acetic acid in water and solvent B was 0.1% acetic acid in acetonitrile. Peptides were eluted over a 2–98% B gradient in 45 min and introduced into the analyzer by electrospray ionization. The MS/MS spectra were acquired using a quadrupole filed ion Trap mass spectrometer (Finningan LCQ) and analyzed with SEAQUEST (Eng *et al.*, 1994). Out of the nine tandem mass peptide spectra collected, two matched spectra from peptides of human and mouse homologs of laminin gamma 1 chain. Two forward (F1: CCC AGC GCC TAT AAC TTT GAC AAT; F2: GTG CTG CAG GAA TGG GTA ACT) and 1 reverse primer (R1: AAG CCA GCT CCC TCA AGC ACA) were designed based on these peptide sequences and used to amplify a cDNA by PCR (Elongase, Gibco/BRL) from a random-primed cDNA library of E10 chick amnion (Stratagene, Cedar Creek, TX). A 3.1-kb band was excised from the agarose gel and cloned into the PCR-script cloning vector (Stratagene). The

nucleotide and deduced amino acid sequence of the 3.1-kb laminin gamma 1 clone was deposited at the GenBank under accession code AF 373,841. *In situ* hybridization using a digoxigenin-labeled cRNA probe made from cloned 3.1-kb cDNA followed the procedure described by Schaeren-Wiemers and Gerfin-Moser (1993).

RESULTS

Basal Lamina Regeneration Induced by Laminin-1/α2-Macroglobulin

As shown previously, the retinal basal lamina can be removed by injecting bacterial collagenase into the vitreous of embryonic chick eyes (n = 155) (Halfter, 1998). The enzyme digests collagen IV and XVIII of the retinal basal lamina within 2 h of injection (n = 7), whereas the noncollagenous basal lamina proteins remain intact. As a consequence of the loss of the collagenous components, the remaining basal lamina proteins dissociate within 5 h of the collagenase injection. The basal lamina does not regenerate by itself, even 10 days after its initial disruption (Halfter, 1998). The regeneration of the basal lamina, however, can be initiated by a second intravitreal injection of matrigel (n = 85) (Halfter *et al.*, 2000), an extracellular matrix extract from a mouse yolk sac tumor (Kleinman *et al.*, 1986).

To screen for proteins that can replace matrigel in the induction of basal lamina regeneration, we chased collagenase-injected eyes with combinations of the collagenase inhibitor α2-macroglobulin (Werb *et al.*, 1974) and individual basal lamina proteins, such as laminin-1, laminin-2, and collagen IV. Staining of sections through the experimental eyes with antibodies to laminin-1, nidogen-1, agrin and collagen IV, and collagen XVIII and perlecan revealed that a combination of α2-macroglobulin and laminin-1 could initiate complete basal lamina regeneration (Figs. 1a and 1b; n = 58). Injections of α2-macroglobulin alone (Fig. 1c; n = 7), or coinjections of α2-macroglobulin with laminin-2 (merosin; n = 6) or collagen IV (n = 10) did not lead to basal lamina regeneration. The critical concentration of laminin-1 to induce basal lamina regeneration was 0.7 mg/ml (n = 4) and higher, with 1 mg/ml used in routine experiments (n = 58). At suboptimal concentrations of 0.5 mg/ml (n = 6) and 0.25 mg/ml (n = 6), only segments of the retinal basal lamina regenerated, and in these segments the new basal lamina was greatly fragmented. Macroglobulin could be omitted from the chase cocktail (n = 15), when the time span between collagenase injection and the chase was more than 10 h.

The regenerated basal lamina was ectopically located 50 μm deep of the vitreal surface of the retina and was not as perfectly continuous and linear as a control basal lamina (Figs. 1a, 1b, and 2a). Its deep location did not change with further incubation, even 5 days after the laminin/macroglobulin chase (Fig. 3; n = 6). Further, a time difference between the collagenase injection and the laminin-1/α2-macroglobulin chase of only 1 h resulted in the

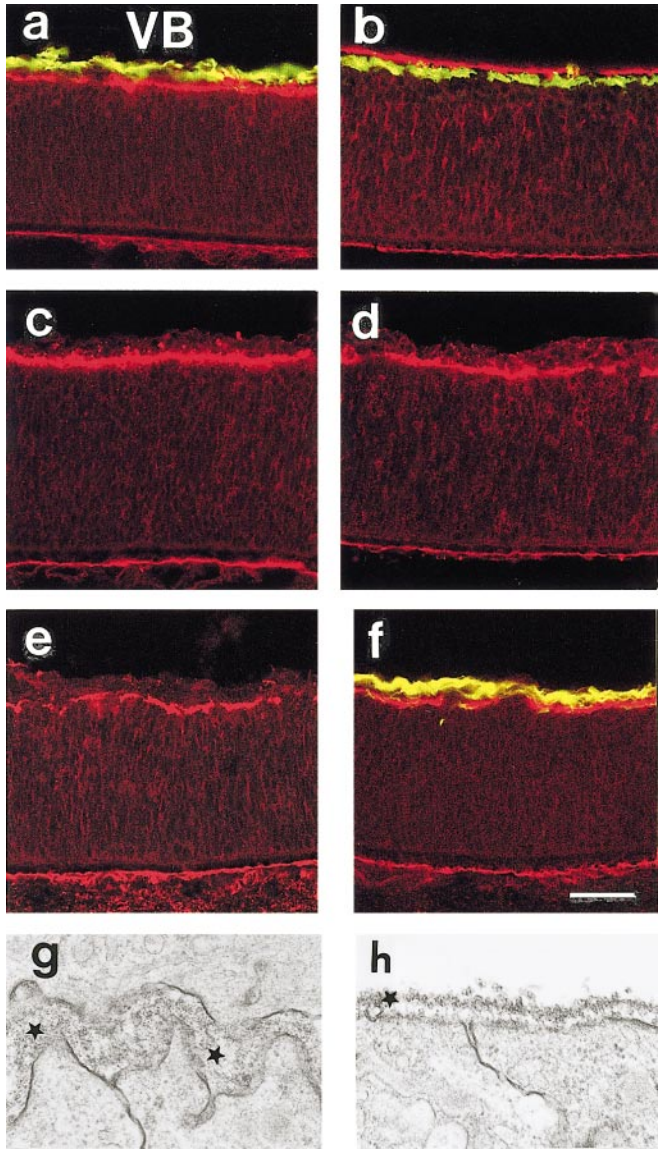

FIG. 2. Fluorescence micrographs showing the ectopic location of the regenerated retinal basal lamina after pulse-chase injections of collagenase and laminin-1/α2-macroglobulin. The collagenase had been injected at E4 followed by the laminin-1/macroglobulin chase 10 h later. The embryo was allowed to survive another 14 h. Double labeling with antibodies to laminin-1 (red) and neurofilament (green) show that the regenerated basal lamina (a) is located underneath the optic fiber layer, whereas in a control retina (b) the basal lamina is on top of the optic fiber layer. The regenerated basal lamina has the full complement of chick basal lamina proteins, such as nidogen-1 (c), collagen XVIII (d), and collagen IV (e). When the laminin/macroglobulin chase followed 1 h after the collagenase injection, the original basal lamina was replaced by a newly generated basal lamina with its ectopic position (f). The ultrastructural appearance of the regenerated basal lamina (stars), as shown in a high-power electron micrograph (g), closely resembled the morphology of the basal lamina from the control retina (h, star). VB: vitreous body. Bar: a–f, 50 μm; g and h, 50 nm.

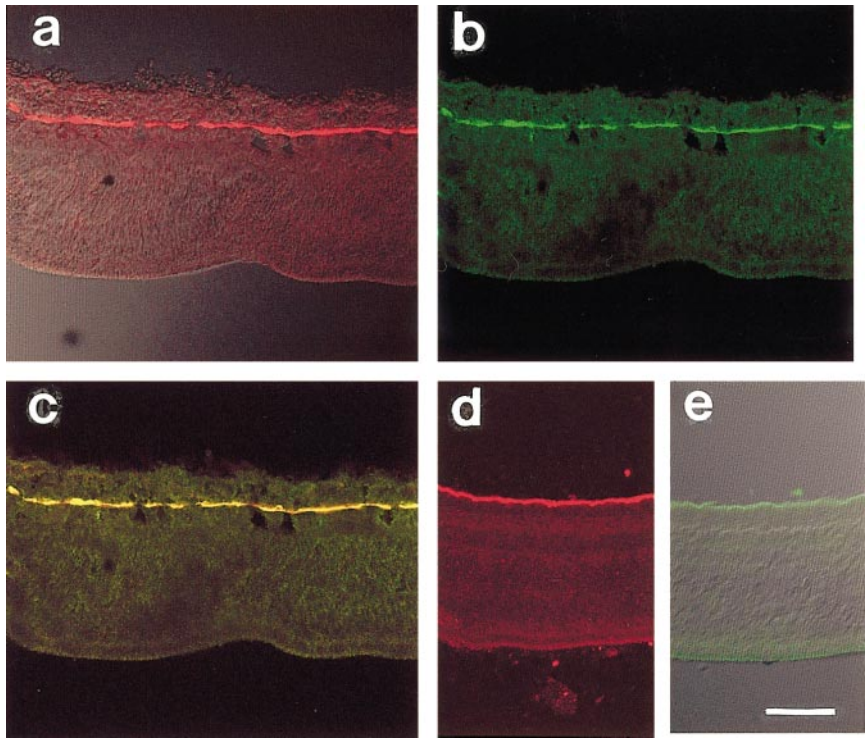

FIG. 3. Fluorescence micrographs showing a cross section of an E10 retina 5 days after pulse-chase injections of collagenase followed by laminin-1 and macroglobulin (a–c). The collagenase had been injected at E4 followed by the laminin-1/macroglobulin chase 10 h later. The embryo was allowed to survive another 5 days. The section had been double-stained with a MAb to chick laminin-1 (a, Cy3) and a polyclonal antiserum to mouse laminin-1 (b, Cy2) to visualize the integration of the mouse laminin-1 in the regenerated chick retinal basal lamina. The distribution of laminin-1 from chick and mouse matched as shown by visualizing both stainings simultaneously (c). Staining of the contralateral control retina with the chick laminin-1 MAb shows a prominent basal lamina labeling (d), whereas the polyclonal anti-mouse laminin-1 antibody shows only a very weak basal lamina staining (e) in the control retina. Bar: 100 μm.

elimination of the original basal lamina and the induction of a regenerated, ectopic basal lamina (Fig. 2f; $n = 19$).

Because the embryonic retina grows at its periphery, the pulse-chase injections resulted in eyes with a regenerated basal lamina in the central portion of the retina and a *de novo*-formed basal lamina at the peripheral parts of the retina. Based on the location and linearity, segments of regenerated basal lamina were easily distinguished from the *de novo*-formed segments of the basal lamina (Fig. 1a). The transition zone of regenerated versus *de novo*-formed basal lamina was in the range of less than 100 μm (see Figs. 8a and 8b below). The length of the regenerated relative to the *de novo*-formed basal lamina was dependent both on the survival time after the laminin chase and on the time difference between collagenase injection and laminin chase. For example, when the collagenase was injected early (E3), the laminin/macroglobulin chase followed a few hours later and the embryos were allowed to survive 2 more days, the stretch of regenerated basal lamina was restricted to only a very small segment of the central retina, whereas the remainder of the retina had a *de novo*-formed basal lamina.

Immunocytochemistry using chick-specific MAbs showed that the regenerated basal lamina was comprised of all known components of a typical chick retinal basal lamina (Halfter *et al.*, 2000), such as laminin-1 (Figs. 1a, 1b, and 2a), nidogen-1 (Fig. 2c), collagen XVIII (Fig. 2d), agrin (not shown), collagen IV (Fig. 2e), and perlecan (not shown). The full complement of basal lamina proteins was also present when the basal lamina regeneration was incomplete, such as in cases when suboptimal concentrations of laminin were injected.

Basal laminae are defined by their ultrastructural appearance as extracellular matrix sheets of 50 nm thickness with a lamina densa and two laminae rarae. Electron microscopy showed that, 14 h after the laminin-1 chase, regenerated basal laminae had a morphology (Fig. 2g; $n = 4$) that was very similar to the morphology of a normal basal lamina from a control retina (Fig. 2h; $n = 4$).

To check whether the mouse laminin-1 from the chase cocktail had contributed to the regeneration of the chick basal lamina, sections through the experimental eyes were stained with a polyclonal antiserum to mouse laminin-1 that, at a dilution of 1:1000, detects mouse laminin-1 much better than chick laminin-1. Immunocytochemistry

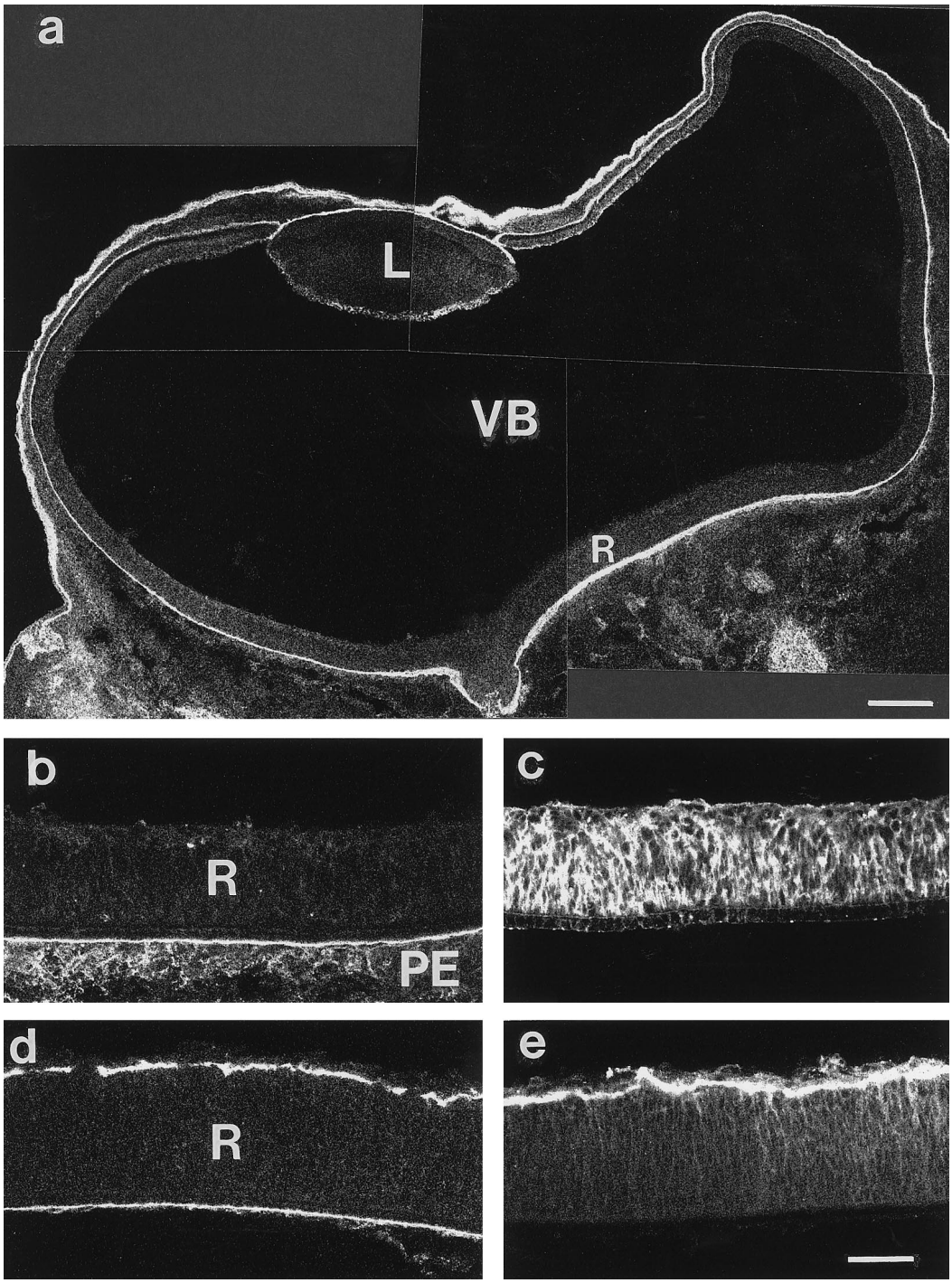

FIG. 4. Fluorescence micrograph of a cross section of an E5 chick eye showing that antibodies to laminin-1 block the regeneration of the retinal basal lamina induced by laminin/macroglobulin. The eye was injected with collagenase at E4, and chased, 10 h later, with laminin/macroglobulin plus polyclonal antiserum to mouse laminin-1. The embryo was allowed to survive for another 14 h. Staining of the experimental eye with a chick-specific MAb to laminin-1 shows that the retinal basal lamina has not regenerated (a). High-power micrographs confirm the absence of the retinal basal lamina (b). Staining with secondary antibody shows the penetration of the injected laminin–antiserum through but not beyond the retina (R; c). A chase of laminin/macroglobulin plus anti-nidogen-1 antiserum does not block basal lamina regeneration (d), and the anti-nidogen-1 antiserum localizes to the site of the newly reconstituted basal lamina (e), as demonstrated by staining with secondary antibody. Note that in the absence of basal lamina regeneration, the newly developed peripheral retina is not covered by a *de novo*-formed basal lamina (compare Figs. 1a and 4a). VB: vitreous body; L: lens. Bar: a, 200 μm; b–e, 50 μm.

showed a strong labeling in the regenerated basal lamina (Fig. 3b), and very little labeling in the exclusively chick-derived basal lamina in the contralateral control retina (Fig. 3e). The perfect match of mouse and chick laminin-1 (Fig. 3c) showed that the regenerated retinal basal lamina was chimeric for chick and mouse laminin-1. Mouse laminin-1 was even detectable 5 days after the laminin-1 chase in the regenerated chick retinal basal lamina (Fig. 3; $n = 6$), indicating a surprisingly slow turnover of laminin-1 in basal laminae. Given that laminin-1 preparations regularly contain traces of nidogen-1, we also checked for integration of mouse nidogen-1 into the regenerated chick retinal basal lamina. Using an antiserum to mouse nidogen-1, we found that mouse nidogen-1 was indeed detectable in the regenerated chick retinal basal lamina (not shown).

To confirm that laminin-1 and not nidogen-1, laminin-2, or any other potential contaminant in the laminin preparations was responsible for basal lamina regeneration, laminin-1 was mixed with antisera to laminin-1, laminin-2, or nidogen-1 and injected into eyes whose basal laminae had been dissolved. As shown by staining for all available basal lamina markers, basal lamina reconstitution was completely blocked by the anti-mouse laminin-1 antiserum (Figs. 4a and 4b; $n = 7$) but was not blocked by the anti-laminin-2 ($n = 5$, not shown) or anti-nidogen-1 antisera (Fig. 4d; $n = 5$). The injected antibody to mouse laminin-1 had penetrated the retina of the injected eye up to the scleral surface, but had not leaked to the adjacent pigment epithelium or into the surrounding periocular connective tissue (Fig. 4c). Antibodies to laminin-2 and nidogen-1 were also restricted to the injected eye but were localized in the retina predominantly to the site of the reconstituted basal lamina (Fig. 4e).

The time course of basal lamina regeneration was established by probing for the first appearance of each of the basal lamina proteins. Two hours after the laminin/macroglubulin chase, a strong labeling for mouse laminin-1 was detected in all embryos ($n = 10$). The most prominent labeling of chick basal lamina proteins was that for nidogen, which was detected in 5 out of 10 cases within 2 h after the chase. At that time point, traces of chick laminin-1, perlecan, and agrin were detectable in these 5 embryos as well. The first detectable signal for collagen XVIII was found 4 h after the chase ($n = 10$), and collagen IV appeared in the new basal lamina only 6 h after the chase. The reconstituted basal lamina was complete 6 h after the laminin-1/macroglobulin chase ($n = 10$). At the 2- and 4-h time points, only segments of the basal lamina were reconstituted and the stretches of reconstituted basal lamina were greatly fragmented. The regenerated basal lamina became more continuous with longer incubation time after the chase. An overnight incubation, for example, resulted in a strongly stained, yet slightly discontinuous basal lamina ($n = 58$), and a basal lamina with almost no interruptions was obtained 48 h after the laminin/macroglobulin chase ($n = 5$).

Laminin Gamma 1 Expression in the Embryonic Chick Eye

To rule out that the disruption of the retinal basal lamina or its induced regeneration is accompanied with an altered or upregulated expression of laminin in the embryonic chick eye, we performed *in situ* hybridization studies using a cRNA probe to the chick laminin gamma 1 chain. The laminin gamma 1 chain is present in 11 out of 12 currently known laminins and is the best marker for the expression of different laminins that may exist in the eye (Colognato and Yurchenko, 2000). Since chick laminin gamma 1 had not been cloned yet, we obtained peptide sequences for laminin gamma 1 from a prominent 200-kD protein band of isolated chick retina basal laminae by SDS–PAGE and mass spectroscopy. Two of the peptide sequences were identical to sequences from the amino terminus of mouse and human laminin gamma 1. Primers were designed from the two peptides, and a cDNA was amplified by PCR. As expected, the 3.1-kb cDNA probe was from the N-terminal part of chick laminin gamma 1 with 75% nucleotide and 81.5% amino acid identity to human laminin gamma 1. *In situ* hybridization showed that in normal E4 to E7 chick eyes, laminin gamma 1 mRNA was most prominently expressed by cells of the ciliary margin of the eye and, to a lesser extent, by the anterior lens epithelial cells (Figs. 5c and 5d; $n = 8$), consistent with a similar distribution pattern in the early embryonic mouse eye (Sarthy and Fu, 1990; Dong and Chung, 1991). There was no detectable labeling of laminin gamma 1 in the retina at these stages of eye development (Figs. 5c and 5e). In chick eyes 24 h after collagenase treatment (Fig. 5a; $n = 5$) or 24 h after a laminin/macroglobulin chase (Fig. 5b; $n = 5$), the distribution and intensity of laminin gamma 1 labeling was indistinguishable from that of the corresponding contralateral control eyes (Fig. 5c) and indistinguishable from that of eyes of untreated embryos ($n = 5$).

Histological Alteration in the Retinal Neuroepithelium after Basal Lamina Removal and Regeneration

Neuroepithelial cells. The principal cells of early retinal neuroepithelium are the neuroepithelial cells. They are spindle-shaped and span the entire tissue by extending fine processes to both the vitreal and the scleral surface (Fig. 6b). Confocal microscopy views of the vitreal surface of retinal whole mounts showed that all labeled endfeet of the neuroepithelial cells are within 1 μm of the focal plane of the basal lamina (Fig. 6c; $n = 6$). The removal of the retinal basal lamina consistently resulted in the retraction of the vitreal endfeet by approximately 50 μm from the retinal surface (Figs. 6e, 6h, and 6i). The retraction precisely paralleled the disruption of the basal lamina, such that 1 h after collagenase injection, the retinal basal lamina was still present and the neuroepithelial cells maintained their nor-

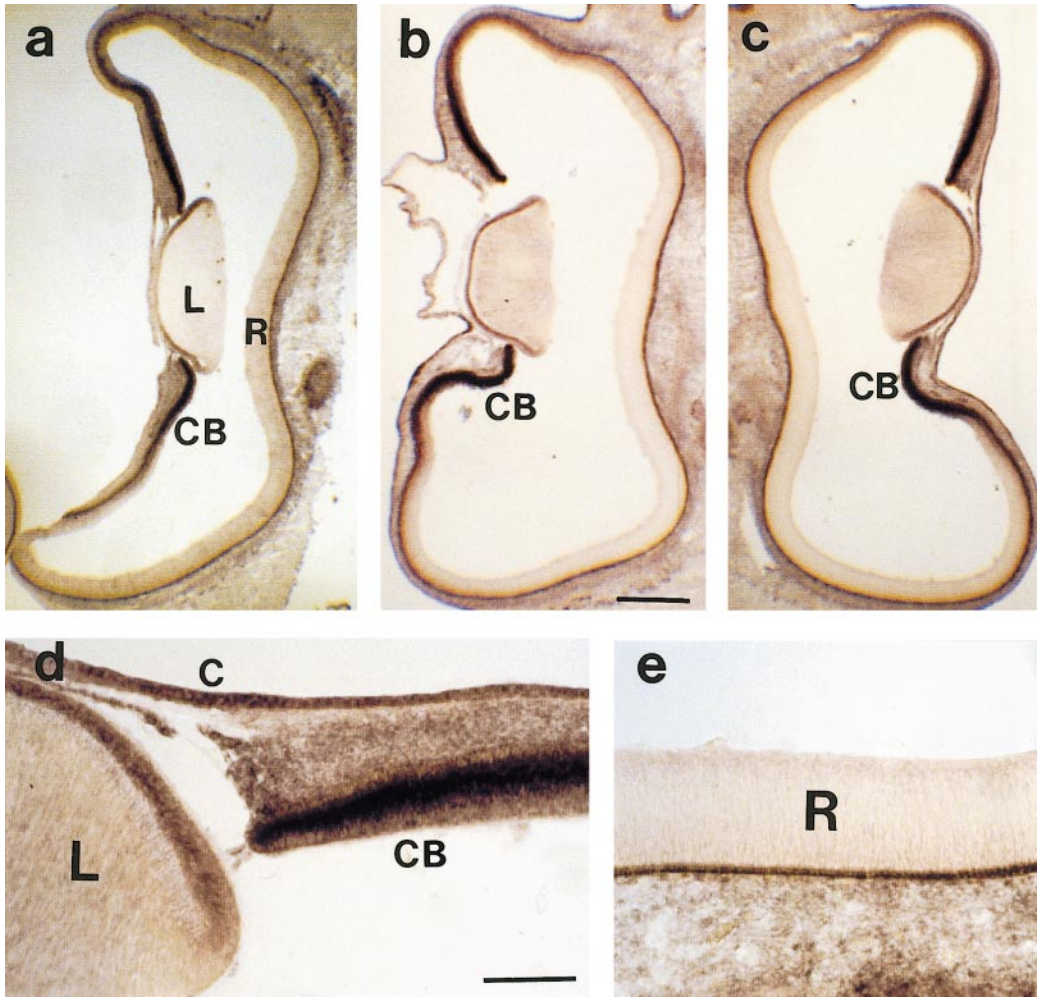

FIG. 5. Light micrographs of cross sections through E5 chick eyes showing the distribution of laminin gamma 1 mRNA in a chick eye 24 h after collagenase injection (a), in an eye that was pulse-chased with collagenase followed by laminin/macroglobulin (b) and in the corresponding control eye (c). In the pulse-chase paradigm, the collagenase had been injected at E4 followed by the laminin-1/macroglobulin chase 10 h later. The embryo was allowed to survive another 14 h. In the experimental and control eyes, the laminin gamma 1 mRNA is present in the epithelium of the lens (L) and, most prominently, in the future ciliary body (CB). The density of staining suggests that the mRNA be expressed in similar quantities in the experimental and the control eyes. The presence of laminin gamma 1 mRNA in the lens and ciliary body and its absence in the retina (R; e) is shown at high power (d, e). C: cornea. Scale bar: a–c, 200 μm; d and e, 100 μm.

mal morphology (not shown; $n = 4$). Four hours after the collagenase injection, the retinal basal lamina was fragmented (Figs. 6d and 6f; $n = 10$), and most of the endfeet of the neuroepithelial had retracted from the vitreal retinal surface by about 50 μm (Fig. 6e; $n = 4$). Whole-mount views of double-stained retinae ($n = 6$) showed that the few neuroepithelial endfeet that were still extended up to the vitreal surface of the retina were always associated with the remaining fragments of the basal lamina (Fig. 6f). Twenty-four hours after the collagenase injection, the basal laminae were no longer detectable, as shown in cross sections (Fig. 6g) and in retinal whole mounts (Fig. 6i). At this time, all neuroepithelial endfeet were retracted from the vitreal surface of the retina by 50 μm, to the same extent as that after the 4 h time point (Figs. 6h and 6i; $n = 6$). Injections of nonspecific proteases, such as dispase ($n = 8$) and chlostripain ($n = 6$) did not disrupt the retinal basal lamina and had no effect on the morphology of the neuroepithelial cells, consistent with earlier results (Halfter, 1998; Halfter *et al.*, 2000). When basal lamina regeneration was induced (Fig. 6k), it reconstituted itself exactly at the level of the retracted neuroepithelial endfeet, 50 μm deep to the vitreal surface of the retina (Figs. 6l and 6m; $n = 15$). Whole-mount views showed that all DiI-labeled neuroepithelial endfeet were located within 1 μm of the focal plane of the regenerated basal lamina (Fig. 6m). The neuroepithelial endfeet remained retracted, even 5 (Fig. 3; $n = 6$) or 10 days after successful basal lamina regeneration ($n = 3$).

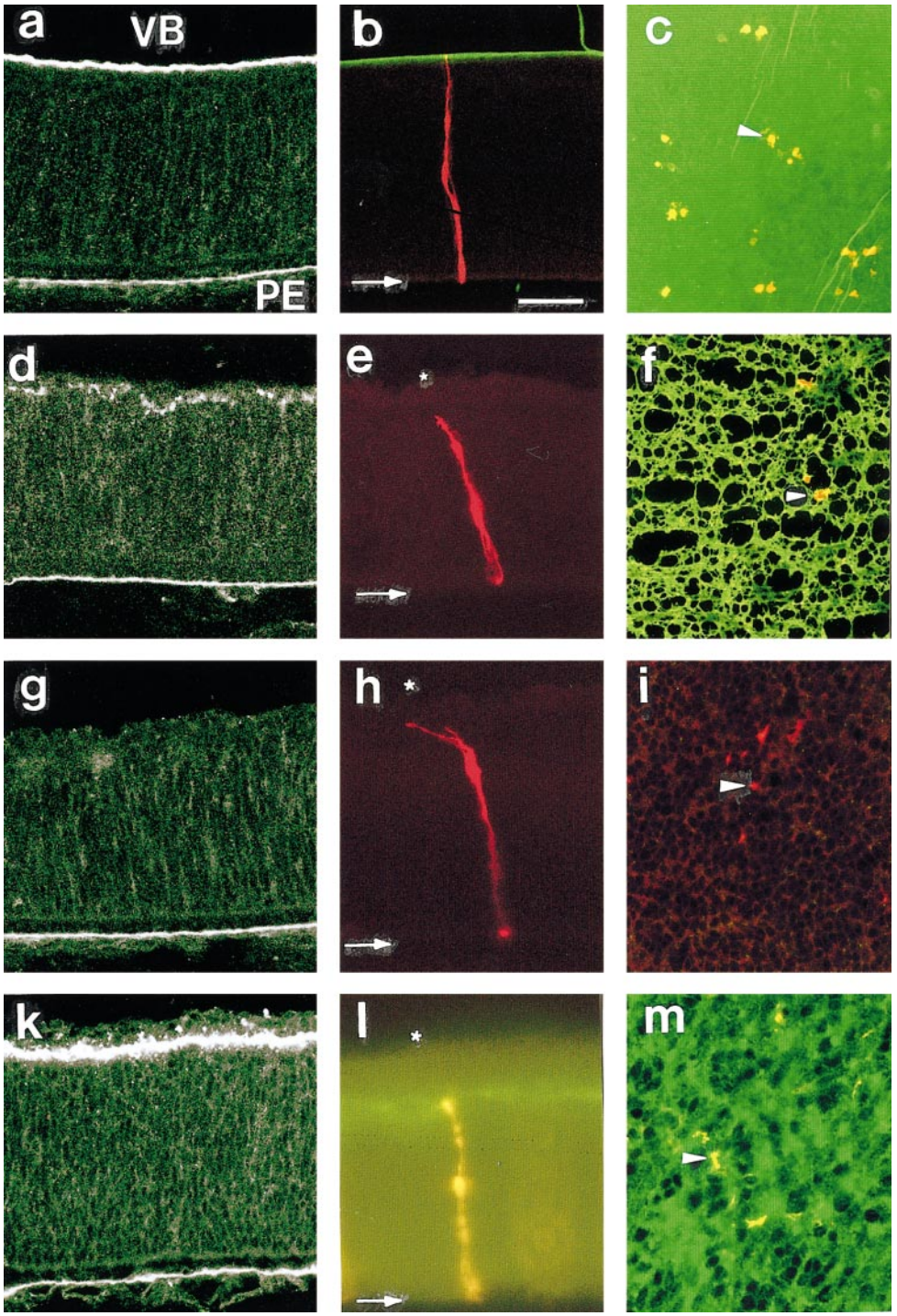

FIG. 6. Change in morphology of individual retinal neuroepithelial cells before (a–c) and after basal lamina disruption (d–i). The status of the retinal basal lamina is shown by staining with a MAb to chick laminin-1 (a, d, g, k; green labeling in b, c, f, l, m). Individual neuroepithelial cells were labeled with DiI (red). In a normal retina with a complete basal lamina (a, b, c), the neuroepithelial cells span the entire width of the retina, as shown in cross sections through the retina (b). Confocal microscopy views of whole mounts (c) confirmed that all DiI-labeled neuroepithelial endfeet (arrowheads) are located within 1 μ of the focal plane of the basal lamina. VB: vitreous body; PE: pigment epithelial basal lamina. Four hours after collagenase injection (d–f), the retinal basal lamina is fragmented (d, f), and most neuroepithelial cells have retracted their vitreal endfeet by approximately 50 μm from the vitreal surface (indicated by white star; e). Whole mounts show the fragmentation of the basal lamina (f), with few of the labeled neuroepithelial endfeet (arrowheads) in the same focal plane. Twenty-four hours after collagenase injection, the retinal basal lamina is no longer detectable (g), and all endfeet are retracted from the vitreal surface of the retina, as shown in cross sections (h) and in whole mounts (i). When the basal lamina was allowed to regenerate (k), it reconstituted itself exactly along the retracted endfeet, as shown in cross section (l) and in whole mounts (m; arrowheads). White arrows indicate the scleral surface of the retina. Bar: 50 μm.

Dystroglycan. A cellular receptor for laminin in the central nervous system is dystroglycan (Henry and Campbell, 1996), a plasma membrane protein that is expressed in the chick and rat retina by neuroepithelial cells (Blank *et al.*, 1997; Moukhles *et al.*, 2000). We localized dystroglycan in normal retina and in retinae at various stages of basal lamina removal and regeneration. As shown in Fig. 7a, dystroglycan in a control retina was clustered at the vitreal surface of the retina ($n = 5$), consistent with earlier data (Blank *et al.*, 1997). When the basal lamina was removed, the dystroglycan clustering at the vitreal surface changed to a diffuse labeling of the entire retinal neuroepithelium (Fig. 7c; $n = 5$). The time course of dystroglycan redistribution followed the time course of basal lamina disruption, such that 1 h after collagenase injection, the basal lamina was still detectable, and dystroglycan was still clustered at the vitreal retinal surface ($n = 4$). Four hours after collagenase injection, both the basal lamina and the dystroglycan clusters had disappeared. Comparing the dystroglycan banding pattern from retinae of collagenase-injected eyes and their contralateral control eyes in Western blots demonstrated that dystroglycan was not degraded by collagenase and its concentration in the experimental and control retinae was similar (Fig. 7c, inset; $n = 2$). During and after basal lamina regeneration, dystroglycan clusters reappeared at the location of the new basal lamina in a time course that followed the reappearance of the basal lamina (Fig. 7e; $n = 10$), that is, dystroglycan clusters reappeared 2 h after the laminin/macroglobulin chase.

Ectopias. During and after basal lamina removal, the vitreal surface of the retina had an even and smooth surface. However, when the basal lamina was allowed to regenerate, distinct cellular ectopias appeared along the vitreal surface of the retina. They were particularly numerous at the border of the regenerated and the *de novo*-formed basal lamina at the retinal periphery and in the central retina next to the optic disc (Figs. 1a and 8). The ectopias were accompanied with two basal laminae, one underneath and one on top of the ectopic cells (Figs. 8a and 8b). The ectopias at the retinal periphery were exclusively of neuroepithelial origin (not shown), but regularly included ganglion cells in the central retina (Fig. 8c).

Ganglion cells. The first postmitotic neurons in the embryonic retina are the ganglion cells. They are generated by E2.5 in the central retina (Kahn, 1973) and establish themselves as a defined cell layer close to the vitreal surface of the retina (Fig. 9). After staining ganglion cell nuclei with the islet I MAb (Austin *et al.*, 1995) and comparing the distribution and number of ganglion cells in experimental and control eyes, we found that the disruption of the basal lamina and its regeneration had an immediate effect on the formation of the ganglion cell layer. One hour after basal lamina removal, the distribution and number of ganglion cells in the retinae of the injected and the control eyes were identical (compare Figs. 9a and 9b; $n = 4$). Four hours after the collagenase injection, the number of ganglion cells in control and experimental eyes were still the same, although the ganglion cells in the experimental retina were slightly more widely dispersed (compare Figs. 9c and 9d; $n = 4$). Twenty-four hours after basal lamina removal, the ganglion cells in the experimental eyes were widely dispersed and their number was increased by the factor of 1.33 ± 1.4 ($n = 7$) relative to the number of ganglion cells in the control eyes (compare Figs. 9e and 9f). When basal lamina regeneration was induced only 1 h after collagenase injection and the embryos allowed to survive for another 24 h, the ganglion cells were also widely scattered in the experimental eyes and their number was increased by a factor of 1.3 ± 0.9 ($n = 9$) compared to that of the controls (compare Figs. 9g and 9h). The ganglion cells were found on top of and underneath the regenerated basal lamina (Fig. 9g).

Injection of laminin and macroglobulin into eyes that had not previously been injected with collagenase had no effect on the distribution of ganglion cells, and their number in the experimental and control eyes, 24 h after the injections, were identical (0.98 ± 0.15; $n = 6$).

Navigation of Optic Axons after Basal Lamina Removal and Regeneration

Staining of the retinae with antibodies to neurofilament showed that 2 days after basal lamina removal the optic fiber layer was wider than that in the control eyes (Figs. 10a and 10b; $n = 20$), consistent with earlier findings (Halfter, 1998). Furthermore, many axons were no longer confined to a defined optic fiber layer but had crisscrossed through the retina, often heading for and reaching the ventricular retinal surface. When basal lamina regeneration was induced 10 h after the collagenase injection and embryos were allowed to survive another 38 h, the axons were prevented from crisscrossing the retina and were confined to a defined, yet wider than normal optic fiber layer (Fig. 10c; $n = 20$).

The removal and the regeneration of basal lamina also had a major effect on the navigation of optic axons at the optic disc, the exit of retinal axons from the retina into the optic nerve. In a normal retina by E6, optic axons from the nasal and the temporal retinal are well separated from each other by the pecten, a segment of connective tissue from the optic stalk that extends into the vitreous and carries the blood vessels into the chick eye (Figs. 11b and 11d; $n = 20$). After basal lamina removal, the pecten does not extend into the vitreous and does not separate nasal and temporal axons, causing the formation of a neuroma at the optic disc (Fig. 11a; $n = 20$). When the basal lamina was allowed to regenerate, the optic pecten did not extend into the vitreous either, and nasal and temporal axons freely interdigitized at the optic disc rather than grew into the optic nerve (Fig. 11c; $n = 20$). Further, although the retinal basal lamina regenerated along the entire length of the retina, it did not regenerate at the optic disc ($n = 20$). Retinal whole mounts stained for neurofilament confirmed the chaos of retinal axons at the optic disc after basal lamina regeneration (Fig. 11e; $n = 10$), contrasting to the well-organized axon convergence at the optic disc of control eyes (Fig. 11f; $n = 10$).

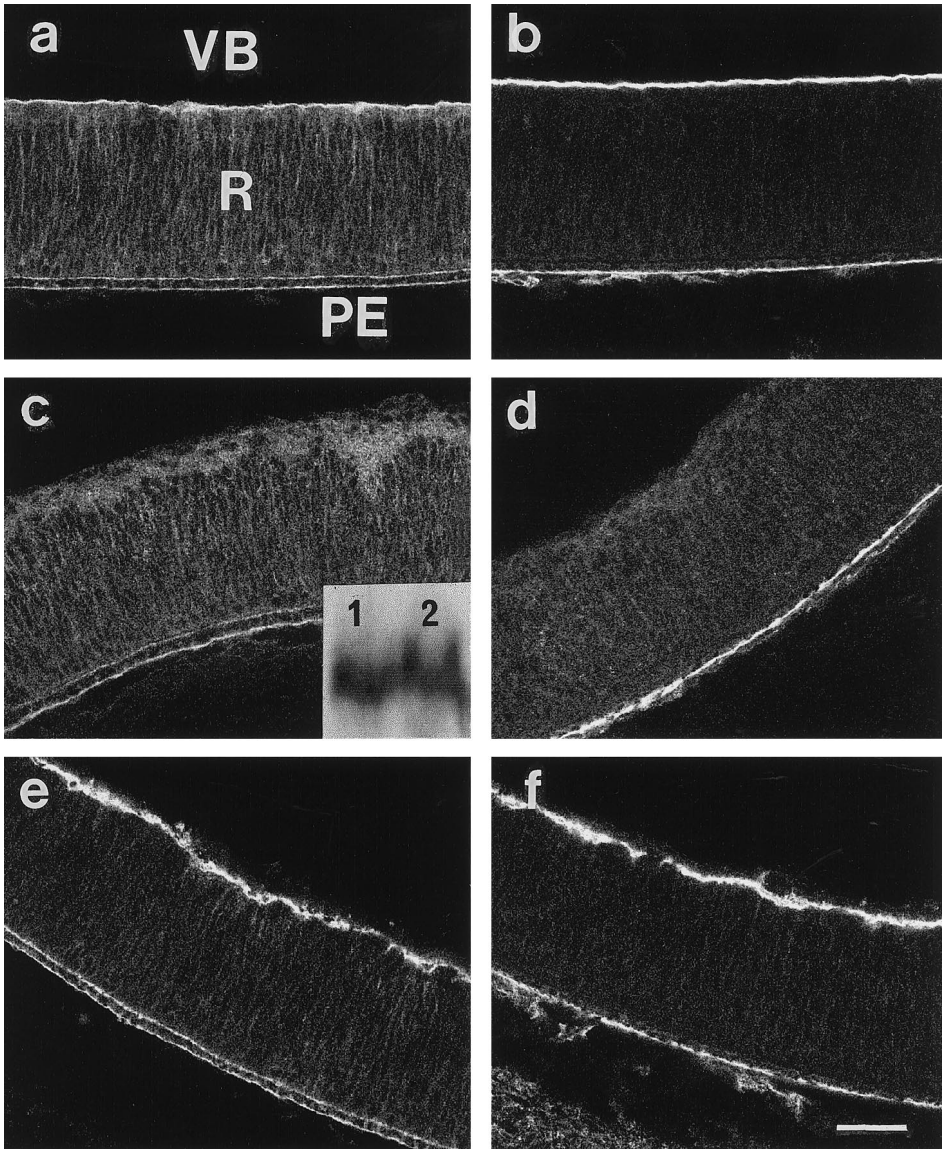

FIG. 7. Fluorescence micrographs of cross sections through the E5 retina showing the location of dystroglycan in a control retina (a), in a retina 24 h after basal lamina disruption (c), and in a retina 24 h after allowing the basal lamina to regenerate (e). Adjacent sections were stained for laminin (b, d, f). In a normal retina (a, b), dystroglycan is concentrated at the endfeet of the neuroepithelial endfeet next to the basal lamina, at the scleral surface, and at the basal surface of the pigment epithelium (PE). Twenty-four hours after basal lamina disruption (c, d), dystroglycan disappeared from the vitreal surface of the retina (R), but remained unchanged at the scleral surface and in the pigment epithelium. Comparing the β-dystroglycan band of retinae from control and collagenase-injected eyes in Western blots (insert in c) showed that the protein was not degraded by collagenase and was present in similar concentrations in the experimental (lane 1) and control retinae (lane 2). Dystroglycan reclustered at the vitreal retinal surface after inducing basal lamina regeneration, whereby the clusters co-localized with the site of basal lamina reconstitution (e, f). The retina shown in e and f was from an embryo 14 h after the laminin–macroglobulin chase. VB: vitreous body. Bar: 50 μm.

DISCUSSION

Initiation of Retinal Basal Lamina Regeneration by Laminin-1

Intraocular injection of collagenase followed by a chase with laminin-1/α2-macroglobulin provides an experimental tool to remove the retinal basal lamina at a specific stage of early eye development and to allow it to reconstitute at any given time point during further incubation. The reconstitution occurs within 4–6 h and leads to a molecularly and morphologically complete basal lamina. The injected α2-macroglobulin in the chase cocktail inactivates the injected

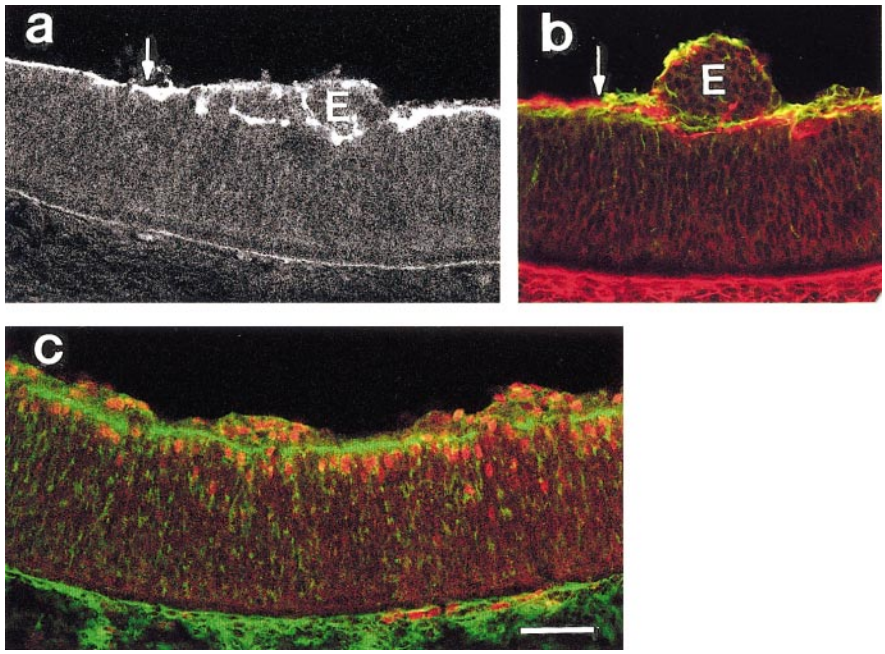

FIG. 8. Fluorescence micrographs of cross sections through E5 chick retinae showing retinal ectopias 14 h after basal lamina regeneration. The sections had been stained with antibodies to laminin-1 alone (a), to laminin (red) and neurofilament (green; b), and to laminin (green) and islet-1 (red); c. Ectopias were usually associated with a doubling of the retinal basal lamina (a, b). They are particularly numerous at the border (arrows) of the regenerated and *de novo*-formed basal lamina (a, b), and they include numerous ganglion cells if they are found in the central part of the retina (c). Bar: 50 μm.

collagenase (Werb *et al.*, 1974) and was found to be dispensable when the regeneration of the basal lamina is induced more than 10 h after the collagenase injection, probably as the activity of the collagenase has waned. The laminin-1 in the chase cocktail serves as the component that is responsible for the initiation of basal lamina reconstitution, whereby laminin-2 or collagen IV cannot substitute for laminin-1. The unique role of laminin-1 in basal lamina reconstitution was confirmed by showing that basal lamina regeneration was blocked by antibodies to laminin-1 but not by antibodies to laminin-2 or nidogen. Given that laminin-1 and laminin-2 differ only by their α chains, it is likely that the binding of the α1 chain to a cellular receptor is a critical step in retinal basal lamina nucleation. Consistent with this view is the finding that the very first step in basal lamina reconstitution is the binding of mouse laminin-1 to the vitreal surface of the retina, followed by the subsequent immobilization of chick nidogen, chick laminin, and the remaining basal lamina components.

The concentration of mouse laminin-1 necessary to induce basal lamina reconstitution is probably higher than the concentration of endogenous chick laminin in the vitreous of the chick eye, and we speculate that a spike of laminin-1 in the vitreous is required to initiate the reclustering of dystroglycan, integrins, and other potential ECM receptors to the endfeet of the neuroepithelial cells, to facilitate the binding of high concentrations of laminin and other members of the basal lamina proteins necessary for basal lamina assembly (Colognato *et al.*, 1999; Lohikangas *et al.*, 2001; Henry *et al.*, 2001). The basal lamina-dependent clustering of dystroglycan at the neuroepithelial endfeet was evident by visualizing its distribution in normal retina, after basal lamina disruption and after its reconstitution. In a normal retina, dystroglycan is concentrated in the plasma membrane of the endfeet of the neuroepithelial cells, next to the retinal basal lamina (Blank *et al.*, 1997). Upon basal lamina removal, the dystroglycan clusters disappeared from the endfeet of the neuroepithelial cells. Western blot analysis showed that the disappearance of dystroglycan clusters was attributed to a redistribution rather than a down-regulation or a proteolytic degradation (Fig. 7). Dystroglycan reclustered to the vitreal surface of the retina within 2 h after a laminin/macroglobulin chase, coinciding temporally and locally with the first traces of mouse and chick laminin immobilization. Dystroglycan was previously implicated as one of the organizers of basal lamina formation (Henry and Campbell, 1999), and the basal lamina-dependent clustering in the retina is consistent with this notion. Other candidate receptors involved in basal lamina formation are the integrins. Previous studies have shown that the integrin β1 chain is prominently expressed on the surface of the neuroepithelial endfeet and disappeared from the endfeet upon basal lamina removal (De Curtis *et al.*, 1991; Cann *et al.*, 1996; Hering *et al.*, 2000), suggesting that clustering of

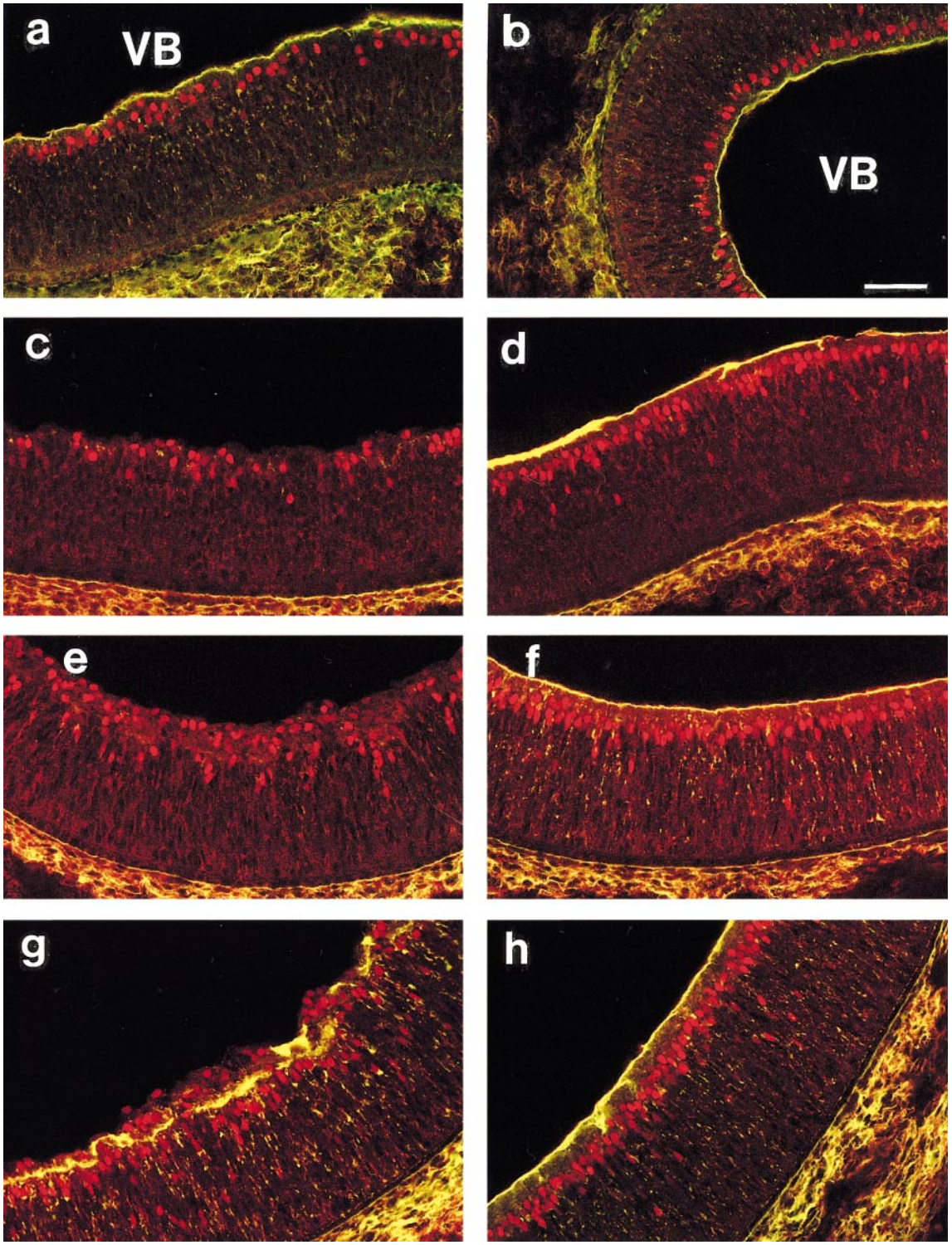

FIG. 9. Fluorescence micrographs of cross section through E4 and E5 chick retinae showing the distribution of ganglion cells 1 h (a, b), 4 h (c, d), 24 h (e, f) after collagenase injection, and 24 h after basal lamina reconstitution (g, h). VB: vitreous body. The sections, comparing injected (a, c, e, g) and their contralateral control eyes (b, d, f, h), were stained with an antibody to islet-1 as marker for ganglion cell nuclei (red) and anti-laminin (yellow). Whereas the distribution and number of ganglion cells in experimental and control retinae are similar at the 1 h (compare a and b) and 4 h (compare c and d) time points, the misalignment and increase of ganglion cells is obvious 24 h after collagenase injection (compare e and f). Dispersion and a greater number of ganglion cells were also found in retinae after the basal lamina had been allowed to regenerate (compare g and h). Bar: 50 μm.

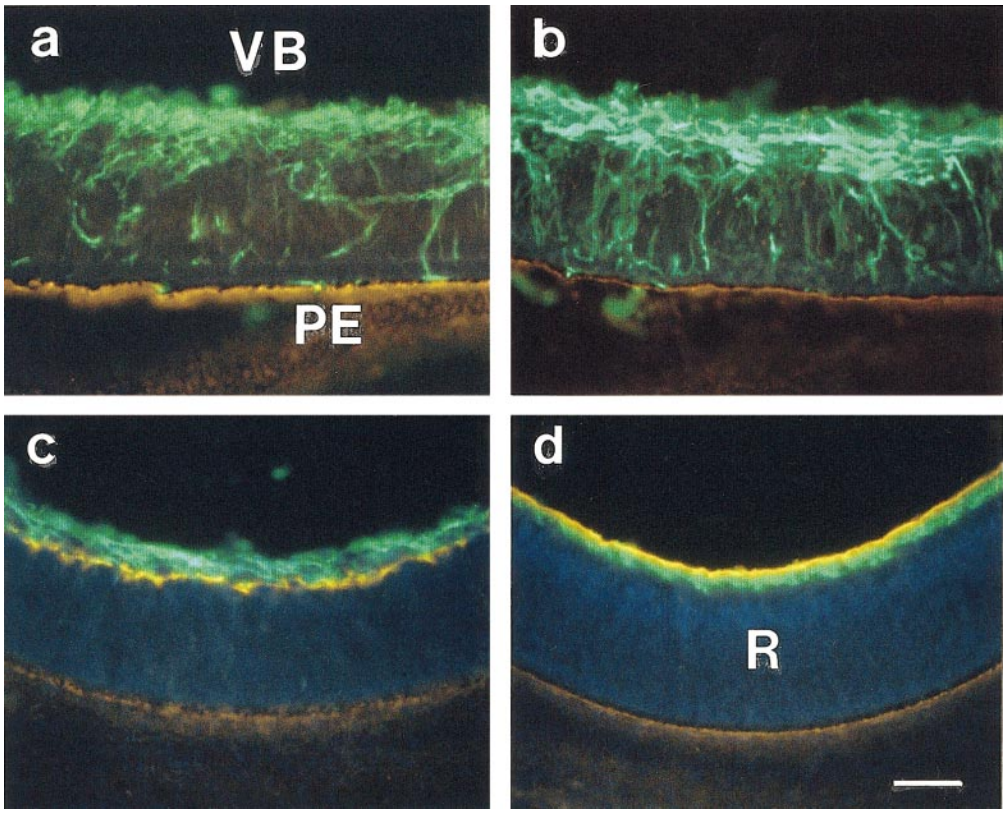

FIG. 10. Cross sections through E7 chick retinae (R) showing the disorganized optic axons 48 h after injection of collagenase (a, b). The retinae had been stained with antibodies to laminin (red) and neurofilament (green). VB: vitreous body; PE: pigment epithelial basal lamina. When the basal lamina had been allowed to regenerate, a wider yet confined optic fiber layer was detected (c) 48 h after the collagenase injection. The contralateral control retina is shown for comparison (d). Bar: 50 μm.

integrins at the endfeet is, like dystroglycan, also dependent on the presence of the retinal basal lamina. In that β1 integrin has also been implicated in basal lamina formation (Sasaki *et al.*, 1998), it is conceivable that dystroglycan and integrins together provide the binding sites necessary for the formation of a complete basal lamina.

Staining for agrin, nidogen, collagen XVIII, collagen IV, and perlecan showed that, whenever a linear laminin-1 staining was present, the other basal lamina proteins were also detectable and the basal lamina was molecularly and morphologically complete, as confirmed by electron microscopy. In addition, we observed a synchrony in the deposition of these proteins that was dependent on the amount of immobilized laminin. For example, in cases of weak laminin-1 labeling, the labeling for all other proteins was also weak. Whereas agrin and nidogen-1 probably bind to the immobilized laminin-1 (Durkin *et al.*, 1988; Mann *et al.*, 1989; Denzer *et al.*, 1995), the recruitment of collagen IV and perlecan to the basal lamina could also be mediated by integrins and dystroglycan.

As shown by *in situ* hybridization, neither the injections of collagenase nor the chase with laminin/macroglobulin led to an altered or visibly elevated expression of the endogenous laminins in the eye. Based on this and previous *in situ* hybridization using probes to collagen IV and perlecan (Halfter *et al.*, 2000), we conclude that the disruption of the retinal basal lamina and its regeneration are not accompanied by an up- or down-regulation of one of the extracellular matrix proteins in the eye.

Function of the Basal Lamina in Retinal Histogenesis and Axonal Navigation

The current experiments demonstrate that the retinal basal lamina is mandatory for retinal histogenesis. One of the functions of the retinal basal lamina is to provide a border to confine axons and cells from invading the vitreous. The presence of multiple ectopias after a temporary absence of the retinal basal lamina is most likely attributable to the fact that the retina has partially lost this confinement. Ectopias, however, became conspicuous only when the basal lamina was partially missing or had regenerated. When the retinal basal lamina was completely dissolved, ectopic cells were found along the entire surface

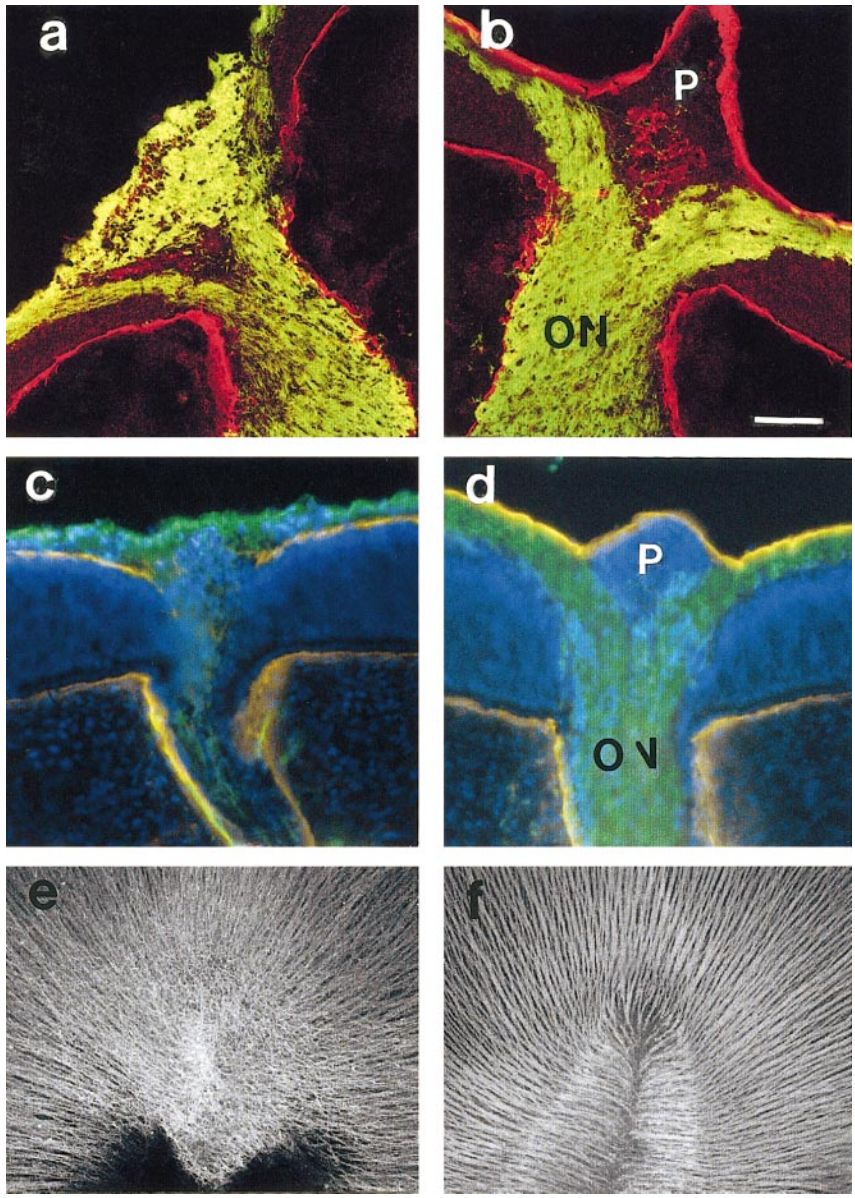

FIG. 11. Cross sections through the optic nerve head of E7 chick embryos 48 h after basal lamina disruption (a) or after basal lamina regeneration (c). The optic nerve heads of the corresponding contralateral control eyes are shown for comparison in (b) and (d). The sections had been stained for laminin (red) and neurofilament (green). Optic axons at the optic disc were disorganized because the pecten (P) does not develop properly in the absence of a complete basal lamina (a). As a result of the absence of pecten, optic axons form a neuroma at the optic disc, and many axons do not enter the optic nerve (ON; a). When the basal lamina is allowed to reconstitute, the pecten does not reemerge, causing the axon to merge at the optic disc as well with fewer axons than normal entering the optic nerve (c). Note that the basal lamina does not regenerate at the optic disc (c). Retinal whole mounts stained for neurofilament show the chaos of axons at the optic disc after basal lamina regeneration (e). The optic disc area of the contralateral control retina is shown in (f) for comparison. Bar: 50 μm.

of the retina and ectopias did not show up as distinctive cell aggregates that stood out from the vitreal retinal surface. Thus, ectopias are indicators of either a localized or a temporarily limited disruption of the basal lamina.

Another function of the retinal basal lamina is to anchor the neuroepithelial cells to the vitreal surface of the retina. As illustrated in Fig. 6, an instant reaction to the loss of the basal lamina is the retraction of the neuroepithelial endfeet from the vitreal surface of the retina. The retraction of the endfeet occurred only when the basal lamina was visibly disintegrated and was not observed after injections of non-specific proteases, such as dispase and chlostripain, both of

which, when injected, did not lead to basal lamina disruption (Halfter, 1998; Halfter *et al.*, 2000). Independent confirmation that the morphology of neuroepithelial cells is dependent on intact basal lamina comes from mouse embryos that have multiple disruptions in the pial basal lamina resulting from a mutation in the laminin gamma 1 chain. In the cortex of these mutant mice, the endfeet in the radial glia cells are retracted in segments where the pial basal lamina is missing and they are normal in segments where the pial basal lamina is intact (W. Halfter, S. Dong, and U. Mayer, unpublished observations). Since the radial extensions of the neuroepithelial cells provide the rail guards along which neuroblasts migrate to their defined layers (Rakic and Caviness, 1995), it is conceivable that any disruptions in the morphology of the neuroepithelial cells cause major histological aberrations that result from an incomplete cell migration. Previous studies confirmed that enzymatic disruption of the pial basal lamina cause an inhibition of neuroblast migration in chick optic tecta (Halfter and Schurer, 1998), whereby cell migration was most dramatically affected at the marginal layer of the tectum where the endfeet had been retracted.

The aberration in the histogenesis in the retina was most obvious in the wide dispersion and the abnormally large number of ganglion cells following a temporary basal lamina loss. The greater number of ganglion cells can be explained by their greater dispersion. Previous studies established that the number of ganglion cells is regulated by the Delta/Notch system (Austin *et al.*, 1995; Henrique *et al.*, 1997), a ligand/receptor combination that requires mutual cell contact. Ganglion cells that are widely separated no longer inhibit each other's differentiation, resulting in a larger than normal ganglion cell population. The disorganized ganglion cell layer may also explain the chaotic crisscrossing of axons throughout the retina. However, aberrant growth of optic axons in the retina no longer occurred when the basal lamina was allowed to regenerate. Ganglion cells were still widely dispersed in these retinae, yet axons were confined to a defined optic fiber layer. The confinement of optic axons after basal lamina regeneration could mean that basal laminae prevent axons from straying away into deeper layers of the retina. However, another explanation is that the presence of a basal lamina leads to a polarization of the neuroepithelial cells that concentrates neurite outgrowth-promoting proteins to the vitreal endfeet and provides a preferred environment for growing axons. As shown by the clustered distribution of dystroglycan at the vitreal endfeet, the neuroepithelial cells are polarized with respect to at least some membrane proteins, consistent with earlier observations (Stier and Schlosshauer, 1995).

Does Basal Lamina Regeneration Reverse the Detrimental Effect of Basal Lamina Removal on Retinal Histogenesis and Axonal Outgrowth?

Some of the effects of basal lamina removal on axonal misguidance were rectified after allowing the basal lamina to reconstitute. For example, the crisscrossing of axons throughout the retina did not occur when the basal lamina was allowed to reconstitute. However, the aberrations in the formation of a defined ganglion cell layer and the axonal guidance problems at the optic disc were not corrected. The reason for the irreversibility of these aberrations is that the endfeet of the neuroepithelial cells, once they have retracted, do not regain contact to the vitreal retinal surface and, consequently, retinal histogenesis and navigation of optic axons into the optic nerve that require intact neuroepithelial cells cannot proceed properly. Surprisingly, disruptions of the retinal basal lamina of only a few hours were already sufficient to completely disorganize the histogenesis of the retina, showing an absolute requirement for the presence of the retinal and pial basal lamina throughout early histogenesis.

The phenotype of the chick retinae after basal lamina disruption and regeneration resemble the phenotype of retina and cortices in many of the mouse and human mutations with defects in basal laminae. Retinal or cortical ectopias were detected in mice with targeted mutations in perlecan (Costell *et al.*, 1999), α3, and α6 integrins (Georges-Labouesse *et al.*, 1998), and in mice with a mutation in the nidogen-binding site of laminin gamma 1 (W. Halfter, S. Dong, and U. Mayer, manuscript in preparation). Human mutations, summarized as lissencephaly II (Gleeson and Walsh, 2000), such as Fukuyama muscular dystrophy (Nakano *et al.*, 1996), muscle–eye–brain syndrome (Hiltia *et al.*, 1997), and Walker–Warburg syndrome (Williams *et al.*, 1984) also show disruptions in the pial and retinal basal lamina that are associated with cortical and retinal ectopias and dysplasias. In all these mutations, the stability of the basal laminae is compromised. We think that our chick model provides an experimental system to study the CNS phenotype of these diseases and allows obtaining further information on the function of basal laminae in the development of the central nervous system.

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