Neuronal Receptors Mediating Responses to Antibody-Activated Laminin-1

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Embryonic retinal neurons lose the ability to extend neurites on laminin-1 (LN-1) with increasing developmental age yet still do so on other laminin isoforms. However, after treatment of LN-1 with antibodies to "short-arm" regions or removal of the short arms proteolytically, LN-1 supports attachment and extension of neurites even by late embryonic retinal neurons. We have mapped a domain for antibody-mediated "activation" of LN-1 to the N-terminal end of the α1 chain. Furthermore, we show that the primary receptors used in the retinal neuron response to "activated" LN-1 are integrins $\alpha 3\beta 1$ and $\alpha 6\beta 1$; these are the same receptors used by these neurons for outgrowth on other LN isoforms. Interestingly, $\alpha 3\beta 1$ is preferentially involved in neurite outgrowth, whereas $\alpha 6\beta 1$ preferentially mediates attachment and spreading. However, in cultures from $\alpha 3$ integrin-deficient mice, $\alpha 6\beta 1$ mediates retinal ganglion cell neurite out-

growth and compensates for the absence of $\alpha 3\beta 1$. Finally, we show that key features of the retinal neuron response to LN-1 also characterize neurons of the hippocampus, thalamus, and cerebral cortex; these include poor response to untreated LN-1, extensive neurite outgrowth on antibody-activated LN-1 or on fragment E8, and dependence of this response on integrin $\alpha 6\beta 1$ and at least one other long arm-binding $\beta 1$ integrin. These data suggest that regulation of LN-1 function via the process of activation could have important consequences for axonal regeneration. Curiously, the data also imply that the mechanism of laminin activation involves enhanced function at sites that cannot be considered cryptic.

Key words: integrin; retina; neurite outgrowth; extracellular matrix; laminin; antibody activation; knock-out; VLA-3; VLA-6; retinal ganglion cell

Signals from the extracellular matrix (ECM) control numerous aspects of cell behavior, including proliferation, survival, gene expression, and morphological differentiation. Regulation of cell–matrix interactions can occur at the level of how cells recognize and respond to ECM molecules, as well as at the level of how ECM molecules are organized in space and presented to cells. Studies of retinal neurons have provided examples of both types of regulation; during embryonic development, retinal neurons lose the ability to attach and extend neurites in response to substrata containing the ECM protein laminin-1 (LN-1), a change that is thought to reflect decreases in the number and/or activation state of integrin receptors (Cohen et al., 1986, 1987; Hall et al., 1987; de Curtis et al., 1991; de Curtis and Reichardt, 1993). Despite this change, however, any of several manipulations of the LN-1 molecule, including the binding of antibodies to short-arm domains or proteolytic cleavages that isolate the long-arm domain, restore the ability of LN-1 to promote neurite

outgrowth by late embryonic retinal neurons, a phenomenon we refer to as laminin "activation" (Calof et al., 1994).

Because LNs are thought to play roles both in axonal development and in regeneration, it is important to understand the mechanisms of laminin activation and the possible physiological significance of such activation. In the present study, we sought to address the following questions. What are the minimal manipulations of the LN-1 molecule required for its activation? What receptors do late embryonic retinal neurons use to respond to activated LN-1, and how do these receptors compare with the receptors that other types of neurons use to respond to native LN-1? Finally, given that induction of LN expression is a common injury response in many parts of the central and peripheral nervous systems (Zak et al., 1987; Brodkey et al., 1993; Frisen, 1997; Fu and Gordon, 1997), we sought to determine whether activation of LN-1 increases its ability to promote neurite outgrowth from CNS neurons other than those of the retina.

MATERIALS AND METHODS

Anti-integrin antibodies. Function-blocking monoclonal hamster anti-rat α1 [clone Ha31/8 (Mendrick et al., 1995)], α2 [clone Ha1/29 (Mendrick and Kelly, 1993)], and β1 [clone Ha2/5 (Mendrick and Kelly, 1993)] integrins were obtained from PharMingen (San Diego, CA) and were used in cell culture experiments at a final concentration of 10 μg/ml. The mouse anti-rat α1 integrin antibody 3A3 (Turner et al., 1989) was a generous gift from Dr. Sal Carbonetto (Montreal, Canada) and was provided as an ascites fluid. Results with 3A3 were identical to those obtained with Ha31/8. The function-blocking rabbit anti-rodent β1 integrin antiserum designated "lenny" was a generous gift of Dr. Clayton Buck (The Wistar Institute, Philadelphia, PA) and was typically used at a 1:200 dilution. The f unction-blocking mouse anti-chicken α2 integrin

monoclonal antibody mep-17 (McNagny et al., 1992; Bradshaw et al., 1995) was the generous gift of Dr. Kelly McNagny (Heidelberg, Germany) and Dr. Amy Bradshaw (University of California at Santa Barbara). A rabbit polyclonal antiserum directed against the cytoplasmic domain of the α3 integrin subunit was the generous gift of Drs. Mike DiPersio and Richard Hynes (Massachusetts Institute of Technology, Cambridge, MA). Hybridoma cells secreting Ralph 3.1, a function-blocking mouse anti-rat α3 integrin antibody (DeFreitas et al., 1995), were the generous gift of Dr. Louis Reichardt (University of California at San Francisco) and were grown in DMEM containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM). The Ralph 3.1 antibody was isolated from spent culture supernatants by ammonium sulfate precipitation (50%) followed by passage over anti-mouse IgG-agarose (Sigma, St. Louis, MO). Antibody was eluted with 100 mM glycine, pH 2.5, neutralized with 1 M Tris, pH 9.0, concentrated by centrifugation in a Centricon 10 (Millipore, Bedford, MA), dialyzed into F12 culture media, sterile filtered, and used at a final concentration of 50 μg/ml. The function-blocking rat monoclonal anti-mouse α6 integrin antibody GoH3 was obtained from AMAC (Westbrook, ME) and was used at a concentration of 2 μg/ml. All antibody reagents were tested for toxic effects using either cultures of retinal neurons derived from noncross-reacting species, cultures of primary chick sensory neurons, or PC12 pheochromocytoma cells grown on various substrata. Normal mouse IgG, rabbit IgG, or nonimmune rabbit sera were also typically included as controls at the same concentrations.

Anti-laminin antibodies. Antibodies to laminin fragments E1′, P1′, recombinant-α1(VI–IVb), recombinant-α1(VI), E8, and E4 were prepared and used as described (Sung et al., 1993; Calof et al., 1994; Colognato-Pyke et al., 1995). Antibodies were applied at a concentration of 10 μg/ml; anti-E4 was an exception and was used as a whole serum diluted 1:100. Nonspecific rabbit IgG or nonimmune rabbit sera were included as controls at the same concentrations. For cross-absorption, antibodies were diluted to their final working concentration in HBSS containing 0.2% BSA and the indicated laminin fragment at 50 μg/ml and were incubated overnight at 4°C before their use.

Preparation of substrata. Murine LN-1 was purified from the Engelbreth–Holm–Swarm tumor as described (Calof et al., 1994). Human merosin (a mixture of LN-2 and LN-4) was purchased from Life Technologies (Gaithersburg, MD). LN-1 fragments E8 and E1′ were prepared as described (Sung et al., 1993; Colognato-Pyke et al., 1995). Proteins were diluted into Ca^{2+}- and Mg^{2+}-free HBSS to a final concentration of 40 μg/ml for LN-1 and LN-2/4 or 20 μg/ml for E8 and E1′ and were applied to 96-well tissue culture plates (#3596; Costar, Cambridge, MA). Typically, proteins were allowed to adsorb overnight at 4°C. Rat LN-5 (Baker et al., 1996) was adsorbed directly onto tissue culture plastic from culture supernatants conditioned by the rat bladder cell line 804G, which were the generous gift of Mark Fitchmun (Desmos, San Diego, CA). After adsorption, substrata were washed a minimum of three times and blocked with HBSS containing 0.2% heat-inactivated BSA (#7030; Sigma). When used, antibodies to substrate-absorbed molecules (see below) were applied in this blocking solution for a minimum of 1 hr at 37°C before the plating of cells.

Cell culture. Cultures of retinal neurons were derived from embryos of timed pregnant Sprague Dawley rats (Charles River Laboratories, Wilmington, MA; Bantin-Kingman, Freemont, CA; or Taconic Farms), CD-1 mice, or chick embryos (Spafas Farms) as described (Calof et al., 1994). Typically, rats at embryonic day 18, mice at embryonic day 16, and chicks at embryonic day 7 or 11 were used. Briefly, the neuroretina was dissected free of surrounding tissues, minced with sharpened forceps, trypsinized briefly, and triturated through a fire-polished Pasteur pipette to obtain a suspension of single cells and/or small clusters of cells. Retinal neurons were plated in DMEM/F12 (1:1) containing N2 supplements (Bottenstein et al., 1979) and 0.5% ultrapure crystalline BSA (ICN Biochemicals, Costa Mesa, CA) on substrata prepared as described above and were cultured overnight at 37°C in a humidified atmosphere containing 5% CO_2. For some experiments, retinal tissue was cut into small pieces with sharpened forceps and placed into culture as explants. Cultures were also established from embryonic day 18 rat cortex and hippocampus as well as embryonic day 14 and 15 mouse cortex and thalamus. These cultures were grown in DMEM supplemented with 0.2% BSA, pen/strep, and B27 (Life Technologies). Cultures were fixed by underlay with warm PBS containing 10% formalin and 5% sucrose and were observed on a Zeiss Axiovert microscope equipped for phase contrast and 35 mm photography. Typically, cultures were scored for the presence of cells or clumps of cells with neurites greater than two cell

body diameters in length. A minimum of 100 cells per well were counted from duplicate wells for each condition scored. In some instances, measurements of neurite length were made with Image1/AT (Universal Imaging Corporation, West Chester, PA) using a Dage-MTI CCD camera housed on a Zeiss Axiovert microscope.

Cell surface biotinylation and immunoprecipitation. After dissociation, cells were allowed to recover from trypsinization overnight in culture media on Petri plastic at a density of 5×10^6 cells/ml. Cells were collected by centrifugation, washed into HBSS containing 1 mM biotin-LC-NHS (Pierce, Rockford, IL), and incubated with gentle rocking at 4°C for 1 hr. The reaction was terminated by the addition of 5 ml of Leibovitz L15 culture media. Cells were then spun through a cushion of L15 containing 4% BSA and lysed with 2 ml of lysis buffer containing 100 mM β-octyl-glucoside, 150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 1 mM $MgCl_2$, 100 μM PMSF, 10 μg/ml N-ethylmaleimide, and 1 μg/ml pepstatin A. This material was precleared by incubation for 15 min at 4°C with 25 μl each of a slurry of 50 mg/ml protein A-Sepharose (Sigma) and 50 mg/ml anti-mouse IgG-agarose, followed by centrifugation. Primary antibodies (5 μg of purified antibody or 2 μl of antiserum) were added to aliquots of this supernatant and incubated overnight at 4°C. Immune complexes were isolated by incubation with 50 μl of either anti-mouse IgG-agarose or protein A-Sepharose for 2 hr at 4°C followed by centrifugation. Beads were washed once with lysis buffer containing 750 mM NaCl and again with lysis buffer. Beads were eluted with 95°C double-strength SDS sample buffer, separated by SDS-PAGE under nonreducing conditions, and transferred to Immobilon P using a semidry blotter (Owl Scientific). Filters were blocked by incubation in TBS containing 0.2% Tween-20, 2% BSA, and 3% goat serum and then were incubated with avidin–HRP (ABC; Vector Laboratories, Burlingame, CA). Biotinylated proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Immunohistochemistry. Immunohistochemistry was performed as described (Ivins et al., 1997) on horizontal sections of embryonic day 18–19 rat embryos and embryonic day 16–17 mouse embryos. Cryostat sections were cut at 20 μm from fresh frozen tissue and post-fixed either with acetone at −20°C for 10 min or with 4% paraformaldehyde in PBS for 10 min at room temperature. In some cases, tissue was fixed overnight in PBS containing 4% paraformaldehyde at 4°C and cryoprotected in PBS containing 20% sucrose before cryosectioning. Sections were blocked for 30 min in TBST (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.2% Tween-20) containing 3% normal goat serum and 2% BSA and then were incubated overnight at room temperature in a humidified chamber in primary antibody diluted in blocking solution. Sections were washed five times with TBST and then incubated in Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) also diluted in block for 1–2 hr at room temperature. Sections were washed five times with TBST and coverslipped with Aquamount (Biomeda) containing ProLong anti-fade reagent (Molecular Probes, Eugene, OR). Sections were observed and photographed on a Zeiss Axiophot photomicroscope equipped for bright field, differential interference contrast, and epifluorescence.

α3β1 integrin "knock-out" mice. Mice harboring a targeted deletion of the α3 integrin gene (Kreidberg et al., 1996) were maintained as heterozygotes through outcrosses with CD-1 mice. Mice were genotyped by PCR performed on DNA isolated from tail biopsies; a PCR protocol generously provided by Dr. H. Gardner (Scripps Research Institute, La Jolla, CA) was used as described (DiPersio et al., 1997). For tissue culture experiments, embryos were harvested from timed pregnancies from heterozygote crosses, and tissue from each embryo was cultured separately. Tissue was also collected from each embryo for DNA isolation and subsequent genotyping. Because the time required to generate dissociated cell suspensions from the retinas of many embryos in parallel was too long to ensure adequate neuronal viability, these experiments were performed with retinal explants rather than with dissociated cells.

RESULTS

A domain for antibody activation maps to the N terminal of the α1 chain of LN-1

Retinal neurons lose the ability to attach and extend neurites on LN-1 substrata with increasing developmental age (Cohen et al., 1986), but decorating substratum-bound LN-1 with antibodies to its short-arm domains allows even late embryonic retinal neurons to extend neurites (Calof et al., 1994). Because neurite outgrowth on activated LN-1 is blocked by antibodies to long-arm domains
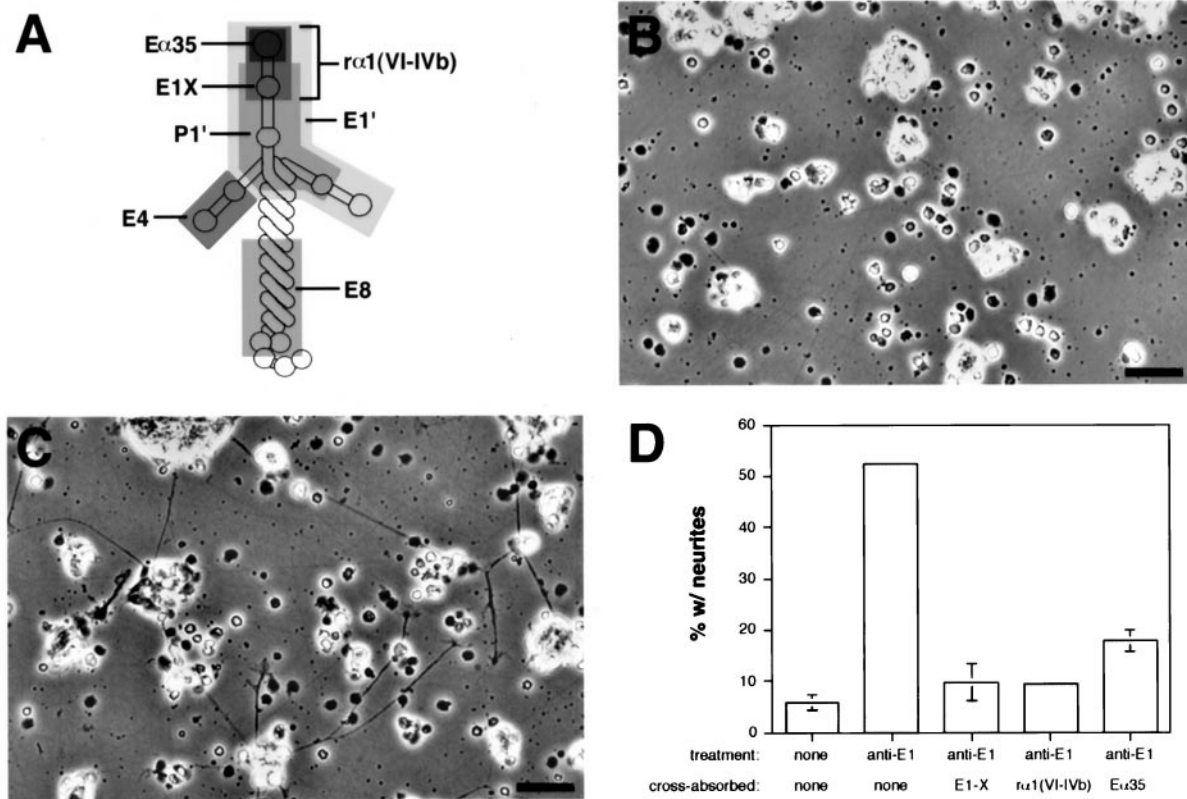
Figure 1. Mapping a domain for antibody activation of LN-1. *A*, The domain structure of LN-1 and the domain-specific antibodies used in this study are shown. *B*, *C*, Dissociated retinal neurons from embryonic day 18 rat retinas were grown on LN-1 treated with anti-E4 (*B*) or with anti-VI–IVb (*C*) antibodies. *D*, Retinal neurons were grown either on untreated LN-1 substrata or on LN-1 that had been decorated with an anti-E1′ antiserum. The anti-E1′ antiserum was further cross-absorbed with LN-1 short-arm fragments VI–IVb, E1X, or Eα35 as indicated. After 18 hr, cultures were fixed and scored for the percentage of cells or clumps of cells with neurites. Scale bars: *B*, *C*, 50 μm.

and because isolated long-arm fragments of LN-1 (fragments E8, C8-9) have the same effects on retinal neurons as does LN-1 that has been antibody-activated, it has been argued that activation of LN-1 is the result of blockade or removal of some type of inhibitory activity that resides in the short-arm domains (Calof et al., 1994).

To date, the antibodies that have been shown to activate LN-1 have been directed against either the E1′ or the slightly smaller P1′ fragments of LN (Fig. 1*A*). Both of these fragments contain parts of all three LN-1 chains ($\alpha1$, $\beta1$, and $\gamma1$). We suspected that the site responsible for antibody activation of LN-1 might reside on the $\alpha1$ or possibly the $\beta1$ chain because late embryonic retinal neurons respond to preparations of merosin, a mixture of LN isoforms $\alpha2\beta1\gamma1$ (LN-2) and $\alpha2\beta2\gamma1$ (LN-4), with vigorous neurite outgrowth (Calof et al., 1994). The simplest explanation for this difference between merosin and LN-1 is that the critical site for activation of LN-1 resides on a chain that is absent in merosin.

To test this hypothesis, we treated substratum-bound LN-1 with affinity-purified polyclonal antibodies specific for an N-terminal 75 kDa fragment of the $\beta1$ chain (fragment E4) or with antibodies to either of two overlapping domains near the N-terminal end of the $\alpha1$ chain. As shown in Figure 1, antibodies against fragment E4 failed to activate LN-1 (Fig. 1*B*), but antibodies against recombinant $\alpha1$ domains VI–IVb [r$\alpha1$(VI–IVb)] rendered LN-1 substrata as effective in promoting late embryonic retinal neurite outgrowth as did anti-E1′ or anti-P1′ antibodies (Fig. 1*C*). Domains VI–IVb represent the N-terminal 120 kDa

portion of the $\alpha1$ chain and are entirely contained within the E1′ fragment and overlap significantly with the P1′ fragment. An antiserum specific for the isolated $\alpha1$ domain VI (Colognato-Pyke et al., 1995) had some activating ability, but it was very weak at all concentrations tested (up to 20 μg/ml; data not shown). As with LN-1 that has been activated with anti-E1′ or anti-P1′ antibodies, the effects of anti-$\alpha1$(VI–IVb)-treated LN-1 on retinal neurons could be blocked by antibodies to the LN-1 long-arm domain E8 (Calof et al., 1994) (data not shown).

To test whether $\alpha1$ chain sites that are sufficient for antibody activation are also necessary for antibody activation, we cross-absorbed an activating anti-E1′ antiserum with preparations of either recombinant $\alpha1$(VI–IVb), fragment E1X ($\alpha1$ domain IVb), or fragment Eα35 ($\alpha1$ domain VI). As shown in Figure 1*D*, each of these fragments strongly inhibited activation of LN-1 by the anti-E1′ antibodies. Together, these data suggest that regions both necessary and sufficient for activation of LN-1 reside on the short arm of the $\alpha1$ chain of LN-1, but these regions may span more than a single domain.

Identification of candidate receptors for retinal neurite outgrowth on activated LN-1

Integrin receptors of the $\beta1$ family are required for the responses of early embryonic retinal neurons to LNs (Hall et al., 1987; Cohen and Johnson, 1991; Neugebauer and Reichardt, 1991; Calof et al., 1994), and we have reported previously that the response of late embryonic retinal neurons to activated LN-1 is
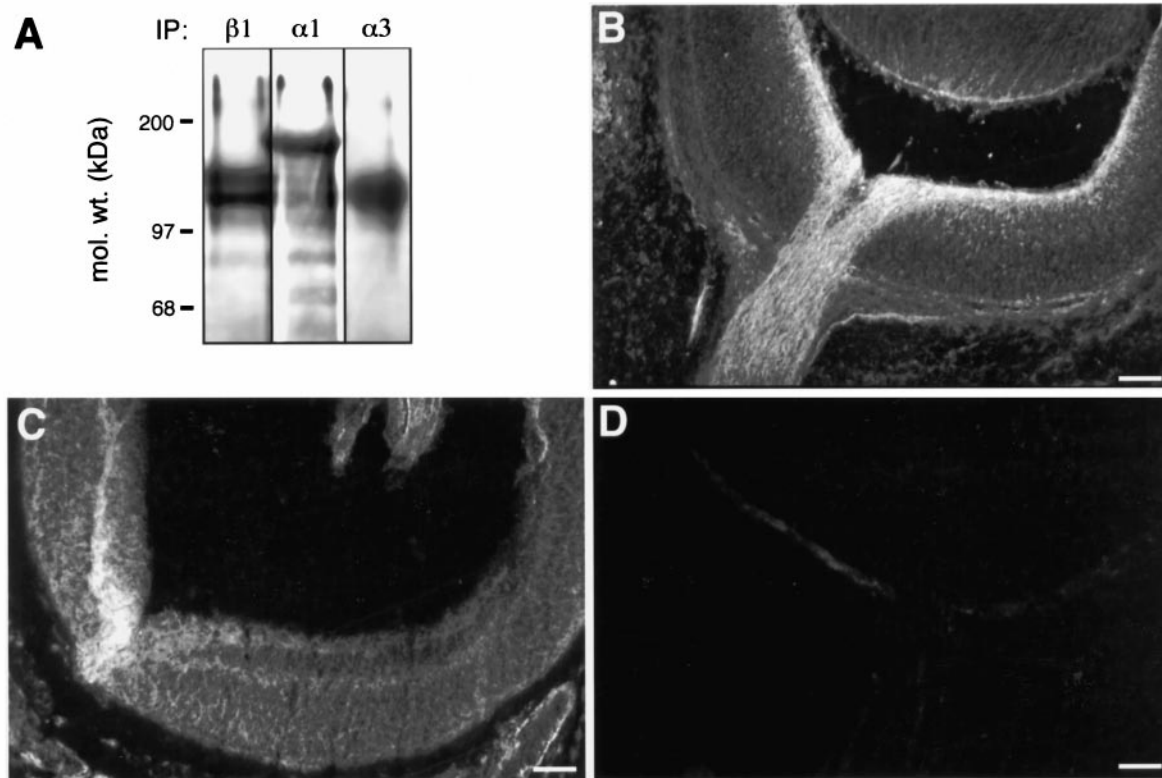
Figure 2. Integrin expression in the rodent retina. *A*, Immunoprecipitations from cell surface-biotinylated retinal neurons performed using the rabbit anti-β1 antibody lenny, the anti-α1 mAb 3A3, and a rabbit antiserum raised against the α3A cytoplasmic domain. Immunoprecipitates were analyzed as described to confirm the cell surface expression of β1 (110 kDa and associated α integrin chains of 130–150 kDa), α1 (185 kDa), and α3A integrin (130 kDa) subunits. *B*, Immunofluorescent localization of α3β1 in a cryosection of an embryonic day 18 rat retina stained with conditioned medium from the Ralph 3.1 hybridoma. Strongest expression is seen throughout the retinal ganglion cell layer and within the optic nerve, although diffuse staining is evident throughout the neuroretina. *C*, Immunofluorescent localization of α6β1 in a cryosection of an equivalent stage (embryonic day 16) mouse retina stained with the GoH3 antibody (5 μg/ml). α6β1 immunoreactivity is present throughout all retinal layers. *D*, Cryosection of embryonic day 18 rat retina stained with nonconditioned medium. The exposure is matched for that shown in *B*. Scale bars: *B–D*, 100 μm.

also β1 integrin-dependent (Calof et al., 1994). The β1 integrins that are known to be able to bind LN-1 include α1β1 and α2β1, which interact near the N terminal of the α chains of both LN-1 and LN-2 (Colognato-Pyke et al., 1995; Colognato et al., 1997), and α3β1, α6β1, and α7β1, which interact at sites on the LN-1 long arm (Hall et al., 1990; Sonnenberg et al., 1990; Tomaselli et al., 1990; Kramer et al., 1991; Gehlsen et al., 1992; Pattaramalai et al., 1996). Three of these integrins have been reported to be expressed in the neural retina during at least some stages of development: α1β1 (Duband et al., 1992), α2β1 (Bradshaw et al., 1995; Cann et al., 1996), and α6β1 (de Curtis et al., 1991; Cann et al., 1996). Expression of α3β1 and α7β1 in the retina has apparently not been studied.

To identify candidate integrin receptors mediating the response of late embryonic retinal neurons to activated LN-1, we performed immunoprecipitations from β-octyl-glucoside extracts of cell surface-biotinylated retinal neurons as well as immunohistochemical localization using α integrin-specific antibodies. The results are shown in Figure 2. Immunoprecipitations with the β1-specific antiserum lenny yielded two major bands with M_r of ~110 and 130 kDa when analyzed under nonreducing conditions by SDS-PAGE and Western blotting; these bands presumably correspond to the β1 chain and one or more associated α chains, respectively (Fig. 2*A*). Immunoprecipitation with the α1-specific monoclonal antibody (mAb) 3A3 (Turner et al., 1989) yielded a

single band with M_r of ~185 kDa (Fig. 2*A*) consistent with the α1 integrin subunit. Immunoprecipitation with an antiserum directed against the cytoplasmic domain of the α3A integrin subunit yielded a single predominant band with M_r of 130 kDa (Fig. 2*A*). Immunoprecipitation with an anti-α7 antibody failed to produce any bands, and *in situ* hybridization, using a partial α7 cDNA derived by reverse transcription-PCR from skeletal muscle RNA, also failed to provide any evidence of expression of this integrin in the retina (data not shown).

Immunoprecipitations with the anti-mouse α6-specific mAb GoH3 also failed to produce any bands (data not shown), but this most likely reflects the known poor ability of this monoclonal antibody to perform in immunoprecipitations. Integrin α6 has been shown previously to be present in the chick neuroretina (de Curtis et al., 1991; de Curtis and Reichardt, 1993). Furthermore, we confirmed the presence of both α3β1 and α6β1 in the retina immunohistochemically. Using the function-blocking anti-rat α3 mAb Ralph 3.1 (DeFreitas et al., 1995) to stain horizontal sections through embryonic day 18 rat retina, we found immunoreactivity in all retinal layers, but with the highest levels of expression associated with retinal ganglion cells and their axons (Fig. 2*B*). An identical staining pattern was observed with an antibody specific for the cytoplasmic domain of the A isoform of the α3 integrin (data not shown). In contrast to this pattern, the anti-
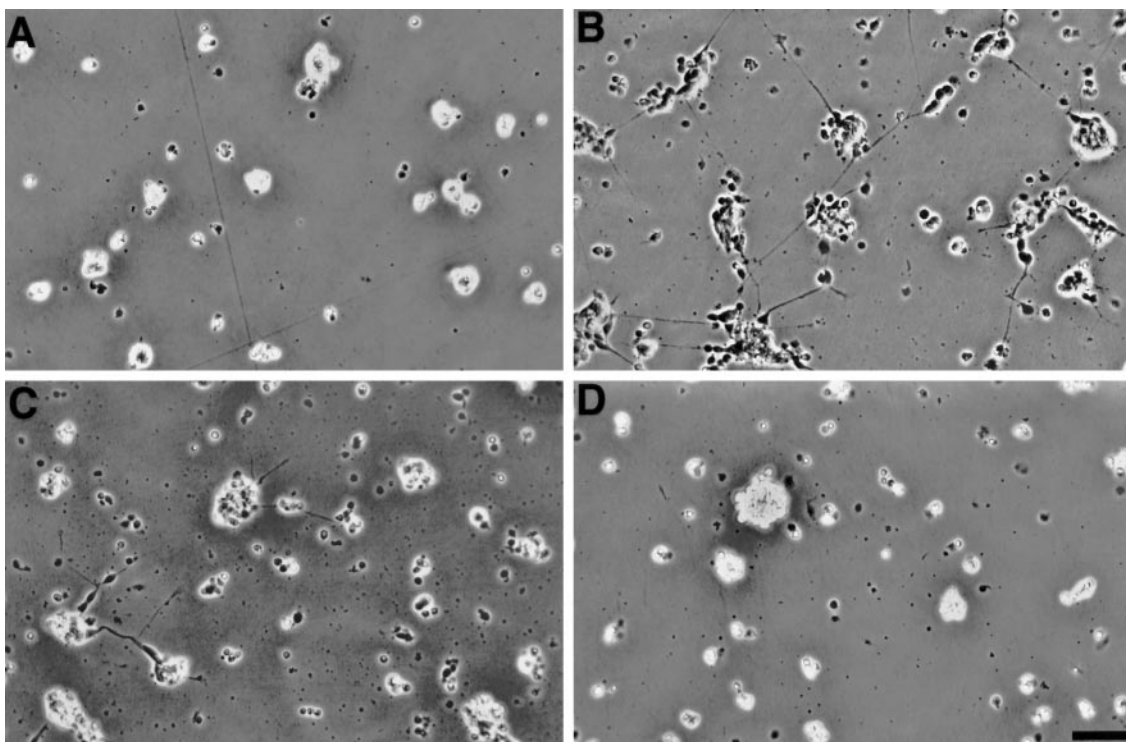
Figure 3. Effect of α3β1 integrin blockade on neurite outgrowth on antibody-activated LN-1. The effect of integrin blockade on the morphology of embryonic day 18 rat retinal neurons growing on an LN-1 substrata in the absence (*A*) or presence (*B–D*) of anti-P1′ antibodies. *A*, Retinal neurons growing on untreated LN-1 attach poorly (remaining phase-bright and rounded) and do not extend neurites. *B*, Decoration of the short arms of LN-1 with anti-P1′ antibodies permits embryonic day 18 rat retinal neurons to attach and extend neurites. Note the phase-dark appearance of most cells, indicating spreading. *C*, Blockade of α3β1 integrin function by Ralph 3.1 (50 μg/ml) inhibits neurite outgrowth while significantly sparing cell attachment and spreading. *D*, Blockade of all β1 integrins with lenny (1:200) inhibits both neurite outgrowth and cell attachment. Scale bar, 75 μm.

mouse α6 antibody GoH3 stained all retinal layers with similar intensity (Fig. 2*C*).

## Functional analysis of laminin-binding integrins in retinal neurons

To test whether α1β1 or α2β1 integrins are involved in the response of retinal neurons to LNs, including antibody-activated LN-1, cultures of embryonic day 11 chick and embryonic day 18 rat retinal neurons were plated on LN-1, LN-2/4 (merosin), LN-5, E8, and LN-1 activated by treatment with anti-E1′ antibodies in the presence or absence of monoclonal antibodies that block the functions of rat α1 [3A3 (Turner et al., 1989)], rat α2 [Ha1/29 (Mendrick et al., 1995)], or chick α2 [mep-17 (McNagny et al., 1992)]. None of these antibodies inhibited the ability of retinal neurons to extend neurites on antibody-activated LN-1, E8, LN-2/4, or LN-5 (data not shown). Furthermore, these antibodies did not alter the inability of retinal neurons to respond to untreated LN-1. These data suggest that α1β1 and α2β1 do not mediate neurite outgrowth on LNs, nor are they responsible for inhibition of neurite outgrowth on LN-1 that has not been activated.

To test for a role for the α3β1 integrin in retinal neurite outgrowth on antibody-activated LN-1, we plated dissociated cultures of embryonic retinal neurons from embryonic day 18 rats on untreated and activated LN-1 substrata in the presence or absence of function-blocking anti-α3 antibodies (Fig. 3). As expected, retinal neurons failed to attach and extend neurites on untreated LN-1 (Fig. 3*A*) but attached, spread, and extended long neurites on LN-1 substrata treated with anti-P1′ antibodies (Fig. 3*B*). The function-blocking anti-α3β1 mAb Ralph 3.1 (50 μg/ml)

substantively inhibited the ability of retinal neurons to extend neurites on antibody-activated LN-1 (Fig. 3*C*, see also Figs. 5, 6). Interestingly, however, the spreading response of the neurons remained unaffected (Fig. 3*C*). Incubation with the function-blocking anti-β1 antibodies lenny (Fig. 3*D*) or Ha2/5 (data not shown) completely inhibited both the attachment and spreading responses as well as all neurite outgrowth. Incubation with non-immune mouse IgG was without effect (data not shown).

Because α3β1 function seemed to be important for retinal neurite outgrowth on antibody-activated LN-1, we also asked whether α3β1 was required for outgrowth on LN-1 that was activated by proteolytic removal of short-arm domains, as well as on other LN isoforms that do not require activation to promote neurite outgrowth from late embryonic retinal neurons (Fig. 4). As described previously (Calof et al., 1994), LN-1 long-arm fragment E8 (Fig. 4*A*) and LN-2/4 (Fig. 4*C*) both promote neurite outgrowth from late embryonic retinal neurons as does thrombospondin-1, an ECM protein known to interact with α3β1 (Fig. 4*G*). Additionally, these cells extend neurites in response to LN-5 (Fig. 4*E*), a LN isoform that shares no chains in common with LN-1 (Marinkovich et al., 1992). However, when retinal neurons were cultured on any of these substrata in the presence of Ralph 3.1 (50 μg/ml), neurite outgrowth was greatly reduced (Figs. 4*B,D,F,H*, 5, 6). This effect can be seen quantitatively both in terms of the number of neurite-bearing cells in the culture (Fig. 5) and in the lengths of neurite-bearing cells (Fig. 6).

These results suggest that α3β1 is a major, although possibly not the only, retinal neuron receptor for antibody-activated LN-1 and further support the idea that antibody activation of LN-1 and
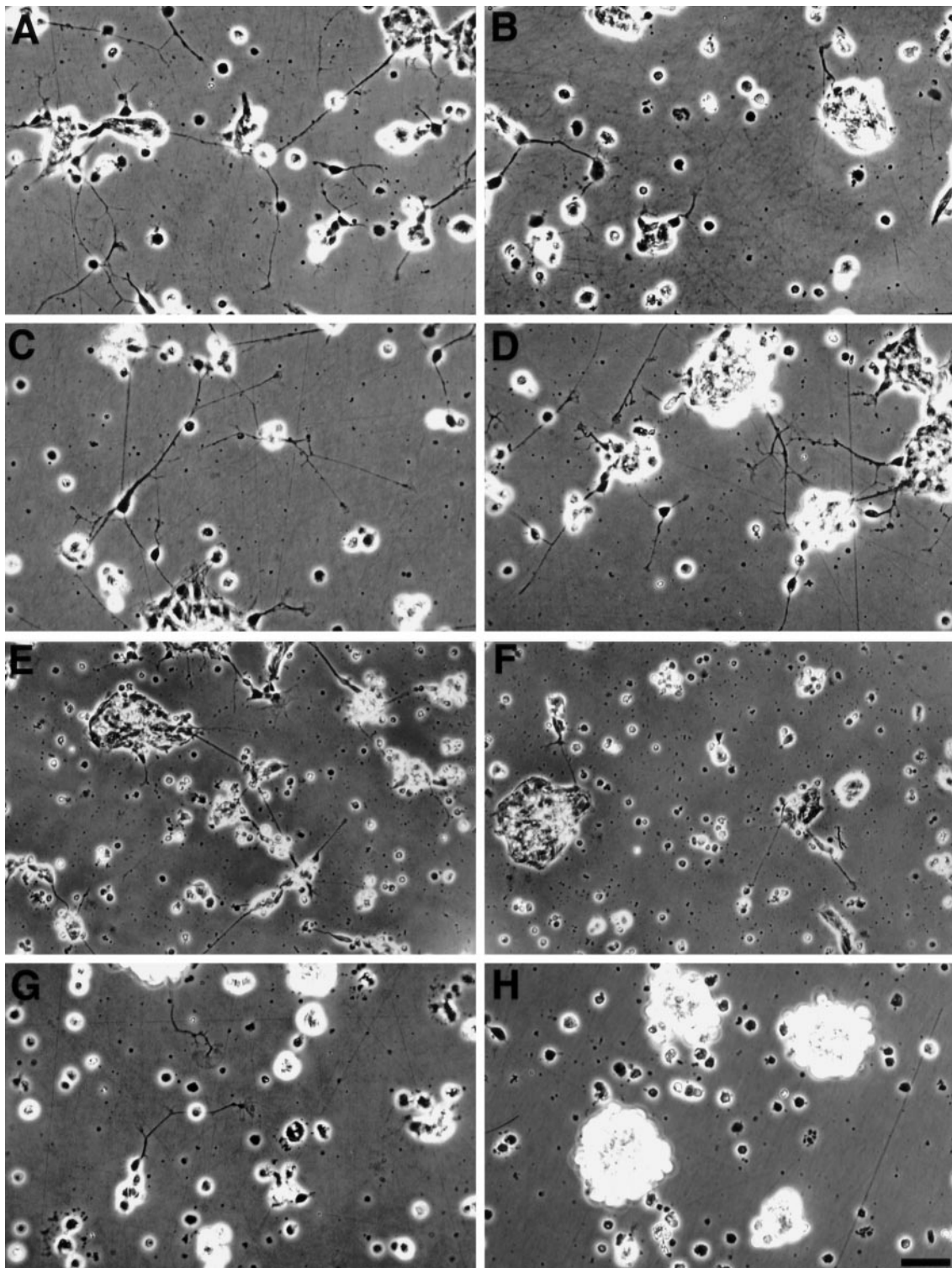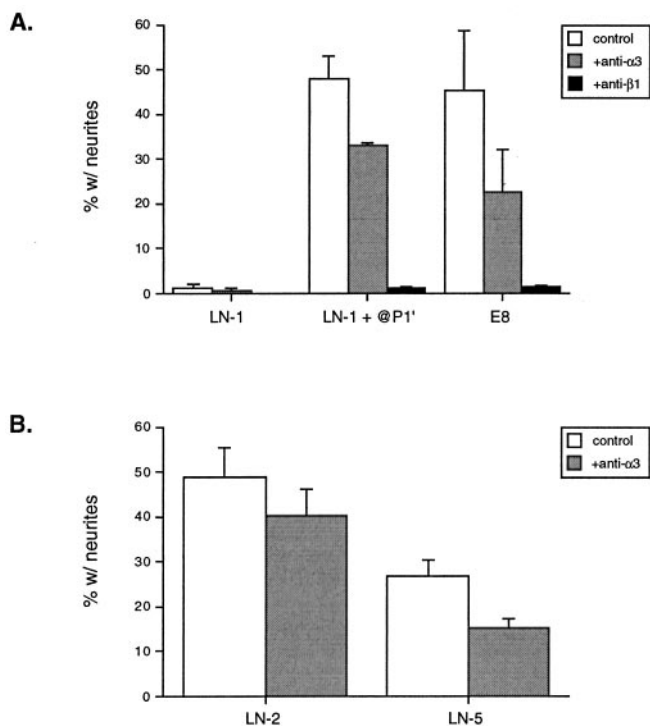
Figure 4. Effect of $\alpha3\beta1$ integrin blockade on neurite outgrowth on LN-1 fragment E8 and on other LN isoforms. Embryonic day 18 rat retinal neurons were grown on substrata as indicated in the absence (*A, C, E, G*) or in the presence (*B, D, F, H*) of the anti-$\alpha3$ function-blocking antibody Ralph 3.1 (50 μg/ml). Ralph 3.1 strongly inhibits neurite outgrowth on the E8 long-arm fragment of LN-1 (*A, B*), on LN-5 (*E, F*), and on thrombospondin-1 (*G, H*) but only has a minor effect on LN-2/4 (*C, D*). Scale bar, 50 μm.

activation by proteolytic removal of short-arm fragments have similar functional consequences for LN-1. The data also support the idea that activation of LN-1 imparts functionality that is "constitutively" present in other LN isoforms.

The role that the $\alpha6\beta1$ integrin plays in the responses of retinal

neuron to LN-1 substrata was also tested with blocking antibodies, this time using embryonic day 16 mouse retinal neurons and the rat monoclonal anti-$\alpha6$ integrin antibody GoH3. Neurons were cultured on a variety of substrata in the presence (Fig. 7*B,D,F*) or absence (Fig. 7*A,C,E*) of the antibody. In contrast to
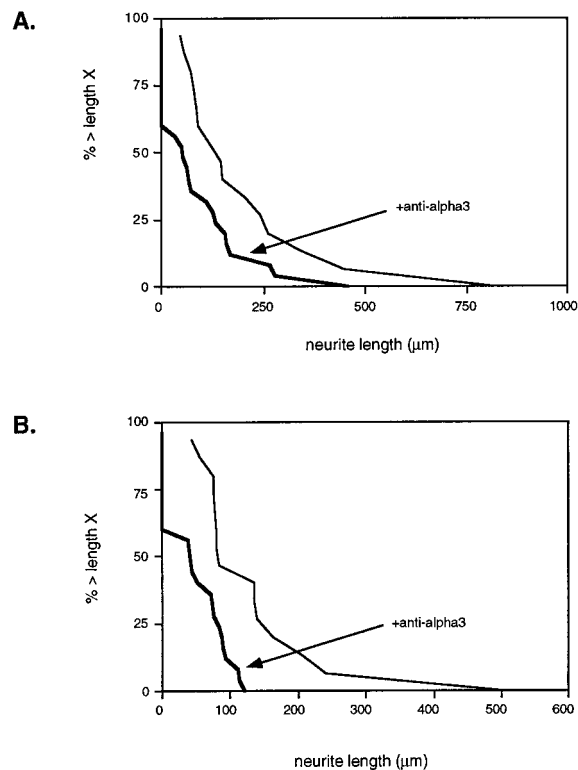
Figure 5. Effect of $\alpha3\beta1$ integrin blockade on numbers of neurite-bearing cells. Cultures of embryonic day 18 rat retinal neurons were grown on the indicated substrata in the presence or absence of antibodies as described. Cultures were fixed after 24 hr and scored for the number of cells or clumps of cells bearing neurites greater than two cell body diameters in length. *A*, Ralph 3.1 (50 μg/ml; *hatched bars*) blocks neurite outgrowth to the same extent relative to controls (*open bars*) on both anti-P1′-treated LN-1 (*LN-1 + @P1′*) and the E8 fragment of LN-1. In each case, cell attachment and neurite outgrowth are completely blocked by anti-$\beta1$ integrin antibodies (*solid bars*). *B*, Ralph 3.1 (50 μg/ml; *hatched bars*) blocks neurite outgrowth on LN-2 and LN-5. The effect of $\alpha3\beta1$ blockade is minor on LN-2 compared with that seen on the other LN substrates. Counts were performed on duplicate wells from a total of six separate experiments, except that for anti-P1′-treated LN-1, two separate experiments were performed. All differences are significant at $p < 0.01$ by Student's *t* test.

the effects seen with $\alpha3\beta1$ blockade on neurite outgrowth, only a slight effect on neurite outgrowth was seen with $\alpha6\beta1$ blockade with GoH3 (2 μg/ml). However, $\alpha6\beta1$ blockade resulted in a marked inhibition of cell attachment and spreading on E8 (Fig. 7*A,B*), on LN-2/4 (Fig. 7*C,D*), and on LN-5 (Fig. 7*E,F*). These results suggest that the $\alpha6\beta1$ integrin is important for the attachment and spreading of retinal neurons on LN substrata but is much less so for neurite outgrowth. The data also demonstrate, however, that activation of LN-1 is characterized not only by an increase in the ability of LN-1 to promote neurite outgrowth but also by an increase in the ability to promote, via a separate mechanism that uses a different receptor, cell spreading.

$\alpha3\beta1$ is required by retinal ganglion cell axons for outgrowth on the long arm of LN-1

The immunohistochemical localization of the $\alpha3\beta1$ integrin suggests that it is predominantly expressed by retinal ganglion cells (Fig. 2*B*). To assess the role of $\alpha3\beta1$ specifically in retinal ganglion cell outgrowth on activated LN-1, we cultured retinas from embryonic day 18 rats as explants. Under these conditions, a number of investigators have shown that the only axons that extend away from the explant are those of retinal ganglion cells


Figure 6. Effect of $\alpha3\beta1$ integrin blockade on neurite lengths. Cultures of embryonic day 18 rat retinal neurons were grown overnight on antibody-activated LN-1 (*A*) or on long-arm fragment E8 (*B*) in the absence (*thin line*) or presence of Ralph 3.1 (50 μg/ml; *thick line*) and then fixed; neurite lengths were measured. Only neurites greater than two cell body diameters in length were included. Neurites that do grow in the presence of $\alpha3\beta1$-blocking antibodies are shorter than controls. All differences are significant ($p < 0.01$) by Student's *t* test.

(e.g., Bates and Meyer, 1993). We observed that, as was the case with dissociated retinal cultures, retinal ganglion cell axons from such explants were unable to extend axons on substrata of LN-1 (Fig. 8*A*). However, these explants exhibited robust axon growth when plated on the long-arm fragment E8 (Fig. 8*B*) or on anti-E1′-treated LN-1 (data not shown). Furthermore, in both cases this outgrowth was strongly inhibited by 50 μg/ml Ralph 3.1 (Fig. 8*C*; data not shown). Interestingly, in the presence of the anti-$\alpha3$ antibody, retinal ganglion cell axons did manage to grow but mainly in highly fasciculated bundles that wrapped many times around the margin of the explant without extending out onto the substratum. These results confirm that $\alpha3\beta1$ is an important receptor for retinal ganglion cell axon outgrowth on activated LN-1.

$\alpha6\beta1$ can support neurite growth when $\alpha3\beta1$ is genetically disrupted

One criticism of the above experiments with blocking antibodies to $\alpha3\beta1$ and $\alpha6\beta1$ is that, because of limitations in the types of antibodies available, it was only possible to block $\alpha3\beta1$ in rat neurons and $\alpha6\beta1$ in mouse neurons. Because each reagent inhibited some of the neuronal response (e.g., attachment and/or neurite outgrowth) to activated LN-1, it is tempting to speculate that the two receptors together account for all of the response, but demonstrating this requires blocking both integrins in the same cells.

To circumvent the lack of appropriate antibodies, we turned to
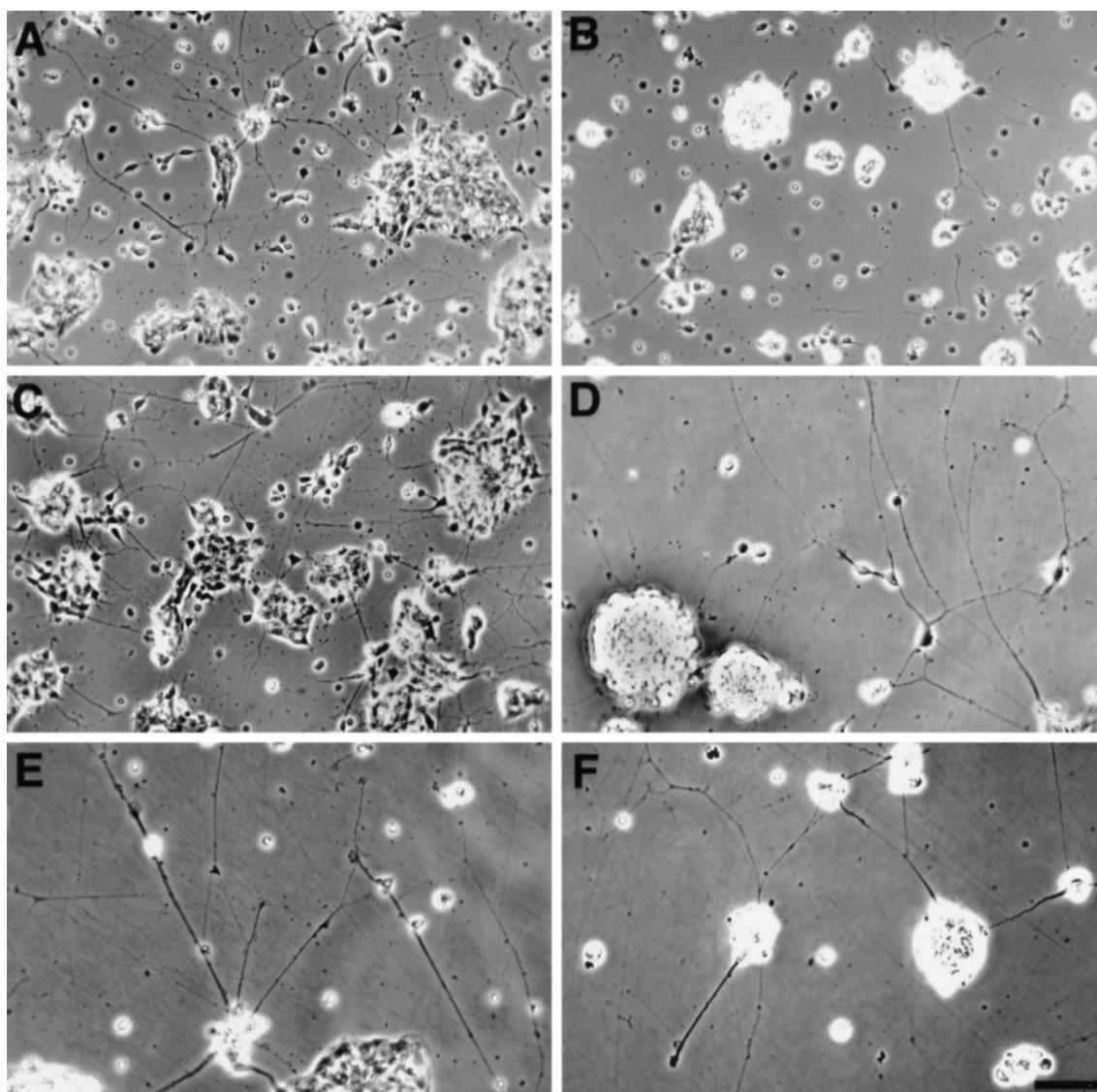
Figure 7. Effect of α6β1 blockade on the spreading response of retinal neurons. Embryonic day 16 mouse retinal neurons were grown on E8 (*A, B*), on LN-2/4 (*C, D*), or on LN-5 (*E, F*) in the presence (*B, D, F*) or absence (*A, C, E*) of the anti-α6β1 antibody GoH3 (2 μg/ml). Cultures were fixed after 24 hr and photographed. In each case, α6β1 blockade inhibited the ability of cells to spread on these LN substrates but still allowed considerable neurite growth. In many cases, dissociated retinal neurons aggregated to form large clumps of cells rather than remaining as single cells or small clumps. Because of this tendency of cells to form large aggregates, the effects of α6β1 blockade on neurite outgrowth were not measured quantitatively. The effect of GoH3 on antibody-activated LN-1 (data not shown) was indistinguishable from that seen here with E8. Scale bar, 50 μm.

a mouse model in which the α3 integrin subunit has been deleted by homologous recombination (Kreidberg et al., 1996). Cultures established from the retinas of littermates derived from α3$^{+/-}$ × α3$^{+/-}$ matings were then grown on the LN-1 long-arm fragment E8 in the presence or absence of the anti-α6β1 antibody GoH3. Explants established from wild-type, heterozygous, and homozygous null retinas all exhibited robust axon growth on E8 (Fig. 9). In agreement with the data presented above, neurite outgrowth produced by wild-type (data not shown) and heterozygote cultures was only partly inhibited by GoH3, but outgrowth by homozygous null retinas was almost completely blocked (Fig. 9). These data support the view that, together, α3β1 and α6β1 mediate the retinal neuron response to activated LN-1.

A surprising feature of the data is that, despite the absence of functional α3β1, the magnitude of the neurite outgrowth response was not noticeably lower in the homozygous null cells than in the wild-type neurons (Fig. 9). This strongly suggests that the mutant cells compensate for the loss of α3β1 by increasing the part of their response that is mediated by α6β1. Whether this is attributable to a change in α6β1 expression or function remains to be determined. However, such compensation may explain why DiI labeling of the visual system of α3$^{-/-}$ mice demonstrates an apparently normal pattern of retinal innervation of the superior colliculus (J. K. Ivins, J. A. Kreidberg, and A. D. Lander, unpublished observations).

Activated LN-1 exhibits enhanced neurite outgrowth-promoting activity for many types of CNS neurons

Integrin α6β1, which was shown above to be one of two integrins used by late embryonic retinal neurons to respond to activated LN-1, is widely expressed in the CNS. This raised the possibility that other types of CNS neurons might also exhibit an enhanced
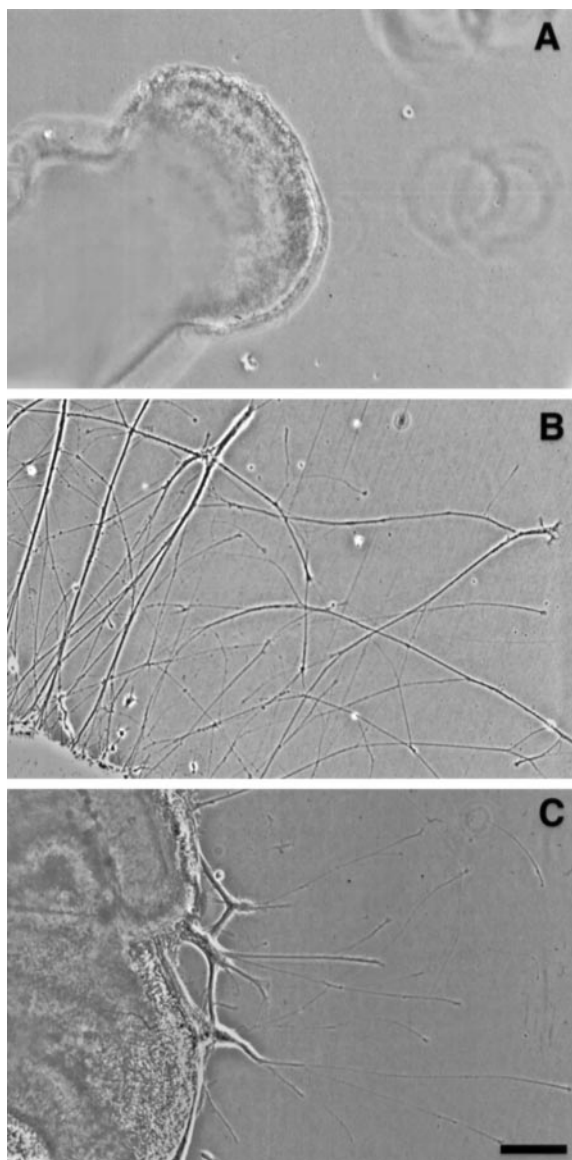
Figure 8. Effect of $\alpha3\beta1$ integrin blockade on retinal ganglion cell outgrowth. Explants of embryonic day 18 rat retina were plated on LN-1 (*A*) or on E8 (*B, C*) in the absence (*B*) or presence (*C*) of Ralph 3.1 and were allowed to grow overnight. In *B*, the edge of the explant is visible in the *lower left corner* of the photograph. Scale bar, 50 μm.

response to LN-1 that has been activated. To test this, we cultured neurons from several CNS regions on LN-1 substrates in the presence or absence of activating antibodies. The results with cultures of embryonic day 18 rat hippocampal neurons are shown in Figure 10. When cultured overnight on untreated LN-1 substrata, dissociated hippocampal neurons attach but tend to form large cell aggregates that exhibit sparse neurite outgrowth (Fig. 10*A*). Treatment of the substratum with anti-E1′ antibodies, however, allowed these neurons to attach individually and to extend extensive neurites (Fig. 10*B*), comparable with that seen on LN-2/4 (Fig. 10*C*). Similar extensive outgrowth was also seen on LN-1 fragment E8 (data not shown). As was the case with retinal neurons, neurite outgrowth on activated LN-1 was completely blocked by function-blocking anti-$\beta1$ integrin antibodies (Fig. 10*D*). Similar results were also obtained with cultures of embryonic day 18 rat cerebral cortical neurons (data not shown).

To test whether these responses are mediated by integrin $\alpha6\beta1$, we repeated the above experiments with mouse cortical neurons, culturing them on E8 in the absence (Fig. 10*E*) or presence (Fig. 10*F*) of mAb GoH3. Treatment with mAb GoH3 resulted in a significant reduction in the number of cells with neurites (78% for control vs 45% for GoH3-treated) as well as a significant reduction in neurite lengths (67.2 ± 6.3 μm for control vs 28.0 ± 3.3 μm for GoH3-treated, mean ± SEM; Student's *t* test, $p < 0.0001$). No outgrowth was observed on untreated LN-1 (data not shown). Neurite outgrowth was completely blocked by anti-$\beta1$ antibodies on all substrates tested (data not shown). Similar results were obtained with cultures of mouse thalamic neurons (data not shown).

In cultures of central neurons, therefore, as in cultures of retinal neurons, neurite outgrowth on activated LN-1 is only partially mediated by the $\alpha6\beta1$ integrin. However, $\alpha3\beta1$ is unlikely to mediate outgrowth in these cultures for several reasons. First, immunohistochemical localization studies by us (data not shown) and others (DeFreitas et al., 1995) have failed to detect the $\alpha3$ integrin subunit in the rodent cerebral cortex, hippocampus, or thalamus. Furthermore, treatment of rat hippocampal or cortical neuronal cultures grown on activated LN-1 with mAb Ralph 3.1 (anti-rat integrin $\alpha3$) had no effect on neurite outgrowth (data not shown). Together, these data show that not only do CNS neurons other than retinal neurons respond to the activation of LN-1 but suggest that at least one $\beta1$ integrin other than $\alpha6\beta1$ and $\alpha3\beta1$ may be involved.

DISCUSSION

In previous studies, treatment of substratum-bound LN-1 with antibodies to its E1′ or P1′ domains rendered LN-1 a potent promoter of neurite outgrowth by late embryonic retinal neurons (Calof et al., 1994). Proteolytic fragments containing the long arm of LN-1 had effects similar to those of antibody-activated LN-1, and antibodies to distal long-arm fragments of LN-1, or to $\beta1$ integrins, blocked the neurite outgrowth activity of both antibody-activated and proteolytically activated LN-1 (Calof et al., 1994). These observations suggest the existence of two discrete activities in LN-1: one located in the short-arm domains, the blockade or removal of which leads to activation, and one located at the end of the long arm, which mediates integrin-dependent neurite outgrowth in the activated LN-1 molecule but fails to do so in native LN-1. In the present study, we have shown that a major site for LN-1 activation resides in domains VI–IVb of the $\alpha1$ chain (Fig. 1). In addition, via the study of retinal neurons (Figs. 2–9) and other CNS neurons (Fig. 10), we have shown that multiple integrins can mediate neuronal responses to activated LN-1, including integrins that, on other cell types, mediate robust responses to unmanipulated LN-1.

Multiple integrins mediate the neuronal response to activated LN-1

In late embryonic retinal neurons, the response to activated LN-1 is mediated by two integrins, $\alpha3\beta1$ and $\alpha6\beta1$ (Figs. 2–9). Both of these interact with the distal long arm of LN-1 (Sonnenberg et al., 1990; Gehlsen et al., 1992; Pattaramalai et al., 1996), consistent with the observation that effects of activated LN-1 are blocked by antibodies to distal long-arm fragments (Calof et al., 1994).

It is noteworthy that, although isolated $\alpha3\beta1$ has been shown to bind LN-1 (DeFreitas et al., 1995) and LN-1-derived peptides (Gehlsen et al., 1992) in biochemical assays, there is little evidence that cells use $\alpha3\beta1$ as a receptor for interactions with LN-1,
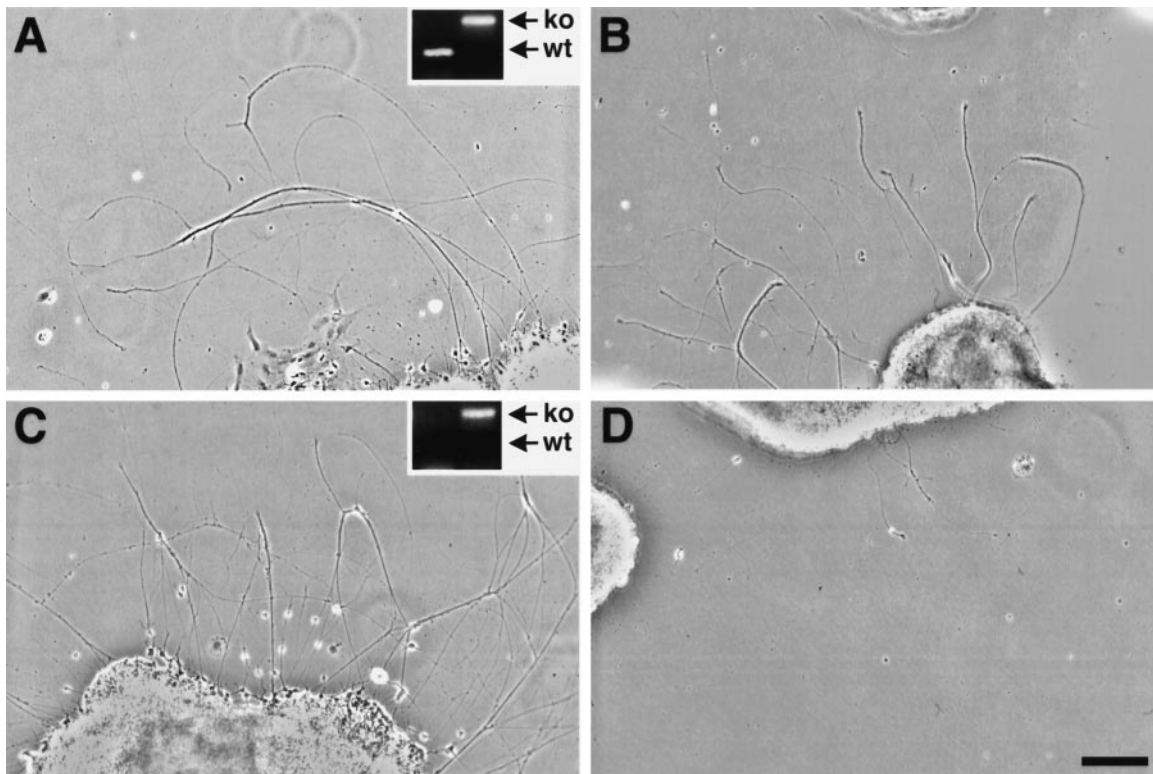
Figure 9. Effect of disruption of the $\alpha3$ integrin gene on retinal ganglion cell outgrowth on the LN-1 long arm. Explants of retina from embryonic day 16 mice derived from crosses of heterozygote $\alpha3$ integrin null mice were plated on E8. Explants were grown in the absence (*A, C*) or presence (*B, D*) of GoH3 (2 μg/ml) to inhibit $\alpha6\beta1$. Explants in *A* and *B* were derived from a heterozygote, whereas explants in *C* and *D* were derived from a null embryo. *Insets* (*A, C*), Embryos were genotyped by PCR. *ko*, Knock-out; *wt*, wild type. Scale bar, 50 μm.

and cells transfected with $\alpha3$ do not acquire the ability to attach to LN-1 (Delwel et al., 1994; Shaw et al., 1996). In contrast, there are numerous cases in which $\alpha3\beta1$ is used by cells, often including neurons, as a receptor for LN-2/4 (Tomaselli et al., 1993), LN-5 (Smith et al., 1996), and LN preparations likely to contain iso-forms other than LN-1 (Gehlsen et al., 1989; Delwel et al., 1994), as well as for other extracellular matrix proteins such as thrombospondin-1 (DeFreitas et al., 1995). These data are con-sistent with the view that there is an $\alpha3\beta1$ binding site on LN-1 but that it is normally not accessible to cells unless LN-1 is altered by antibody or proteolytic activation.

Interestingly, whereas the inability of retinal neurons to use $\alpha3\beta1$ to respond to unaltered LN-1 could potentially be ascribed to inaccessibility of the receptor binding site, the same cannot be said for $\alpha6\beta1$, which late embryonic retinal neurons also seem to use to respond only to activated, and not untreated, LN-1. This is because $\alpha6\beta1$ is known to be used by many cell types, including neurons, as a receptor for (unmanipulated) LN-1 (Hall et al., 1990; Sonnenberg et al., 1990; Calof et al., 1994). Indeed, $\alpha6\beta1$ has been identified as the major LN-1 receptor used by early embryonic retinal neurons before they lose LN-1 responsiveness (de Curtis et al., 1991; de Curtis and Reichardt, 1993). Accord-ingly, there is little reason to believe that the $\alpha6\beta1$ binding site on LN-1 is inaccessible to cells in unmanipulated LN-1 molecules. Thus, activation of LN-1 seems to affect the interaction of cells with receptor binding sites that cannot normally be considered cryptic. It is interesting that only late embryonic, and not early embryonic, retinal neurons require LN-1 to be activated before $\alpha6\beta1$-mediated responses can be elicited (de Curtis et al., 1991; de Curtis and Reichardt, 1993). This difference might reflect

developmental changes in molecules that control the cell surface exposure or activation state of integrins, such that some threshold for signaling is no longer reached by unactivated LN-1.

Structurally, the $\alpha3$ and $\alpha6$ integrin subunits are more closely related to each other than to other integrin α subunits (Schwartz et al., 1995), yet on late embryonic retinal neurons, $\alpha3\beta1$ is concerned mainly with the promotion of neurite growth, whereas $\alpha6\beta1$ preferentially mediates attachment and spreading (Figs. 2–7). This may represent an intrinsic functional difference be-tween $\alpha3$ and $\alpha6$, or it may reflect differential usage in neurons of the alternative "A" and "B" type cytoplasmic domains that either of these integrins can possess (Tamura et al., 1991; de Curtis and Reichardt, 1993; Schwartz et al., 1995). Interestingly, despite the functional difference observed between $\alpha3$ and $\alpha6$ in normal retinal neurons, the experiments with $\alpha3$-deficient mice (Fig. 9) or with neurons derived from other CNS regions (Fig. 10) suggest that, at least under some circumstances, $\alpha6\beta1$ can mediate neurite outgrowth as effectively as $\alpha3\beta1$. It will be interesting to see whether this effect reflects differences in the level of expression or splicing of $\alpha6$.

Potential mechanisms underlying LN activation

It has been suggested previously that activation of LN-1 reflects the blockade or removal of an inhibitory, or suppressive, domain residing in the short arms of LN-1 (Calof et al., 1994). The mapping of a domain involved in LN-1 activation within the N-terminal portion of the $\alpha1$ chain (Fig. 1) is consistent with the observation that LN isoforms that lack the $\alpha1$ chain [e.g., merosin ($\alpha2\beta1/2\gamma1$) and LN-5 ($\alpha3\beta3\gamma2$)] do not require any activating
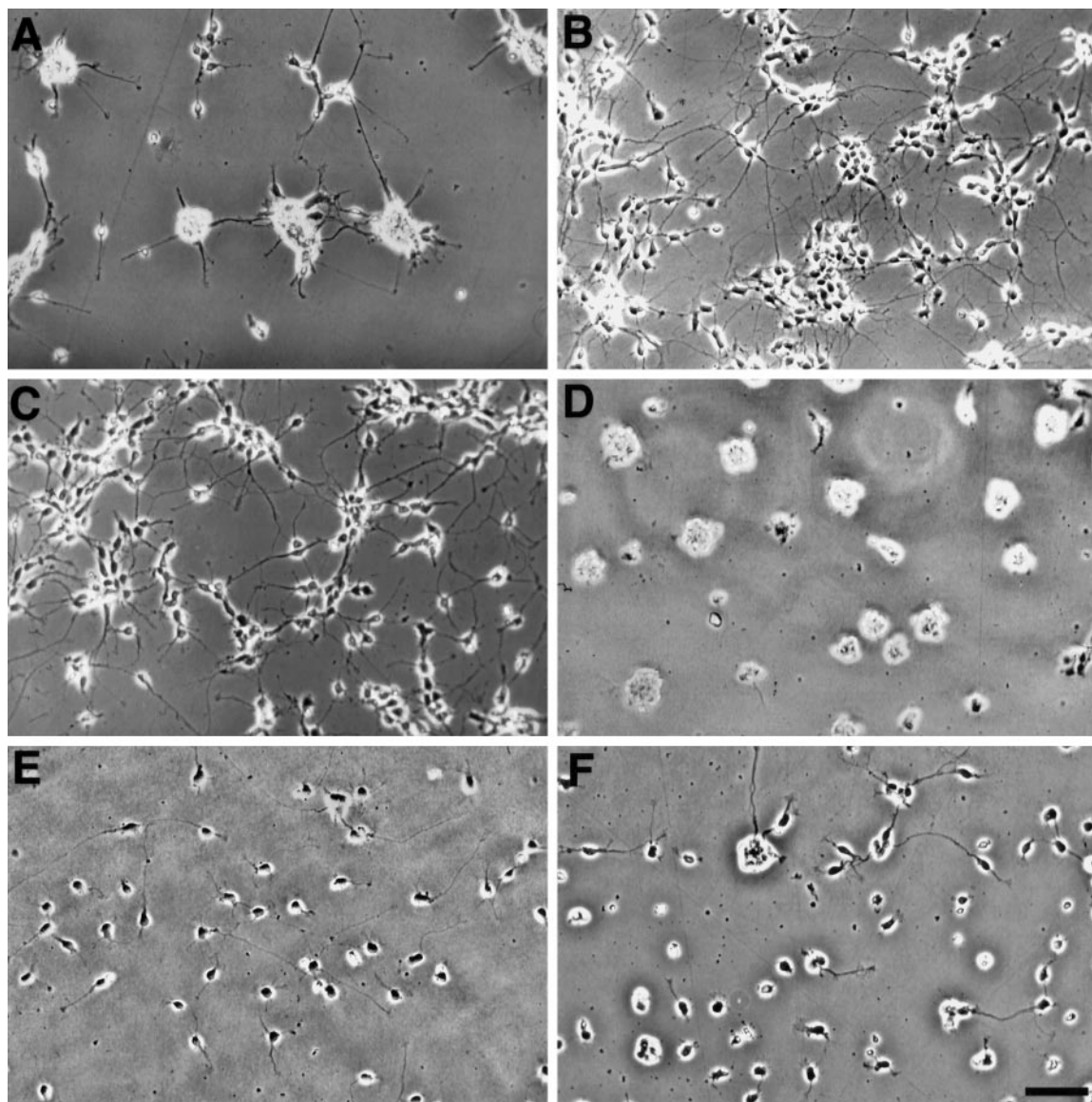
Figure 10. Effect of antibody activation of LN-1 on the neurite outgrowth of other CNS neurons. *A–D*, Hippocampal neurons from embryonic day 18 rats were plated on LN-1 in the absence (*A*) or presence (*B, D*) of anti-E1′ antibodies or on LN-2/4 (*C*). The culture in *D* was additionally treated with anti-β1 function-blocking antibody Ha2/5 (10 μg/ml). Like late embryonic retinal neurons, hippocampal neurons respond to activated LN-1 with vigorous neurite outgrowth. *E, F*, Embryonic day 14 mouse cortical neurons were grown overnight on E8 in the absence (*E*) or presence (*F*) of the anti-α6 mAb GoH3 (2 μg/ml). GoH3 treatment resulted in a significant reduction both in the number of cells with neurites (78 vs 45%) and in neurite lengths (67.2 $\pm$ 6.3 vs 28.0 $\pm$ 3.3 μm, mean $\pm$ SEM; Student's *t* test, $p < 0.0001$). No outgrowth was seen on untreated LN-1 (data not shown). Outgrowth was completely blocked by anti-β1 antibodies (data not shown). Scale bar, 75 μm.

manipulations to promote neurite outgrowth by late embryonic retinal neurons (Figs. 3, 4, 6).

If the activation domain of LN-1 does have an inhibitory function, the mechanism of action must involve either an effect of this domain on cells (altering the way cells respond to the rest of the LN-1 molecule) or an effect of this domain on the structure or accessibility of other parts of LN-1. If this domain acts on cells, it apparently does not do so via signaling mediated by integrins α1β1 and α2β1 (the only known integrin receptors for this part of the LN-1 molecule), because blocking antibodies to these integrins did not render late embryonic retinal neurons capable of extending neurites on LN-1.

If the activation domain acts via an influence on LN-1 structure, it must alter the activity of integrin binding sites located

50–75 nm away, at the distal end of the long arm (Sonnenberg et al., 1990; Gehlsen et al., 1992; Pattaramalai et al., 1996), affecting the activities of binding sites for α3β1 (Figs. 3–6), α6β1 (Fig. 7), and probably at least one more β1 integrin (Fig. 10). One large-scale structural change that LN-1 is known to undergo is polymerization, a process that involves end-to-end interactions of the short arms (Schittny and Yurchenco, 1990; Colognato-Pyke et al., 1995). However, LN activation is unlikely to involve changes in the polymerization state of LN-1, because antibodies to short-arm domains of LN-1 (including those that activate LN-1) generally have little effect on polymerization (H. Colognato and P. D. Yurchenco, unpublished observations). Furthermore, chemically treated LN-1 that cannot polymerize also fails to promote neurite outgrowth from late embryonic retinal neurons but is

activated by anti-E1′ antibodies (J. K. Ivins, H. Colognato, P. D. Yurchenco, and A. D. Lander, unpublished observations).

Recently, a phenomenon strikingly similar to LN-1 activation was described for LN-5. Cleavage of LN-5 at a site near the short-arm–long-arm junction of the γ2 chain changed LN-5 from a molecule that promotes only epithelial cell attachment to one that promotes α3β1-integrin-dependent epithelial cell migration (Giannelli et al., 1997). Intriguingly, this new activity mapped to an epitope that has since been shown to be located at the distal end of the long arm (V. Quaranta, personal communication). It will be interesting to determine whether the mechanism of activation of LN-5 shows similarity to that underlying activation of LN-1.

Does LN activation occur *in vivo*?

Whether endogenous, physiological activators of LN-1 exist is not known. Although antibodies to the short arms of LN-1 are unlikely to be present *in vivo*, at least one extracellular matrix protein, fibulin-2, is thought to interact with LN-1 via domain IVb of the α1 chain (Utani et al., 1997), within the region defined here for antibody activation of LN-1. Thus LN-1 activation may be a function of the ECM molecules with which LN-1 is complexed. Alternatively, activation may occur as the result of endogenous proteolytic cleavage. Indeed, the LN-5 activation phenomenon referred to above has been shown to result from an matrix metalloproteinase-2-mediated cleavage event that can be detected in LN-5 extracted from multiple tissues (Giannelli et al., 1997).

It is noteworthy that parenchymal LN-1 is primarily present in the CNS only during development [in locations that include the optic pathways followed by retinal ganglion cell axons (Cohen et al., 1986; Halfter and Fua, 1987)], but it frequently reappears after neural injury (Zak et al., 1987; Brodkey et al., 1993; Frisen, 1997; Fu and Gordon, 1997). If the CNS neurons that encounter LN-1 at injury sites behave like the CNS neurons that were tested here (retinal, thalamic, cortical, and hippocampal), then the state of LN-1 activation could have considerable influence over whether the ECM at those sites succeeds or fails to promote the regeneration of axons. Likewise, if LN-1 is not normally activated at sites of neural injury, then the possibility of activating it with exogenous agents (e.g., specific anti-short-arm antibodies) might have potential therapeutic value.

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