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An LRE (Leucine–Arginine–Glutamate)-dependent Mechanism for Adhesion of Neurons to S-laminin

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S-laminin is a homolog of laminin that is concentrated in the synaptic cleft of the neuromuscular junction. We previously showed that the tripeptide LRE is a crucial determinant for binding of ciliary motoneurons to recombinant s-laminin. Here, we describe a neuroblastoma–spinal neuron hybrid cell line, NSC-34, that binds to an LRE-containing s-laminin fragment and to a synthetic LRE–protein conjugate. NSC-34 cells exhibit several properties of motoneurons; other cell lines tested were not motoneuron-like and did not display LRE-dependent adhesion. We therefore used NSC-34 cells to characterize the LRE-dependent adhesion mechanism. Inhibition studies with a series of 20 tripeptide LRE analogs showed that the cells exhibit a high degree of selectivity for LRE, and suggested that ligand binding requires a combination of electrostatic and hydrophobic interactions. The effects of cations on LRE-dependent adhesion are unlike those of previously described adhesion molecules including the integrins, a family of receptors for extracellular matrix proteins, including laminin. Specifically, adhesion to LRE does not require divalent cations and is inhibited by Ca^{2+} (but not by Mg^{2+}) in the physiological range. In contrast, adhesion of NSC-34 cells to laminin is LRE- and Ca^{2+} independent but Mg^{2+} dependent, and appears to be mediated by integrins. Additionally, experiments using mixed substrates demonstrated that LRE–protein conjugates inhibit neurite outgrowth promoted by laminin. Finally, we show that, under ionic conditions that minimize integrin-dependent adhesion, NSC-34 cells bind to s-laminin–rich basal laminae in tissue sections in an LRE-dependent manner. Together, these results suggest that LRE comprises a motoneuron-selective adhesion site that is accessible in native basal laminae and that acts to inhibit neurite outgrowth.

Following nerve injury, motor axons preferentially reinnervate original synaptic sites on skeletal muscle fibers (Tello, 1907; Gutmann and Young, 1944). At these sites, the axons arborize, and their branches differentiate into functional nerve terminals. From the topographic specificity of reinnervation and differentiation, it is evident that there must be factors closely associated with synaptic sites to which regenerating axons can respond. Axons selectively contact and differentiate at synaptic sites on muscle fiber basal lamina sheaths, even in the absence of muscle fibers, arguing that some of these factors are associated with basal lamina rather than (or in addition to) the muscle cell itself (Sanes et al., 1978, 1990b; Glicksman and Sanes, 1983).

In searching for such factors, we discovered s-laminin, a laminin-like glycoprotein that is concentrated in synaptic basal lamina and is adhesive to ciliary motoneurons (Hunter et al., 1989a). Then, using recombinant proteins and synthetic peptides, we mapped a motoneuron-selective adhesive site on s-laminin to a tripeptide, LRE, located ~10 kDa from the C-terminus of the 190 kDa protein (Hunter et al., 1989b). Subsequently, we noted that the LRE sequence is present in four proteins that are concentrated in synaptic basal lamina and for which sequence data are available: s-laminin (three occurrences in rat), the A-subunit of laminin (one occurrence in human), AChE (one occurrence each in *Torpedo* and human, but none in mouse), and agrin (two occurrences each in *Torpedo* and rat) (sequences: Sasaki et al., 1988; Hunter et al., 1989a; Rachinsky et al., 1990; Soreq et al., 1990; Rupp et al., 1991; R. Scheller, personal communication; localization: McMahan et al., 1978; Reist et al., 1987; Sanes et al., 1990a). Although LRE is expected to occur occasionally by chance alone (once per ~3000–5000 residues; Hunter et al., 1989b), its abundance in synaptic basal lamina (seven occurrences in four polypeptides totaling ~8000 residues) supports the hypothesis that it serves as ligand for motor axons. Additionally, neurotactin, a recently described neural adhesion molecule from *Drosophila* (Barthalay et al., 1990), contains three LREs in its short (~50 kDa) predicted extracellular domain (de la Escalera et al., 1990; Hortsch et al., 1990).

These suggestions that LRE may serve as a developmentally important recognition signal have motivated the detailed characterization of LRE-dependent adhesion that we report here. First, because ciliary ganglion cells are available in limited numbers, we sought a cell line that displayed LRE-dependent adhesion. Of several cell lines tested, the one that best met this criterion, called NSC-34, is a motoneuron-like line derived from the fusion of spinal cord neurons to neuroblastoma cells (Cashman et al., 1987). The ability of NSC-34 cells to adhere to LRE-containing peptides supports the hypothesis that the LRE-dependent system is motoneuron selective. Second, we used NSC-34 cells to characterize LRE-dependent adhesion in

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ways that are prerequisite to isolation of cellular LRE receptors. Specifically, we determined the dependence of adhesion on time, temperature, and ions, as well as its sensitivity to a large series of LRE analogs. Interestingly, the ion dependence of adhesion not only differs from that expected for integrins (a large class of cellular receptors for extracellular matrix molecules; Akiyama et al., 1990; Albelda and Buck, 1990; Hemler, 1990), but also suggests ways in which adhesive strength might be linked to synaptic activity. Third, we asked whether LRE-containing ligands are capable of promoting process outgrowth from NSC-34 cells. In fact, LRE–protein conjugates inhibit the outgrowth that laminin promotes, raising the possibility that the several LRE-containing molecules at synaptic basal lamina in muscle could cooperate in causing regenerating motor axons to stop growing at original synaptic sites. Finally, we used a "cryoculture" assay (Covault et al., 1987) to determine whether cells can adhere to native basal laminae via an LRE-dependent mechanism. We show that, under appropriate conditions, NSC-34 cells adhere selectively to s-laminin–rich areas in cryostat sections of adult rat tissue and that this adhesion is inhibited by soluble LRE. Together, these results define an apparently novel adhesion system on motoneuron-like cells that specifically recognizes LRE-containing ligands in native basal laminae and that may act to inhibit neurite outgrowth.

Materials and Methods

Cells. B35 and B104 cells were obtained from David Gottlieb (Washington University). PC12 cells, grown with or without nerve growth factor, were provided by Eugene Johnson and Pat Lampe (Washington University) (see Hunter et al., 1989b). C2 cells were provided by John Merlie (Washington University). Glomerular epithelial cells were a generous gift of David Salant (Boston University Medical School). C6 and A10 cells were purchased from American Type Culture Collection (Rockville, MD). N18TG2 cells were the gift of Marshall Nirenberg (NIH). Production of NSC-6, -19, and -34 cells has been reported in abstract form (Cashman et al., 1987) and will be described in detail elsewhere (N. Cashman et al., unpublished observations).

Reagents. Nitrocellulose (BA85) was purchased from Schleicher and Schuell (Keene, NH). Laminin and fibronectin were from Collaborative Research (Bedford, MA). A recombinant fragment of s-laminin (pET-RK36) was prepared as described by Hunter et al. (1989b). Poly-L-lysine was from Sigma (St. Louis, MO). Peptides were synthesized by solid-phase techniques (Barany and Merrifield, 1979) as previously described (Hunter et al., 1989b). Peptide–protein conjugates were prepared by coupling cysteinyl-peptides to keyhole limpet hemocyanin (KLH; Sigma) with *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Pharmacia) according to the manufacturer's instructions.

Adhesion assays. Adhesion assays were performed as previously described (Lagenauer and Lemmon, 1987; Hunter et al., 1989b), with minor modifications. Potentially adhesive compounds were spotted onto nitrocellulose-coated wells of 12-well tissue culture dishes, allowed to bind for 20 min, and aspirated. The wells were rinsed with 75 μg/ml KLH in phosphate-buffered saline (PBS: 150 mM NaCl and 15 mM sodium phosphate, pH 7.2) and then incubated with 750 μg/ml KLH in PBS for 1 hr at room temperature. After two rinses with PBS, 0.2–0.4 ml of adhesion medium was added to each well. Initial screening of cell lines used Dulbecco's Modified Eagle's Medium containing 10 mg/ml bovine serum albumin (BSA). For most of the assays on NSC-34 cells (including those illustrated in Figs. 1–4, 6–8), the adhesion medium consisted of Ca^{2+}- and Mg^{2+}-free Hanks' Balanced Salt Solution supplemented with 0.8 mM $MgCl_2$, 2 mg/ml $NaHCO_3$, and 10 mg/ml BSA. Modifications to this medium are noted in the text. The plates were brought to 37°C, and 40,000 cells were added to each well, bringing the total volume of medium to 0.5 ml. Except as noted in figure captions, the plates were then incubated for 90 min at 37°C in a humidified incubator with a 5% CO_2, 95% air atmosphere. Subsequently, unbound cells were removed by two rinses in PBS that had been warmed to 37°C. Finally, bound cells were fixed in 2% glutaraldehyde plus 2% paraformaldehyde in PBS. Because the absolute number of cells bound varied among assays, we present data from representative experiments, but repeated each experiment several times with qualitatively similar results.

Cryoculture assays were performed as described in Covault et al. (1987). Briefly, pieces of adult rat kidney were frozen in liquid nitrogen–cooled isopentane and sectioned at 5 μm in a cryostat. Sections were mounted on 12 mm ethanol-sterilized coverslips, which were then placed in the wells of 24-well cluster dishes. NSC-34 cells were suspended at 10^5 per ml in Hanks' Balanced Salt Solution, supplemented with 10 mg/ml BSA and 2 mg/ml $NaHCO_3$, and 0.5 ml was added to each well. The dishes were incubated at 37°C for 1 hr, then washed and fixed as described above.

Results

LRE-dependent adhesion of motoneuron-like cells

We showed previously that a bacterially produced, 20 kDa C-terminal fragment of s-laminin is adhesive for neurons from chick ciliary ganglia. Ciliary neurons were used because they form cholinergic neuromuscular junctions on striated muscle fibers *in vivo*, and thus are motoneurons. The s-laminin fragment pET-RK36 contains the sequence LRE, and neuronal adhesion to it is inhibited by soluble LRE. Other neuronal types tested did not bind to the LRE-containing s-laminin fragment, and LRE did not inhibit attachment of ciliary neurons to non-LRE-containing substrates. Together, these results defined LRE as a major determinant of a motoneuron-selective attachment site on an s-laminin–derived polypeptide and implied that motoneurons bear an LRE receptor (Hunter et al., 1989b).

Because ciliary neurons are available in limited quantity, we began the present study by seeking a cell line that displayed LRE-dependent adhesion. To this end, we tested the binding of several cell lines to pET-RK36 and to laminin in a short-term assay of cell adhesion. Tissue culture dishes were coated with a thin nitrocellulose film (Lagenaur and Lemmon, 1987) and then with a potentially adhesive protein. Free sites were blocked with BSA or KLH, and cells were incubated over the substrata for 90 min at 37°C. The plates were then washed, and the adherent cells were counted (see Materials and Methods for details). Cells of several lines adhered well to laminin in this assay, but did not adhere detectably to pET-RK36. These included PC12 rat pheochromocytoma cells, which resemble adrenal chromaffin and/or sympathetic cells (Tischler and Greene, 1975); B35 and B104 cells, which are neuron-like cells derived from chemically induced brain tumors in rats (Schubert et al., 1974, 1986); C6 rat glioma cells (Benda et al., 1968); C2 murine myogenic cells (Yaffe and Saxel, 1977); A10 rat aorta-derived smooth muscle–like cells (Kimes and Brandt, 1976); GEC rat renal glomerular epithelial cells (Kreisverg et al., 1978; Quigg et al., 1988); and N18TG2 murine neuroblastoma cells (Minna et al., 1972).

Of all cell lines tested, only one adhered well to pET-RK36. This line was derived from the polyethylene glycol–mediated fusion of embryonic murine spinal cord neurons with N18TG2 neuroblastoma cells. Forty clonal cell lines were isolated from this fusion; several of them exhibited neuron-like properties (Cashman et al., 1987), and one, NSC-34, resembles spinal motoneurons in several respects (see Discussion). We tested three of these lines, NSC-6, NSC-19, and NSC-34. All adhered well to laminin (Fig. 1*a*). However, few NSC-6 or NSC-19 cells attached to pET-RK36 in our short-term adhesion assay. In contrast, a large fraction of NSC-34 cells, up to ~50% in some assays, attached to pET-RK36 (Fig. 1*b*). This adhesion was specific in that it was inhibited by a monoclonal antibody to s-laminin; anti–s-laminin did not inhibit adhesion to other substrates such as fibronectin or laminin, and a control antibody

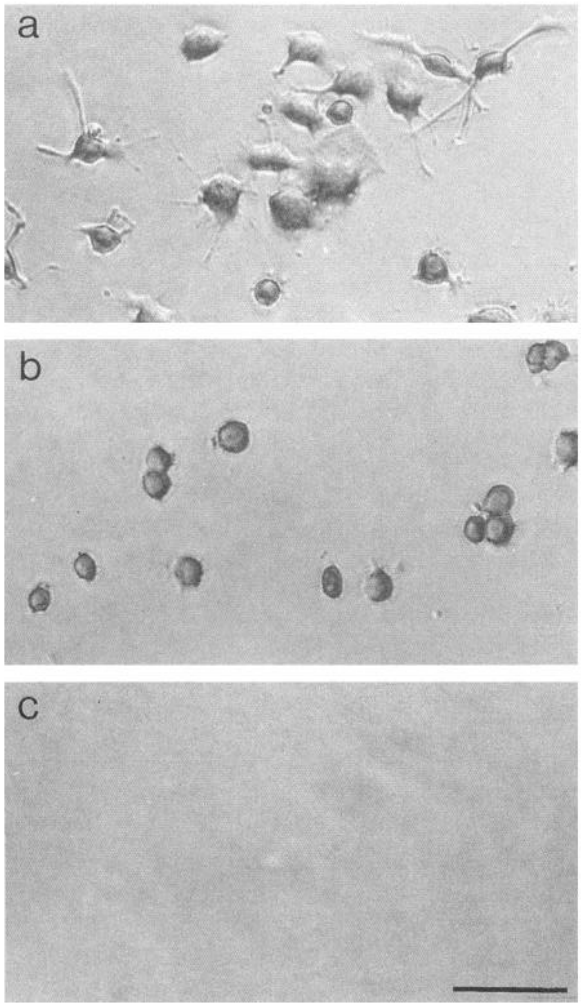

Figure 1. Adhesion of NSC-34 motoneuron-like cells to nitrocellulose coated with laminin (*a*), the recombinant s-laminin fragment pET-RK36 (*b*), or the inert "blocking" protein KLH (*c*). Cells were incubated in coated tissue culture dishes for 90 min, and then unbound cells were washed off and bound cells were fixed and photographed under phase optics. Scale bar, 100 μm.

did not inhibit adhesion to pET-RK36 (Fig. 2*a*). In addition, NSC-34 cells did not adhere significantly to KLH alone (Figs. 1*c*, 2*a*). Adhesion to both laminin and pET-RK36 was complete in 90 min at 37°C (Fig. 2*b*); however, cells spread on laminin but remained rounded on pET-RK36 (Fig. 1*a,b*; see also below).

Two observations demonstrated that adhesion of NSC-34 cells to pET-RK36 is mediated by specific, protein-associated cell surface receptors. First, adhesion to laminin and to pET-RK36 was prevented by lowering the temperature to 4°C, whereas NSC-34 cells adhered to poly-L-lysine comparably well at 4°C and 37°C (Fig. 2*c*). Second, treatment of NSC-34 cells with trypsin abolished their ability to adhere to laminin or pET-RK36 but not to poly-L-lysine; reincubation in trypsin-free medium for 4–6 hr restored the cells' adhesive properties (data not shown). Thus, adhesion to laminin and to pET-RK36 was "active" by the criteria of Grinnell (1978) and Turner and Flier (1989) in that it appeared to be energy dependent and mediated by cell surface receptors; in contrast, adhesion to poly-L-lysine was "passive" by these criteria.

We tested the LRE dependence of the adhesion of NSC-34

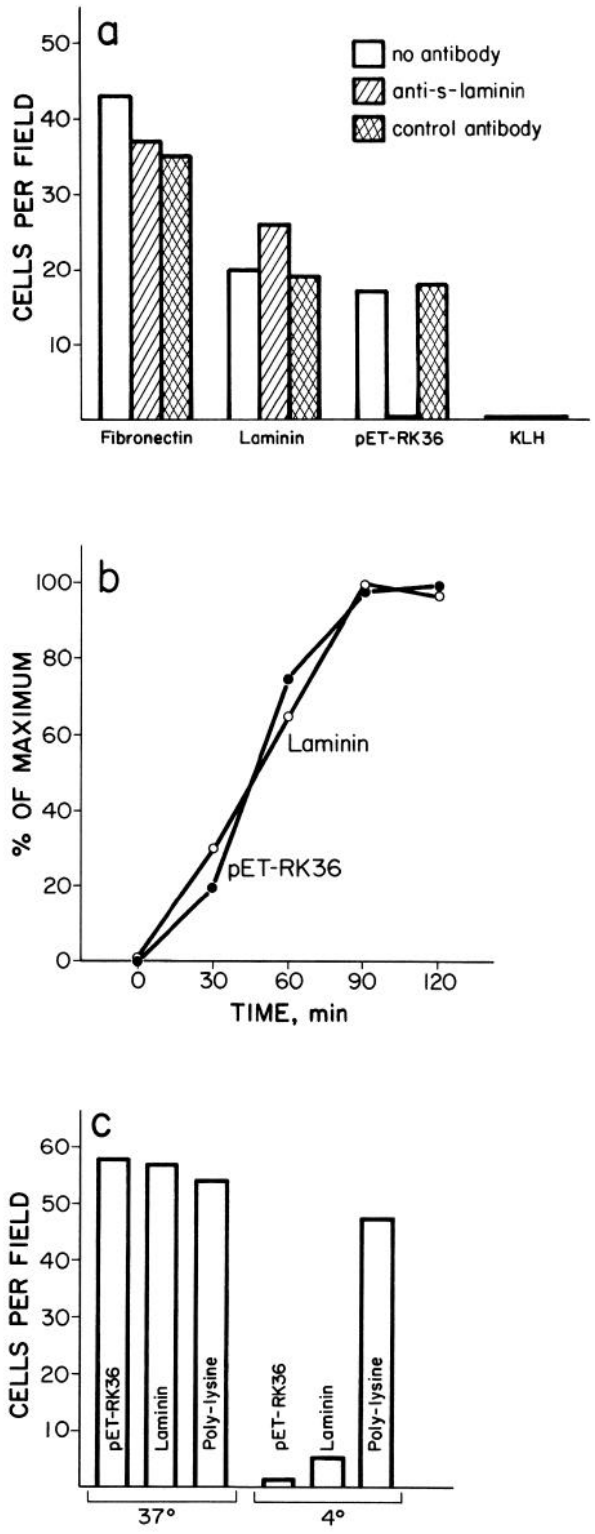

Figure 2. Adhesion of NSC-34 cells to pET-RK36 is specific, rapid, and active. *a,* Anti–s-laminin monoclonal antibody D5 inhibits adhesion to pET-RK36 but not to laminin or fibronectin. An irrelevant monoclonal antibody of the same class (C14; Sanes and Chiu, 1983) is inactive, and no adhesion to KLH is detectable. *b,* Adhesion to both pET-RK36 and laminin is detectable in 30 min and complete by 90 min under the conditions used. *c,* Fewer cells adhere either to pET-RK36 or to laminin at 4°C than at 37°C. In contrast, adhesion to poly-L-lysine is similar at 4°C and 37°C.

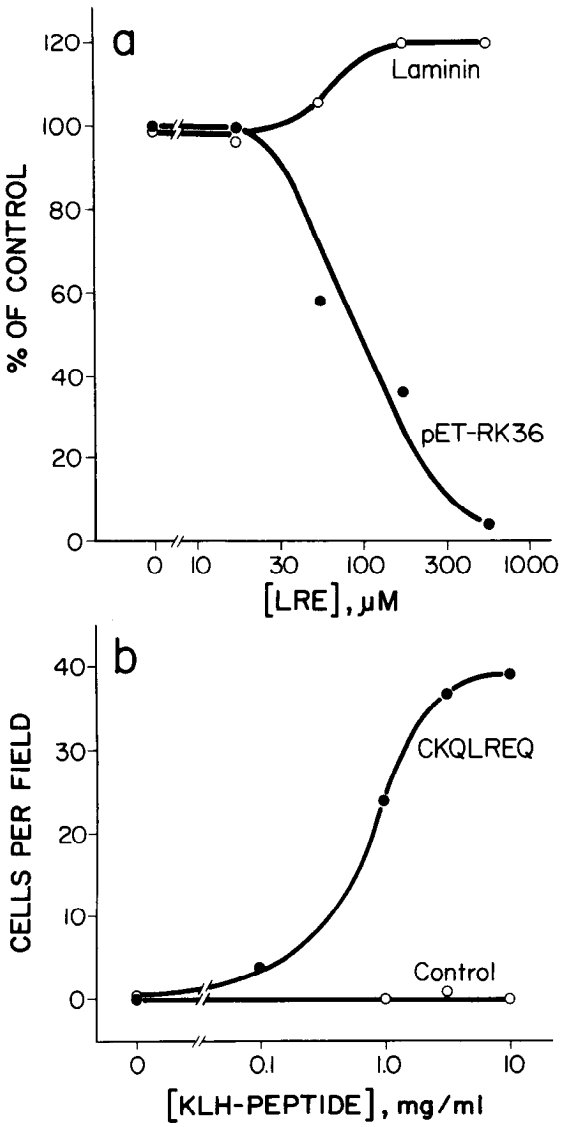

Figure 3. NSC-34 cells use an LRE-dependent adhesion mechanism. *a,* Soluble LRE inhibits adhesion to pET-RK36, but has no marked effect on adhesion to laminin. *b,* NSC-34 cells adhere to KLH that had been coupled to an LRE-containing peptide (KLH–CKQLREQ; *solid circles*); a control KLH–peptide conjugate (KLH–RYAAPVNRRKSRC; *open circles*) and KLH alone (see Fig. 2*a*) are inactive.

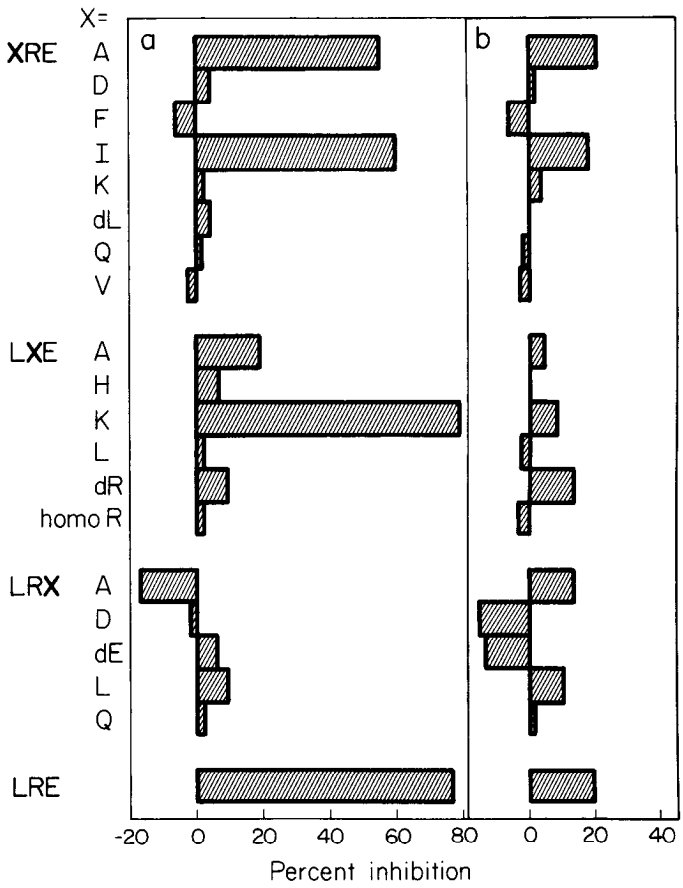

Figure 4. Stereoselectivity of the LRE-dependent adhesion mechanism. Adhesion of NSC-34 cells to pET-RK36 (*a*) or to laminin (*b*) was measured in control medium or in the presence of 100 μg/ml (~200 μM) of the indicated LRE-related tripeptides. *X* indicates the position of the residue substituted. None of the peptides inhibited adhesion to laminin significantly (<20%), whereas ARE, IRE, and LKE, as well as LRE, reduced adhesion to pET-RK36 by >50%. *d* prefix indicates dextro form. Values are means of four separate experiments, each of which included duplicate measurements.

cells to pET-RK36 in two ways. First, we asked whether soluble LRE inhibited adhesion. Indeed, LRE did block adhesion when added to the culture medium, with >90% inhibition at ~500 μM and an IC_{50} of ~80 μM (Fig. 3*a*). These concentrations are about two- to threefold higher than those that inhibited adhesion of ciliary neurons to pET-RK36 (Hunter et al., 1989b). LRE had no consistent effect on adhesion of NSC-34 cells to laminin (Fig. 3*a*; see also Fig. 4*b*), indicating that the action of the peptide was specific and did not interfere generally with NSC-34 metabolism or integrity. Second, we tested the ability of NSC-34 cells to bind to nitrocellulose coated with either of two LRE–protein conjugates (KQLREQ coupled to KLH via a cysteinyl residue added to the peptide's C- or N-terminus). Cells bound to both conjugates in a dose-dependent fashion, whereas no binding was seen to KLH alone or to a conjugate of KLH with an irrelevant peptide (Fig. 3*b*). Thus, NSC-34 cells are capable of specific LRE-dependent adhesion to LRE-containing substrata.

Selectivity of the LRE receptor

To assess the selectivity of the LRE-dependent adhesion system, we synthesized and tested a series of tripeptide LRE analogs in which each residue was systematically replaced by other amino acids. The tripeptides were dissolved in medium, and their ability to inhibit adhesion of NSC-34 cells to pET-RK36- or laminin-coated substrata was assayed. Initially, the peptides were tested at 100 μg/ml, or ~200 μM; this concentration of LRE is sufficient to inhibit adhesion of NSC-34 cells to pET-RK36 by ~80% and is twice the concentration required for 50% inhibition (Fig. 3*a*). At this concentration, only 3 of the 19 analogs tested—ARE, IRE, and LKE—significantly inhibited adhesion to pET-RK36 (Fig. 4*a*). In each case, the inhibition was specific, in that adhesion to laminin was not significantly affected (Fig. 4*b*). Dose–response curves indicated that the IC_{50} values were ~800 μM for ARE and IRE and ~200 μM for LKE; thus, these analogs were approximately 10% and 50% as effective as LRE by this measure. Of the other 16 analogs, 15 inhibited adhesion to pET-RK36 by <10% at 200 μM and are thus likely to be at least

sixfold less effective than LRE (10% inhibition at ~30 μM). The remaining analog, LAE, inhibited adhesion by ~15% at 200 μM; we do not know if this inhibition is specific.

Our interpretation of these results is that LRE-dependent adhesion involves a cellular receptor that recognizes specific features of each residue in the tripeptide. When leucine is replaced by isoleucine or alanine in the first position, a partial but significant decrease in activity is observed. Both of these residues are similar to leucine in terms of their hydrophobic and conformational properties, but differ in their precise shapes. Interestingly, replacement with valine, which has similar hydrophobicity but different conformational propensities, results in an inactive compound. Conformational and/or stereochemical requirements in this position are further corroborated by the observation that the D-leucine replacement was inactive. In the second position, the positive charge presented by the guanidino-group of arginine is clearly important for activity, in that the only replacement that retains activity is lysine, which bears a positive charge on its ε-amine group. The position of this positive charge is apparently critical, as reflected in the inactivity of the homo-arginine derivative, which has one less methylene group than arginine between its peptide backbone and guanidino-group. Again, the stereochemistry at this position is important, as D-arginine is inactive. Finally, the importance of the glutamate residue is demonstrated by the findings that conservative substitutions that retain charge (aspartate) are isosteric and able to form hydrogen bonds (glutamine), or simply have different stereochemistry (D-glutamate), are all inactive.

In summary, the cellular LRE receptor apparently binds to its ligand by a combination of electrostatic and hydrophobic interactions, such that the charge and shape of each of the three residues must be maintained for proper recognition. In these respects, the selectivity of the LRE receptor for LRE resembles that of some integrins for the RGD sequence that comprises a crucial determinant of cell-binding site in several extracellular matrix molecules (Ruoslahti and Pierschbacher, 1987). In both cases, the receptor recognizes a tripeptide domain on a macromolecule, the recognition has strict stereochemical requirements, and the binding site is formed by the conjunction of a positive, a negative, and a hydrophobic residue.

Ionic requirements of LRE-dependent adhesion

Recent molecular biological analyses have revealed that many cell adhesion molecules can be classified into a small number of families; members of each family have related sequences and are thought to be evolutionary relatives as well (Albelda and Buck, 1990). In addition, some such families have distinctive requirements for divalent ions. Thus, the cadherins require calcium ions to function effectively (Takeichi, 1988), integrins generally require magnesium or calcium ions (Dransfield and Hogg, 1989; Loftus et al., 1990), and the immunoglobulin superfamily members are cation independent (Edelman, 1988). To learn whether the cellular LRE receptor might belong to one of these families of adhesion molecules, we asked whether adhesion of NSC-34 cells to pET-RK36 is cation dependent.

In the first experiment of this series, adhesion to fibronectin, laminin, and pET-RK36 was assayed in medium that contained 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+}, in the same medium plus 5 mM EDTA to reduce the concentrations of free Ca^{2+} and Mg^{2+} to <0.1 μM, and in the EDTA-containing medium supplemented with 20 mM Mg^{2+} (Fig. 5*a*). Addition of EDTA completely abolished adhesion of NSC-34 cells to fibronectin and laminin; adhesion was completely restored when excess Mg^{2+} was added back. These results are consistent with a large body of data showing that many cells, including neurons, use Mg^{2+}-dependent integrins to adhere to these extracellular matrix molecules (Albelda and Buck, 1990; Hemler, 1990; Reichardt and Tomaselli, 1991). In contrast, more cells adhered to pET-RK36 in the presence of EDTA than in its absence, and this potentiation was not greatly affected by the presence of excess Mg^{2+}. Thus, adhesion to pET-RK36 did not require Mg^{2+} or Ca^{2+} at ≥1 μM and appeared to be inhibited by "physiological" concentrations of Ca^{2+}.

Based on these results, we varied the concentrations of Mg^{2+} and Ca^{2+} systematically to test their effects on adhesion. Adhesion of NSC-34 cells to laminin required Mg^{2+} ions at >0.1 mM, whether or not Ca^{2+} was present, but was unaffected by Ca^{2+}, whether or not Mg^{2+} was present (Fig. 5*b,d*). In contrast, adhesion to pET-RK36 was Mg^{2+} independent whether or not Ca^{2+} was present but was inhibited by Ca^{2+} whether or not Mg^{2+} was present (Fig. 5*c,d*). The degree of inhibition varied somewhat from experiment to experiment, but was generally ~50% at 0.3–0.5 mM and sometimes reached 80% at 1–3 mM (Fig. 5*d*).

Two general classes of mechanism can be envisioned by which Ca^{2+} might inhibit adhesion of cells to pET-RK36. In one, the affinity of the receptor–ligand interaction differs depending on whether or not Ca^{2+} is bound to some element of the adhesion mechanism, for example, the substrate, the cellular LRE receptor, or another cell surface component. In these cases, inhibition by Ca^{2+} would be expected to be readily reversible. Alternatively, the presence of Ca^{2+} might reduce the number of cellular binding sites or substrate molecules available for adhesion, for example, by activating a Ca^{2+}-dependent protease that degrades the substrate or the LRE receptor, or by inducing internalization of the receptor. In these cases, inhibition by Ca^{2+} would be irreversible, at least until the cells could regenerate new surface components. To distinguish these alternatives, we exposed either the NSC-34 cells or the substrate (pET-RK36) to 5 mM calcium for 90 min, then chelated excess Ca^{2+} with EDTA before measuring adhesion during the subsequent 90 min. In no case did preincubation with Ca^{2+} detectably affect adhesion; that is, equal numbers of NSC-34 cells adhered to pET-RK36 regardless of whether the cells, the substrate, both, or neither had been preincubated with Ca^{2+} directly before addition of EDTA (data not shown). Thus, inhibition of binding by Ca^{2+} is readily reversible. Furthermore, binding of NSC-34 cells to the LRE–protein conjugate (KLH–CKQLREQ) was inhibited by Ca^{2+} (data not shown); because it is unlikely that the conformation of this substrate is markedly Ca^{2+} dependent, this result suggests that Ca^{2+} acts by binding to the cells rather than to the substrate. Together, these results are most consistent with the idea that calcium ions modulate the affinity of a cellular LRE receptor for pET-RK36.

Effects of LRE-dependent adhesion on neurite outgrowth

Laminin is a potent promoter of neurite outgrowth (Sanes, 1989), and laminin-derived peptides have been shown to bear outgrowth-promoting activity (Tashiro et al., 1989; Beck et al., 1990). It was therefore interesting to ask whether LRE-dependent adhesion might affect process outgrowth. As shown above, we were able to use a relatively short LRE-containing peptide (CKQLREQ), coupled to a carrier protein, as a substrate for adhesion. To assay the effects of this peptide on neurite out-

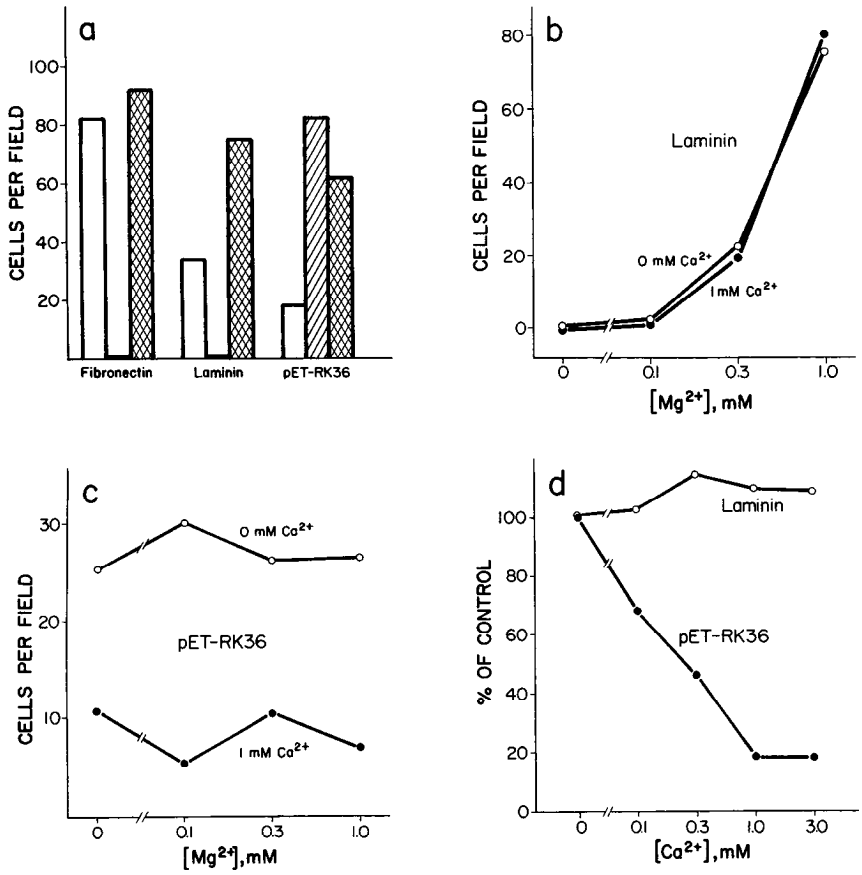

Figure 5. Cation dependence of the adhesion of NSC-34 cells to pET-RK36 and to laminin. *a,* Adhesion was measured in medium containing 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+} (*open bars*), in the same medium to which 5 mM EDTA had been added (*hatched bars*), or in the EDTA-containing medium to which 20 mM Mg^{2+} had been added (*cross-hatched bars*). Adhesion to laminin (and to fibronectin) was inhibited by chelation of divalent cations, whereas adhesion to pET-RK36 was enhanced. The inhibition but not the enhancement was reversed by readdition of Mg^{2+}. *b,* Adhesion to laminin is Mg^{2+} dependent, in the presence or absence of 1 mM Ca^{2+}. *c,* Adhesion to pET-RK36 is unaffected by Mg^{2+}, in the presence or absence of 1 mM Ca^{2+}. *d,* In the presence of 0.8 mM Mg^{2+}, adhesion to pET-RK36 is inhibited by Ca^{2+}, whereas adhesion to laminin is unaffected by Ca^{2+}.

growth, we examined the morphology of NSC-34 cells plated on laminin, KLH–CKQLREQ, and mixtures of the two.

Representative fields of NSC-34 cells plated on pET-RK36, the KLH–CKQLREQ conjugate, or laminin are shown in Figure 6. Some cells adhered to all substrates within 30 min; the cells remained rounded on pET-RK36 (Fig. 6*a*) or KLH–KQLREQ (not shown) but spread rapidly on laminin (Fig. 6*b*). By 90 min, processes had begun to appear on laminin, whereas cells plated on the s-laminin fragment or peptide remained rounded and bore no processes (Fig. 6*c,d*). By 180 min, the different effects of the substrata were marked (Fig. 6*e,f*). These results show that, although KLH–CKQLREQ, pET-RK36, and laminin all support adhesion, only laminin supports rapid process outgrowth.

In order to determine whether the s-laminin peptide inhibited process outgrowth, rather than merely failing to support it, we examined outgrowth on laminin substrates overlaid with KLH–peptide conjugates. The striking result was that KLH–CKQLREQ inhibited process outgrowth on laminin, whereas a control KLH–peptide conjugate and KLH alone were without detectable effect (Figs. 6*g,h*; 7*b*). A concentration–effect curve for KLH–CKQLREQ indicates that it inhibited process outgrowth in the same range of concentrations that promoted adhesion (Fig. 7*c*; cf. Fig. 3*b*). Because the KLH–peptide conjugates were added to surfaces that were already coated with laminin, the effects observed cannot be attributed to competition between laminin and KLH–peptide for binding sites on the nitrocellulose substrate. Additionally, similar numbers of cells adhered to all substrata (Fig. 7*a*), indicating that the reduction in the number of cells with processes reflects a reduction in the percent of adherent cells with processes, rather than a reduction in total adhesion. Thus, activation of the LRE receptor appears to inhibit outgrowth on laminin.

LRE-dependent adhesion to native basal laminae

The experiments reported thus far involve adhesion of cells to LRE-containing recombinant proteins or synthetic peptides that are derived from the primary sequence of s-laminin. An important issue that this work does not address is whether LRE is exposed and active in the environments that neurons or neurites actually encounter *in vivo*. In fact, some adhesive sites demonstrable on fragments of laminin are now believed to be inaccessible or "latent" in the native molecule (Nurcombe et al., 1989), and others may be masked in the complexes that laminin forms with other matrix molecules (e.g., Muir et al., 1989).

To address this issue, we used a "cryoculture" assay in which cells are plated on cryostat sections of unfixed adult tissues (Covault et al., 1987). We previously showed that neurites of chick ciliary neurons grow along basal laminae in several tissues in this assay, and that the neurites stop growing at synaptic sites on sections of skeletal muscle. For the present work, we used adult kidney as a source of tissue, because sections of renal cortex contain large, easily discerned areas of glomerular basal laminae, which are rich in s-laminin, as well as large areas of tubular basal laminae, which are devoid of s-laminin (Fig. 8*a,b*; Hunter et al., 1989a; Sanes et al., 1990).

When NSC-34 cells were plated on cryostat sections of rat kidney in Mg^{2+}-containing culture medium and incubated at

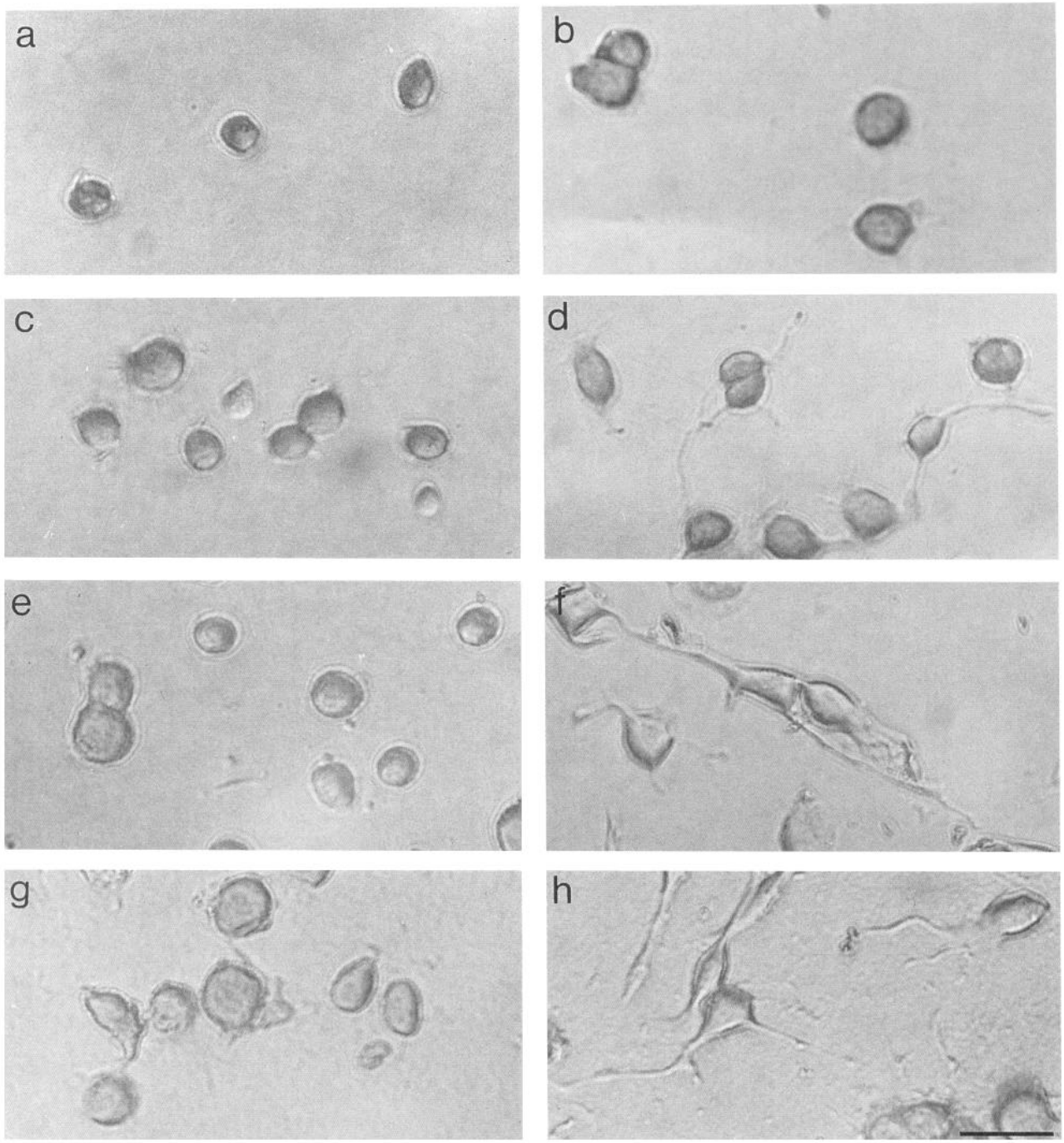

Figure 6. Substrate-dependent process outgrowth from NSC-34 cells. Cells were incubated on pET-RK36 (*a, c, e*), laminin (*b, d, f*), laminin overlaid with KLH–CKQLREQ (*g*), or laminin overlaid with KLH–RYAAPVNRRKSRC (*h*). After 30 (*a, b*), 90 (*c, d*), or 180 min (*e–h*), unbound cells were rinsed off and bound cells were photographed. NSC-34 cells extended processes on laminin but not on pET-RK36. KLH–CKQLREQ inhibited the process outgrowth that laminin promoted, whereas the control KLH–peptide conjugate was inactive. Cells plated on KLH–CKQLREQ alone were indistinguishable in morphology from those plated on pET-RK36. Scale bar, 100 μm.

37°C for 1 hr, numerous cells adhered to both tubules and glomeruli (Fig. 8*c,d*). In contrast, when the cells were incubated in Ca^{2+}- and Mg^{2+}-free medium, many cells adhered to glomeruli but few adhered to tubules (Fig. 8*e,f*). In the assay illustrated in Figure 8 *e* and *f*, for example, the density of bound cells was 39.7 ± 4.8 per mm^2 over glomeruli and 2.04 ± 0.24 per mm^2 over tubules (mean ± SEM for 15 10× microscope fields), or a 20-fold "preference" for glomeruli. Qualitatively similar results were obtained in five separate experiments, although the absolute number of adherent cells varied from experiment to experiment. In light of the studies on ion dependence detailed above, a reasonable explanation of this result is that adhesion to laminin-rich tubular basal lamina is mediated by Mg^{2+}-requiring integrins, whereas adhesion to s-laminin–rich glomerular basal lamina is mediated at least in part by an Mg^{2+}-independent, Ca^{2+}-inhibited LRE receptor. To test this idea, we assayed the adhesion of NSC-34 cells to cryostat sections in the presence of LRE. LRE had no discernible effect on adhesion in

control medium (not shown), but almost completely blocked the preferential adhesion of cells to glomeruli in Ca^{2+}- and Mg^{2+}-free medium (Fig. 8*g,h*; in this experiment, the "preference" of neurons for glomeruli over tubules was 1.6-fold in the presence of LRE, compared to 20-fold in its absence). Thus, NSC-34 cells can adhere to native basal laminae via an LRE-dependent mechanism, indicating that LRE-like ligands are accessible and active in native basal laminae.

Discussion

We have characterized the binding of NSC-34 cells to pET-RK36, a 20 kDa, LRE-containing fragment of s-laminin. Adhesion appears to depend critically on the LRE sequence within the fragment, in that it is inhibited by soluble LRE and mimicked by substrata coated with an LRE–protein conjugate. The LRE-dependent adhesion system is "active" (temperature dependent), protein associated (protease sensitive), stereochemically specific (as determined by use of a panel of LRE analogs), and capable of recognizing ligands in native basal laminae (as shown in the cryoculture assay). All of these features support the idea that NSC-34 cells bear a specific receptor that recognizes the LRE tripeptide within proteins. NSC-34 cells are a promising source for the isolation of LRE receptors, and the pharmacological analysis presented here (e.g., on the ionic dependence and stereochemical features of the binding) will be useful in assessing whether LRE-binding moieties correspond to the physiological receptor.

LRE-dependent adhesion of motoneuron-like cells

Of 10 cell lines tested, the only one that displayed robust LRE-dependent adhesion was NSC-34. In independent experiments, which will be detailed elsewhere (N. R. Cashman et al., unpublished observations), NSC-34 cells were shown to differ from their N18TG2 parents in ways that suggest they may have been derived from a spinal motoneuron. In brief, NSC-34 cells (1) express high levels of ChAT, (2) synthesize and store ACh, (3) release ACh in response to depolarization, (4) express cell surface antigens that are also present on cultured primary motoneurons, and (5) form morphologically stable contacts with and induce contractile activity in cocultured myotubes. In short, of many cell lines tested, the one that adheres best to pET-RK36 is the one that exhibits several properties of motoneurons.

The finding that NSC-34 cells exhibit LRE-dependent adhesion supports our previous suggestion that LRE comprises a motoneuron-selective attachment site on s-laminin. So far, the only other cells that we have found to be capable of LRE-dependent adhesion are chick ciliary neurons, which innervate striated muscle *in vivo* and thus are motoneurons (Hunter et al., 1989b). Other primary cells tested, as well as other cell lines, do not adhere appreciably to LRE-containing s-laminin fragments, even under conditions where they bind well to laminin. Moreover, although it is a somewhat circular argument, the observation that NSC-34 cells bear an LRE-dependent adhesion system supports the contention that they are motoneuron-de-

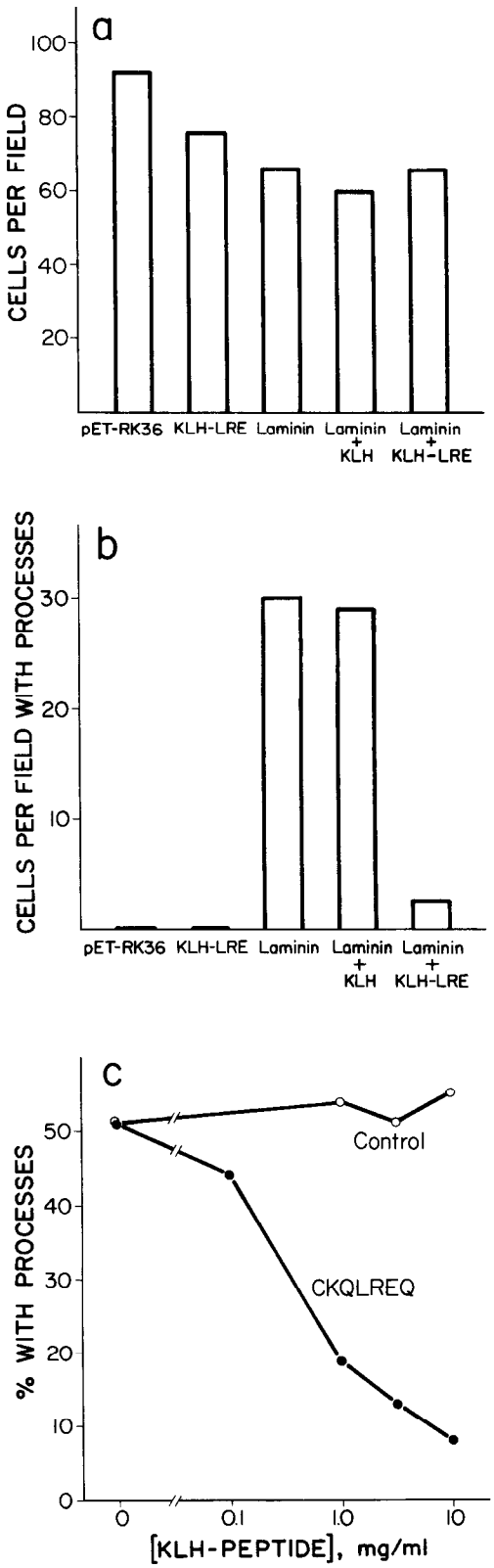

Figure 7. Inhibition of process outgrowth from NSC-34 cells by an LRE–protein conjugate. *a* and *b*, Cells were incubated for 180 min on the indicated substrates, and then unbound cells were rinsed off and bound cells were counted and scored for process outgrowth. Values are averaged from two experiments; similar results were obtained in three additional experiments. Processes were counted if they were more than one cell body diameter in length. *a*, Similar numbers of cells adhered to all substrates tested. *b*, Many cells extended processes on laminin overlaid with KLH, whereas few cells extended processes on pET-RK36, KLH–CKQLREQ, or laminin overlaid with KLH–CKQLREQ. *c*, In another experiment, laminin was overlaid with varying concentrations of KLH–CKQLREQ or KLH–RYAAPVNRRKSRC (control). The LRE-containing conjugate inhibited process outgrowth at 1 mg/ml, whereas the control conjugate was without effect at 10 mg/ml.

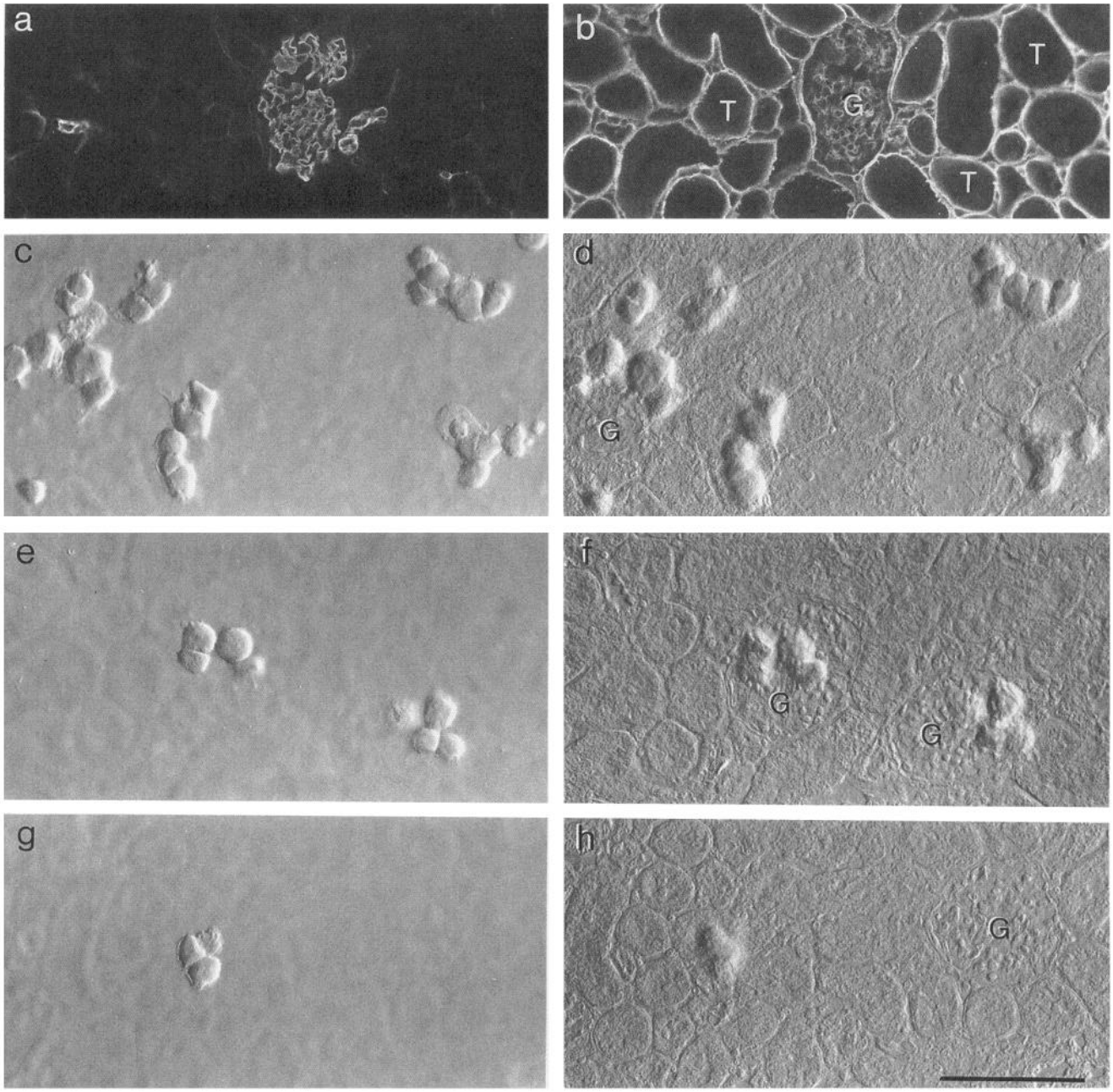

Figure 8. Attachment of NSC-34 cells to cryostat sections of kidney in the "cryoculture" assay. *a* and *b,* This section was double labeled with a monoclonal antibody to s-laminin (*a*) and a polyclonal antibody to laminin (*b*), plus appropriate second antibodies, to show that glomeruli (*G*) have s-laminin–rich basal laminae, whereas tubules (*T*) have s-laminin–poor basal laminae. Tubules and glomeruli are easily distinguished with Nomarski optics in *d, f,* and *h. c* and *d,* Adhesion in Mg^{2+}-containing medium. Cells adhere to both tubules and glomeruli. *e* and *f,* Adhesion in Mg^{2+}- and Ca^{2+}-free medium. Many more cells adhere to glomeruli than to tubules. *g* and *h,* Adhesion in Mg^{2+}- and Ca^{2+}-free medium in the presence of 200 μM LRE. Adhesion to glomeruli is reduced. Both micrographs in each pair show the same field; the first (*c, e, g*) is focused on the cells, while the second (*d, f, h*) is focused on the section. Fewer cells than usual are attached in *c* and *d*; this field was chosen to permit visualization of the underlying section. Scale bar, 100 μm.

rived cells. On the other hand, some points of uncertainty prevent unqualified acceptance of three conclusions. First, we have not yet shown that adhesion to native s-laminin is LRE dependent. To do so will require elucidation of the oligomeric structure of s-laminin and preparation of pure s-laminin under nondenaturing conditions; these studies are in progress (Engvall et al., 1990; Green et al., 1990). Second, we have not yet demonstrated LRE-dependent adhesion of primary spinal motoneurons. Finally, in that s-laminin is present in several extramuscular basal laminae (Sanes et al., 1990a) and in developing cortex (Sanes et al., 1990b), it is reasonable to expect that other cell types will be found that recognize it. In fact, we have recently shown that some photoreceptors adhere to pET-RK36 (Hunter et al., 1990). Nonetheless, it remains attractive to speculate that the LRE receptor, when isolated, will prove to be a motoneuron-specific marker within defined regions of the nervous system, and that motoneurons will prove to be capable of interactions with native s-laminin.

Inhibition of LRE-dependent adhesion by calcium ions

Because several families of adhesion receptors have distinctive requirements for divalent cations, we studied the effects of Mg^{2+} and Ca^{2+} on the LRE-dependent adhesion system. Results presented in Figure 5 indicate that adhesion is unaffected by Mg^{2+} and inhibited by Ca^{2+}. These results are noteworthy in two respects. First, they suggest that the cellular LRE receptor is not one of the previously characterized members of the integrin superfamily. We had suspected that integrins might mediate adhesion to LRE because integrins serve as receptors for several extracellular matrix molecules, because several different integrins have been implicated in the adhesion of neurons to laminin, and because some integrins are known to recognize short peptide determinants (e.g., RGD) in their ligands (Ruoslahti and Pierschbacher, 1987). However, although patterns of divalent cation dependence vary considerably among integrins, all those studied to date require millimolar concentrations of a divalent cation, most are able to use Mg^{2+} for adhesion, and none are inhibited by Ca^{2+} in the absence of other divalent cations (Dransfield and Hogg, 1989; Albelda and Buck, 1990; Hemler, 1990; Loftus et al., 1990; Reichardt and Tomaselli, 1991; but see Lallier and Bronner-Fraser, 1990, for one possible exception). Furthermore, an antibody to the chick integrin β_1-subunit, JG22 (Greve and Gottlieb, 1982), inhibits adhesion of chick ciliary neurons to laminin but not to pET-RK36 (data not shown; antibodies to murine integrins were not available for tests of NSC-34 cells). While definitive classification of the LRE receptor awaits its purification, our results to date are not consistent with its assignment to any heretofore described family of adhesion molecules, including the integrins.

Second, the inhibition of LRE-dependent adhesion by Ca^{2+} occurs in the physiological range (the concentration of Ca^{2+} is 0.5–2 mM in most culture media, and ~1 mM in serum) and might therefore be physiologically relevant. For example, theoretical calculations (Attwell and Iles, 1979) and measurements *in vivo* (Krnjevic et al., 1982; see also Betz et al., 1989) suggest that extracellular Ca^{2+} concentrations may fall near synapses consequent to Ca^{2+} influx during synaptic transmission (but see Ginsburg and Rahamimoff, 1983). If such local decreases in $[Ca^{2+}]_o$ occurred during development, they might lead to strengthening of LRE-dependent adhesion. Interestingly, NSC-34 cells that bind to pET-RK36 in the absence of Ca^{2+} remain attached when Ca^{2+} is subsequently added back to the medium (B. Porter and J. R. Sanes, unpublished observations), suggesting that LRE-dependent adhesion may become Ca^{2+} insensitive (or be supplanted by a Ca^{2+}-insensitive mechanism) following an initial phase of attachment. Thus, a transient decrease in $[Ca^{2+}]_o$ could result in a long-lasting increment in adhesion that in turn could lead to stabilization and/or maturation of the synapse, a consequent increase in the strength of synaptic transmission, a further increment in LRE-dependent adhesion, and so on. In this way, the LRE receptor could provide a mechanism for the known activity dependence of synaptic strength (e.g., Shatz, 1990).

Inhibition of process outgrowth by LRE-containing ligands

Experiments in which laminin and LRE-containing peptides were mixed indicate that occupancy of the LRE receptor inhibits process outgrowth promoted by laminin (Figs. 7, 8). These results provide the first clue to the functional consequences of LRE-dependent adhesion. However, a variety of processes could result in an apparent inhibition of neurite outgrowth (Patterson, 1988; Muir et al., 1989; Keynes and Cook, 1990; Schwab, 1990), and further experiments will be required to distinguish among them. For example, activation of an LRE-dependent system could prevent the initiation of neurites, decrease their rate of elongation, or cause their retraction. These alternatives can be distinguished by studying process outgrowth on patterned substrates (cf. Letourneau, 1975), for example, by asking whether neurites growing on laminin stop at, cross onto, or turn away from stripes on which laminin is overlaid with KLH–CKQLREQ or s-laminin. Additionally, "stripe" experiments could, in principle, reveal whether interaction with LRE affects the local differentiation of a growing neurite into a nerve terminal, as either a cause or an effect of growth inhibition. Such experiments are perhaps better performed with ciliary neurons than with NSC-34 cells, because the neurites, terminal specializations, and synapses that ciliary neurons form *in vitro* have been characterized in detail (e.g., Covault et al., 1987; Dubinsky and Fischbach, 1990; Lupa et al., 1990). In fact, preliminary experiments have revealed that LRE-containing ligands can inhibit outgrowth from ciliary neurons, as they do from NSC-34 cells (Weis et al., 1989). Experiments to study the basis of this inhibition are now in progress.

A possible role for the LRE-dependent adhesion system in neuromuscular interactions

Our interest in the LRE-dependent adhesion system stems from studies that indicated that components of the basal lamina at the neuromuscular junction direct the preferential reinnervation of original synaptic sites by regenerating motor axons (Sanes et al., 1978). We previously showed that s-laminin is concentrated in synaptic basal lamina (Hunter et al., 1989a), and that LRE is a crucial determinant of an adhesion site on an s-laminin–derived fragment (Hunter et al., 1989b). Here, we have documented three features of LRE-dependent adhesion that support the notion that it plays a role in the interactions of motor axons with the synaptic cleft. (1) LRE-dependent adhesion is apparently motoneuron selective: it is displayed by two motoneuron-like cell types, ciliary motoneurons and NSC-34 cells, but not by any of a variety of other primary cells or cell lines tested. Correspondingly, motor axons can innervate or reinnervate striated muscle fibers, whereas other intramuscular axons (sensory and sympathetic) apparently cannot. (2) LRE-rich substrata not only fail to promote outgrowth from NSC-34 cells, but appear to inhibit outgrowth promoted by laminin. During reinnervation, many terminal branches of motor axons do not grow beyond synaptic sites, but instead differentiate there into nerve terminals. (3) Cells can adhere to s-laminin–rich areas in unfixed tissue sections via their LRE-dependent system, thus demonstrating that the LRE sequence is exposed and active in native basal laminae. Together with the observation that at least four proteins concentrated in synaptic basal lamina contain the LRE sequence (see introductory remarks), these results support the hypothesis that binding of an LRE receptor on motor axons to LRE at synaptic sites may mediate nerve–muscle interactions important for the formation and/or maintenance of synapses.

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