Development 113, 1069–1084 (1991)
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Avian neural crest cell attachment to laminin: involvement of divalent cation dependent and independent integrins

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Summary

The mechanisms of neural crest cell interaction with laminin were explored using a quantitative cell attachment assay. With increasing substratum concentrations, an increasing percentage of neural crest cells adhere to laminin. Cell adhesion at all substratum concentrations was inhibited by the CSAT antibody, which recognizes the chick β_1 subunit of integrin, suggesting that β_1-integrins mediate neural crest cell interactions with laminin. The HNK-1 antibody, which recognizes a carbohydrate epitope, inhibited neural crest cell attachment to laminin at low coating concentrations (>1 $\mu g\,ml^{-1}$; Low-LM), but not at high coating concentration of laminin (10 $\mu g\,ml^{-1}$; High-LM). Attachment to Low-LM occurred in the absence of divalent cations, whereas attachment to High-LM required >0.1 mM Ca^{2+} or Mn^{2+}. Neural crest cell adherence to the E8 fragment of laminin, derived from its long arm, was similar to that on intact laminin at high and low coating concentrations, suggesting that this fragment contains the neural crest cell binding site(s). The HNK-1 antibody recognizes a protein of 165 000 M_r which is also found in immunoprecipitates using antibodies against the β_1 subunit of integrin and is likely to be an integrin α subunit or an integrin-associated protein. Our results suggest that the HNK-1 epitope on neural crest cells is present on or associated with a novel or differentially glycosylated form of β_1-integrin, which recognizes laminin in the apparent absence of divalent cations. We conclude that neural crest cells have at least two functionally independent means of attachment to laminin which are revealed at different substratum concentrations and/or conformations of laminin.

Key words: extracellular matrix receptors, cell adhesion, laminin, integrin, neural crest, quail.

Introduction

Interactions between the extracellular matrix (ECM) and cell surface receptors are thought to play pivotal roles in cell movement and axon guidance. Antibodies that disrupt adhesion of cells to the extracellular matrix have been used to define a class of cell surface receptors in the integrin family. As a class, integrin receptors mediate adhesion of cells to a variety of extracellular matrix molecules including fibronectin, laminin, vitronectin, various collagens and tenascin (Horwitz *et al.* 1985; Buck *et al.* 1986; Hynes, 1987; Tomaselli *et al.* 1988; Bourdon and Ruoslahti, 1989). There is evidence that these transmembrane glycoproteins also interact with the cytoskeleton, thereby coupling the extracellular matrix to the cell's intracellular machinery (Buck and Horwitz, 1987; Burn *et al.* 1988). While some integrin receptors have specificity for a single molecule, several have been shown to bind multiple extracellular ligands (Buck and Horwitz, 1987).

An integrin receptor is a heterodimer composed of two non-covalently linked transmembrane subunits, α and β. There are at least twelve α and five β subunits that have been wholly or partially characterized. Different α subunits share a significant amount of sequence identity to each other, as do different β subunits, suggesting that these are families of related molecules. The α and β subunits have distinct and unrelated amino acid sequences. Three major classes of integrin receptors have been defined by their use of a common β subunit. The matching of one α subunit with one β subunit is thought to determine the specificity of the receptor complex. α subunits possess binding sites for divalent cations that are thought to be required for function (Cheresh *et al.* 1987). All α-β integrin heterodimers described to date have been shown to require the divalent cations Ca^{2+}, Mg^{2+} and/or Mn^{2+} for ligand binding (Edwards *et al.* 1988; Ignatius and Reichardt, 1988; Smith and Cheresh, 1988). The β subunits contain regions of abundant intrachain disulfide linkages, resulting in an apparent increase in relative molecular mass (M_r) by

SDS–PAGE under reducing conditions when compared with non-reducing conditions (Horwitz *et al.* 1985; Hynes, 1987).

Antibodies that perturb cell–substratum adhesion have been useful for defining the interactions important for cell movement and neurite outgrowth. The CSAT monoclonal antibody recognizes a β_1 integrin subunit on avian cells (Horwitz *et al.* 1985), and inhibits chick neurite outgrowth on laminin, fibronectin and type IV collagen *in vitro* (Bozyczko and Horwitz, 1986; Hall *et al.* 1987; Letourneau *et al.* 1988; Neugebauer *et al.* 1988). In addition, the CSAT antibody disrupts the attachment of neural crest cells to laminin and fibronectin *in vitro* (Bronner-Fraser, 1985) and the migration of cranial neural crest cells *in vivo* (Bronner-Fraser, 1986). The HNK-1 antibody can also perturb cell adhesion. The HNK-1 antibody recognizes a carbohydrate epitope present on leukocytes, neural crest cells, various neurons and neuron-supporting cells (Abo and Balch, 1981; Tucker *et al.* 1984). This epitope is found on several adhesion-related molecules, including N-CAM, Ng-CAM, myelin-associated glycoprotein, and tenascin/cytotactin (Kruse *et al.* 1984, 1985; Grumet *et al.* 1985; Hoffman and Edelman, 1987; Tan *et al.* 1987). The HNK-1 epitope also has been reported to be present on the avian β_1 subunit of integrin (Pesheva *et al.* 1987). The HNK-1 antibody perturbs cranial neural crest cell migration *in vivo* (Bronner-Fraser, 1987) and inhibits neural crest cell attachment to laminin *in vitro* but, unlike CSAT antibodies, it does not disrupt attachment to fibronectin (Bronner-Fraser, 1987). The HNK-1 epitope may be involved in heparin-dependent neurite outgrowth from dorsal root ganglion cells (Dow *et al.* 1988) and in cell adhesion and neurite outgrowth in the mouse cerebellum (Kunemund *et al.* 1988).

Neural crest cells are a convenient experimental system for studying the cell interactions involved in cell migration and differentiation because they migrate widely in embryos along pathways containing numerous ECM molecules and give rise to diverse cells types (LeDouarin, 1982). Interactions between neural crest cells and their surrounding extracellular matrix have been proposed to influence both their movement and differentiation (Newgreen and Thiery, 1980; Bronner-Fraser, 1985, 1986; Rogers *et al.* 1986; Runyan *et al.* 1986; Tan *et al.* 1987; Perris *et al.* 1989). One approach for characterizing cell–matrix interactions that may be important in neural crest development is to determine which receptors on the neural crest cell surface mediate interactions with the extracellular matrix. In the present study, we utilize a centrifugation assay (McClay *et al.* 1981) to analyze the adhesive interactions of neural crest cells with laminin and fibronectin. In contrast to typical qualitative migration assays, this approach provides a reproducible and quantitative measure of cell attachment for small cell numbers to defined substratum molecules at varied surface concentrations. The CSAT and HNK-1 antibodies were used to inhibit neural crest cell–matrix attachments at a variety of substratum and divalent cation concentrations. The results suggest that neural crest cells attach to laminin by at least two different mechanisms: one mediated by a divalent cation-independent integrin(s), which is blocked by the HNK-1 antibody, and the other by a divalent cation-dependent integrin(s), which is not blocked by the HNK-1 antibody.

Materials and methods

Materials

Extracellular matrix glycoproteins (mouse laminin and human fibronectin) were purchased from Collaborative Research Inc. (Bedford, MA). Rat laminin was purchased from Telios (La Jolla, CA). Mouse laminin–nidogen complex and purified laminin fragments (E8, E1′ and P1) were the generous gift of Dr Matts Paulsson; these molecules were tested for purity by HPLC (Paulsson *et al.* 1987). Heparin, dextran sulfate (M_r 500 000), poly-D-lysine and ovalbumin were purchased from Sigma (St Louis). CSAT antibodies were the generous gift of Dr A. F. Horwitz. The HNK-1 antibody producing hybridoma cell line was purchased from ATCC (Rockville, MD). The 20B4 antibody producing hybridoma cell line was obtained through Developmental Studies Hybridoma Bank (Iowa City, Iowa).

Purification of IgMs using protamine Sepharose beads

Preswollen Sepharose 4B (Pharmacia) was activated using 10 mg ml^{-1} cyanogen bromide at pH 11.0 for 5 min, and thoroughly washed using distilled water, followed by 100 ml borate-buffered saline. Protamine sulfate (8 mg ml^{-1}) in 100 mM $NaHCO_3$, pH 8–9, was added to these beads and incubated with gentle stirring for 12 h at 4°C. The beads then were washed thoroughly with 100 mM PBS, pH 7.4 (Hudson and Hay, 1980).

Before use, these beads were washed with 80 mM PBS, pH 7.4, containing 77 mM NaCl. Solution containing IgMs was added to the protamine–Sepharose beads and incubated with stirring for 3 h at 4°C. The beads then were packed into a column, washed thoroughly with 80 mM PBS, pH 7.4 containing 77 mM NaCl, and the IgMs were eluted using 80 mM PBS pH 7.4 containing 1.1 M NaCl. IgMs in solution then were precipitated by mixing equal volumes of the IgM containing solution and a 55 % solution of $(NH_4)_2SO_2$. The precipitate was centrifuged at 1500 *g* and the supernatant discarded. The pellet was resuspended in distilled water of a volume equal to that of the original sample, and the precipitation was repeated. The final pellet was resuspended in a minimal volume of distilled water and dialyzed against 100 mM PBS, pH 7.4, at 4°C for 48 h. The purity of the resulting IgM solution was ascertained by reducing and non-reducing SDS–PAGE. A single major band was seen with a molecular mass greater than 500 000 M_r under non-reducing conditions, and three bands were seen of 70 000, 25 000, and 20 000 M_r under reducing conditions by silver stain and on immunoblots using an anti-IgM antibody conjugated to horseradish peroxidase. These bands are characteristic of IgMs.

Small fragments of IgMs

An IgM solution (1 mg ml^{-1} in PBS pH 7.4) was digested with 0.01 mg ml^{-1} TPCK trypsin (Gibco) in 50 mM Tris pH 8.0 containing 150 mM NaCl and 20 mM $CaCl_2$ for 5 h at 37°C (Matthew and Reichardt, 1982; Bidlack and Mabie, 1986). 2-Mercaptoethanol was added to the solution to a final concentration of 10 mM and incubated for 5 min at 37°C. Soybean trypsin inhibitor was added to a final concentration

of 0.1 mg ml^{-1} and incubated for 5 min at 37°C to stop the enzymatic cleavage. Iodacetamide was added to a final concentration of 60 mM and incubated for 10 min at 25°C. IgM fragments then were dialyzed to 100 mM PBS at 4°C for 48 h. Fragments were separated by size using a Biogel P-200 column calibrated with catalase, IgG, BSA and ovalbumin (232 000, 150 000, 68 000 and 44 000 M_r respectively). Only fragments of >70 000 M_r were used in the present study. Fragments were concentrated using Amicon centricon tubes to a concentration of 1 mg ml^{-1}.

Neural crest cell primary cultures

Primary neural crest cultures were prepared from the neural tubes of Japanese quail embryos (*Coturnix coturnix japonica*). Embryos were incubated for 48 h, at which time their developmental age was comparable to that of chick stages 13–15. The region of the trunk consisting of the six to nine most posterior somites as well as the unsegmented mesenchyme was dissected away from the embryo. The neural tubes were isolated by trituration in 160 units ml^{-1} of collagenase (Worthington Biochemical, Freehold, NJ). After stopping the enzymatic reaction with MEM containing 15 % horse serum and 10 % chick embryo extract, neural tubes were plated onto fibronectin-coated culture dishes. After several hours, the neural crest cells migrated away from the explant.

Neural crest cultures for use in quantitative attachment assays were labelled by the addition of [^{3}H]leucine (10–50 μCi ml^{-1}) to their culture media 4 h after explantation. The cells were allowed to incorporate labelled leucine for 16 h before use in the assay. Cultures were scraped to remove the neural tube, notochord and occasional somite cells. The remaining neural crest cells were rinsed five times with blocking MEM [bMEM; minimal essential media (Gibco) containing 0.5 mg ml^{-1} ovalbumin (Sigma)]. Cells were removed by incubation in 5 mM EDTA in bMEM for 10 min at 37°C.

Cell attachment assay

Cell–substratum adhesion was measured as described by McClay *et al.* (1981). Briefly, fibronectin, laminin or ovalbumin were coated at the indicated concentrations in carbonate buffer (100 mM, pH 8.0; containing 1 mM $CaCl_2$ and 500 μM $MgCl_2$) onto polyvinyl chloride (PVC) microtitre plates by adsorption for 12 h at 25°C. Wells were washed extensively with PBS (5 times) and incubated with bMEM for 2 h at 25°C. Ovalbumin was routinely added to block non-specific binding. Wells were rinsed and filled with either bMEM containing the antibody to be tested, or blocking phosphate buffer (bPBS; 10 mM phosphate buffer, pH 7.4 containing 150 mM NaCl and 0.5 mg ml^{-1} ovalbumin) containing the divalent cation concentration to be tested. Aliquots of [^{3}H]leucine-labelled neural crest cells, rinsed to remove excess EDTA, were added to each test well. In those cases where cell adhesion was measured in the absense of divalent cations, cells were resuspended in Ca^{2+}/Mg^{2+}-free PBS containing 0.5 mg ml^{-1} ovalbumin and 1.5 mM EDTA. Neural crest cells were brought into contact with the substratum molecules by a centifugal force of 150 *g* for 5 min. Chambers were sealed, and incubated for 15 minutes at 37°C. Neural crest cells that failed to adhere strongly to the substratum molecules were removed by a centrifugal force of 50 *g* for 5 min. The chambers were quickly frozen in a methanol/dry ice bath. The tops (unbound cells) and bottoms (bound cells) of each well were removed and placed separately into scintillation vials. Samples were analyzed for ^{3}H cts min^{-1} using a scintillation counter (Beckman LS 5801). Individual wells contained between 5000–10 000 cts min^{-1} with background counts on the order of 20–50 cts min^{-1}. The percentage of bound neural crest cells per well was determined as follows:

$$\%\ \text{cells bound} = \%\ \text{cts min}^{-1}\ \text{bound} = \frac{\text{cts min}^{-1}\ \text{bound to substratum (bottom)} * 100\ \%}{\text{Total cts min}^{-1}\ \text{(bottom + top)}}$$

Within these experiments, non-specific binding to ovalbumin ranged from 5–15 % of the counts present within a given well. Variability between experiments using identical conditions was ~10 %. This variability is likely to be due to slight differences within the population of neural crest cells.

Comparisons between two values for percentage of neural crest cell attachment were deemed significantly different when the P value, determined by Student's one-sided t-test, was <0.05.

Measurement of substratum attachment to PVC plates

Radiolabelling of proteins (fibronectin, laminin and ovalbumin) was performed by reductive methylation as previously described by Tack *et al.* (1980). Briefly, proteins at final concentrations between 1 and 10 mg ml^{-1} were dialyzed against 200 mM borate buffer, pH 8.9, and 0.5 ml were placed in glass vials on ice for 1 h. 0.05 ml of 0.18 M formaldehyde was added to the sample, followed by 0.1 ml [^{3}H]$NaBH_4$ (2 mCi in 0.1 N NaOH) and allowed to react for 1 h on ice. The unbound [^{3}H]$NaBH_4$ was removed by running the sample through a disposable Sephadex G-25M column (Pharmacia). Protein concentrations were assayed using the BioRad protein assay following the manufacturer's instructions. Labelled proteins were serially diluted in carbonate buffer, pH 8.0, applied to wells in PVC microassay plates, and allowed to bind for 12 h at 25°C. Wells were washed five times with carbonate buffer, cut into individual wells and placed into scintillation vials for analysis.

Phalloidin staining of neural crest cells

Neural crest cells were isolated as described for cell attachment assays. Cells were brought into contact with fibronectin and laminin substrata in 35 mm polystyrene dishes (Falcon) under conditions similar to those employed in the cell attachment assay. Fifteen minutes after cells were introduced to the substrata, non-adherent cells were rinsed from the substrata with three washes of 100 mM PBS, while the adherent cells were fixed in a solution of 4 % paraformaldehyde in 100 mM PBS at 25°C for 1 h. Cells were labelled with FITC–phalloidin (Sigma) in a solution of 100 mM PBS containing 0.1 % saponin and 1 % horse serum for 1 h. Cells then were rinsed with 100 mM PBS and viewed using an Olympus Vanox epifluorescence microscope.

Immunoprecipitations

Immunoprecipitations were performed on cultures of surface labelled neural crest cells. Cells were isolated as described above (Neural Crest Cell Primary Cultures) and removed from their substratum by incubation in 5 mM EDTA for 5 min. Cells then were washed four times in labelling buffer (140 mM NaCl, 8 mM KCl, 2 mM glucose, 0.8 mM $MgSO_4$, 1.5 mM $CaCl_2$, 6 mM $NaHCO_3$, pH 7.4) and labelled with biotin as described by von Boxberg *et al.* (1990). Briefly, a solution of 50 μg ml^{-1} biotin-X-NHS (biotin-e-aminocaproic acid-*N*-hydroxy-succinimide ester, Calbiochem) in DMSO was added to a volume 5 % of the total volume of the cell suspension and incubated for 5 min at 25°C. Cells were washed 4 times with labelling buffer containing 100 mM NH_4Cl. Proteins then were extracted from these cells using an extraction buffer (20 mM Tris pH 7.2, 150 mM NaCl, 1 mM $CaCl_2$, 1 % Triton X-1000,

1 mM PMSF) for 1 h at 4°C. Extracts were cleared twice by incubation with Sepharose 4B for 1 h at 4°C. Antibodies were added to the extracts, incubated for 12 h at 4°C, followed by incubation with protein A-Sepharose for 1 h at 4°C. Antibody–antigen–protein A complexes were washed extensively with wash buffer (20 mM Tris pH 7.2, 150 mM NaCl, 1 mM $CaCl_2$, 0.1% Triton X-100, 1 mM PMSF). HNK-1 immunoprecipitations were performed using purified antibody covalently coupled to CNBr-activated Sepharose (Pharmacia) prepared using the manufacturers' instructions. In some cases, immunoprecipitations were performed in the presence of 5 mM EDTA and 1% SDS in order to disrupt integrin complexes. Immunodepletions were performed by repeating three cycles of immunoprecipitation with a given antibody on one sample, and then immunoprecipitating with a second antibody on that same sample. For immunoblots with the HNK-1 antibody, immunoprecipitations were performed on samples from trunk tissue dissected from 2.5 day quail embryos.

Immunoprecipitates then were separated by SDS–polyacrylamide gel electrophoresis (PAGE), carried out using a vertical slab gel system (Hoeffer; dimensions of 83 mm×57 mm×.75 mm) with a 4% stacking gel, utilizing the buffer system described by Laemmli (1970). Eight percent acrylamide gels were chosen to achieve optimal separation of proteins with M_r in the range of 250 000 to 50 000. Extracts of quail tissues were solubilized in buffer containing 4% SDS, 10% glycerol, 0.04 M EDTA and 0.1% PMSF in 0.125 M Tris–HCl. Some samples included 5% β-mercaptoethanol to reduce disulfide bonds before boiling for 5 min, though non-reducing conditions were used extensively to resolve integrin α subunits better. Proteins were electrophoretically transferred to nitrocellulose membranes using a Tris-glycine buffer system (Burnette, 1981). Apparent relative molecular masses were estimated by comparison to pre-stained high range molecular weight standards (BRL). Membranes were blocked with 5% BSA prior to incubation with ^{125}I- streptavidin (Amersham) for 1 h in TBST (10 mM Tris pH 7.2, 150 mM NaCl, and 1% Tween 20) containing 1% BSA. Membranes were air dried, and allowed to expose X-ray film (XAR5, Kodak) at −70°C, which was developed using D19 developer (Kodak).

Immunoblots

Immunoprecipitations of 2.5 day trunk tissue were performed using the CSAT antibody; proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes as described above. Membranes were incubated with the HNK-1 antibody (1 μg ml^{-1}) for 1 h in TBST (10 mM Tris pH 7.2, 150 mM NaCl, and 1% Tween 20) containing 1% BSA. Membranes were rinsed extensively, and incubated in ^{125}I-anti-mouse IgM/IgG/IgA secondary antibodies (0.1 μg ml^{-1}: Amersham) in TBST with 1% BSA for 1 h. Membranes were air-dried and allowed to expose X-ray film (XAR5, Kodak) as described above.

Results

To analyze neural crest cell adhesion to various coating concentrations of fibronectin and laminin, we have used a quantitative centrifugation attachment assay that allows reproducible measurement of the adhesive properties of small populations of cells. Roughly 10^5 cells per experiment were isolated from primary neural crest cell cultures within 24 h of explantation from the embryo. This corresponds to a time well before their detectable differentiation, thus providing a population of 'migrating' neural crest cells. The centrifugation attachment assay makes it possible to draw meaningful conclusions from diverse assay conditions using small numbers of cells. Radioactively labelled neural crest cells were placed into fluid-filled chambers to which antibodies, divalent cations, or heparin could be added. The chambers were sealed and a constant centrifugal force was used to bring the cells into contact with the fibronectin- or laminin-coated substrata. Non-adherent cells were removed by inverting the chamber and centrifuging at 50 *g*. Because cells were allowed to settle onto their substratum for 15 min prior to removing unbound cells, we define attachment as initial adhesion plus cell spreading.

Effects of laminin and fibronectin coating concentration on neural crest cell attachment

To quantitate the amount of substratum binding to the polyvinyl chloride (PVC), radioactively labelled fibronectin, laminin or ovalbumin were added to PVC wells at various coating concentration. The binding of all three radiolabelled molecules appeared sigmoidal with increasing coating concentrations, saturating between 10 and 100 μg ml^{-1} (Fig. 1).

The percentage of neural crest cells binding to fibronectin or laminin increased with increasing substratum coating concentration, over the range of 0.1 to 10 μg ml^{-1} (Fig. 2A and B). The maximal percentage of adhering cells (~85%) was observed between coating concentrations of 1 and 10 μg ml^{-1} for fibronectin and 3 and 10 μg ml^{-1} for laminin.

Effects of the monoclonal antibodies CSAT, HNK-1 and 20B4 on neural crest cell attachment

The CSAT antibody, which recognizes the β_1 subunit of the integrin α/β heterodimer, inhibited neural crest cell attachment to fibronectin at coating concentrations

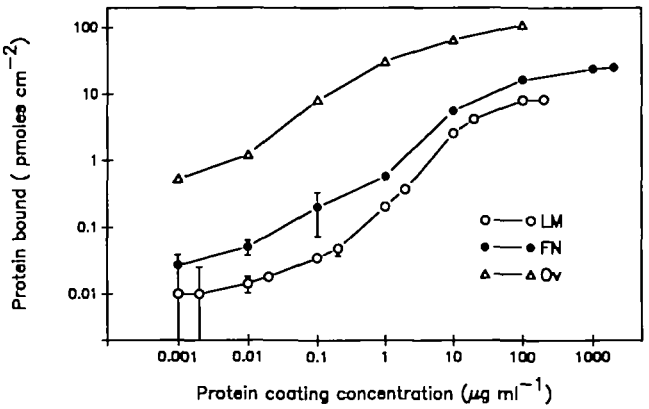

Fig. 1. Measurement of protein binding to polyvinyl chloride wells. Various concentrations of ^{3}H-labelled protein were coated to wells and their relative binding was assayed. Points represent the mean of eight experiments and the error bars represent the standard error of the mean (S.E.M). Proteins tested were fibronectin (FN), laminin (LM) and ovalbumin (Ov).

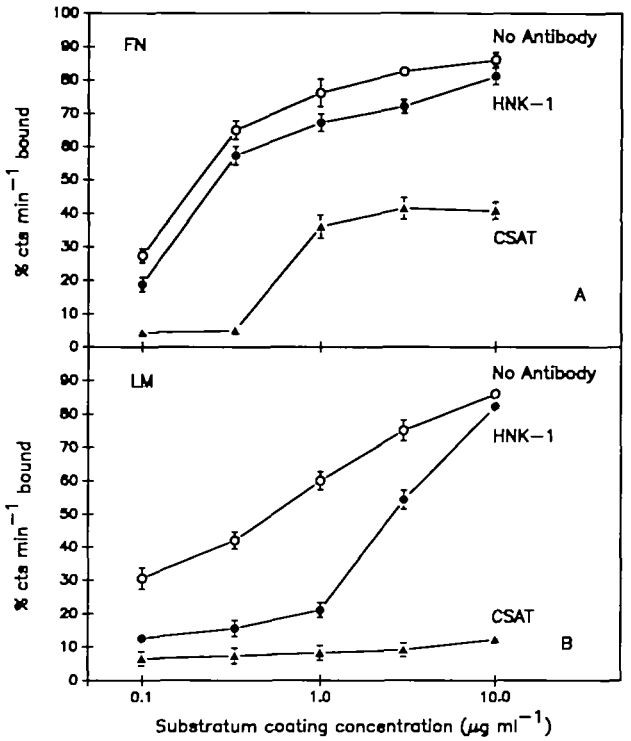

Fig. 2. Neural crest cell attachment to various concentrations of fibronectin and laminin in the presence of the CSAT and HNK-1 antibodies. Fibronectin (A) and laminin (B) were assayed for their abilities to promote neural crest cell attachment over coating concentrations ranging from 0.1 to 10 $\mu g\,ml^{-1}$. CSAT and HNK-1 antibodies (50 $\mu g\,ml^{-1}$) were added to test their ability to perturb cell adhesion over a range of substratum/coating concentrations. Points represent the mean of at least six experiments and the error bars represent the S.E.M.

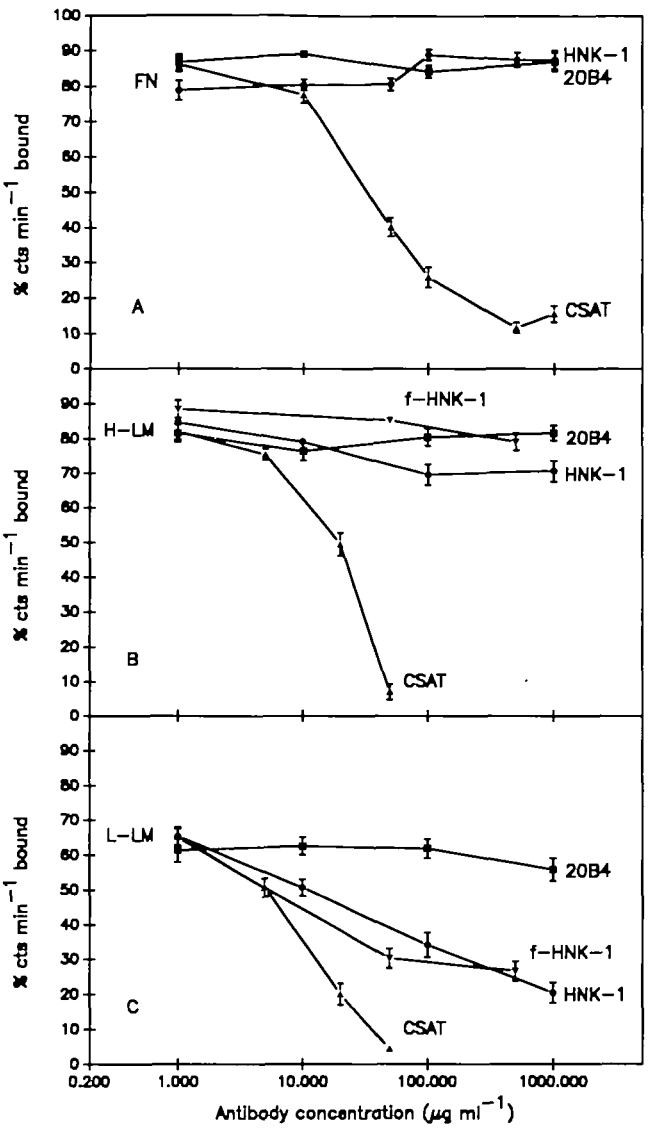

Fig. 3. Neural crest cell attachment to (A) 10 $\mu g\,ml^{-1}$ of fibronectin, (B) 10 $\mu g\,ml^{-1}$ of laminin, and (C) 1 $\mu g\,ml^{-1}$ of laminin were assayed in the presence of varied concentrations of CSAT, HNK-1, fragments of HNK-1 (>70 000 M_r), and 20B4 antibodies. Points represent the mean of at least six experiments and the error bars represent the S.E.M.

of 0.1 to 10 $\mu g\,ml^{-1}$ (Fig. 2A; 3A); inhibition was dependent on antibody concentration, with total abolition of cell binding to fibronectin at 500 $\mu g\,ml^{-1}$ of antibody (Fig. 3A). Neither HNK-1 nor 20B4 antibodies, which recognize epitopes on the neural crest cell surface (Tucker *et al.* 1984; Stern *et al.* 1989), inhibited neural crest cell adhesion to fibronectin at antibody concentrations of up to 1 $mg\,ml^{-1}$ (Fig. 3A).

Adhesion to all laminin substratum concentrations was completely abolished by the CSAT antibody (Fig. 2B) at a concentration of 50 $\mu g\,ml^{-1}$ (Fig. 3B and C), suggesting the involvement of β_1-integrin receptors. In contrast, neural crest cells exhibited differential sensitivity to the HNK-1 antibody at different coating concentrations of laminin (Fig. 2B). At low coating concentrations of laminin (Low-LM; $\leq 1\,\mu g\,ml^{-1}$), neural crest cell adhesion was inhibited in a dose-dependent manner with maximal inhibition (70%) occurring at 1 $mg\,ml^{-1}$ of HNK-1 antibody (Fig. 3C). With increasing laminin coating concentrations ($>1\,\mu g\,ml^{-1}$), neural crest cells became progressively less sensitive to the HNK-1 antibody and were unaffected at high coating concentrations of laminin (High-LM; 10 $\mu g\,ml^{-1}$) by antibody concentrations of up to 1 $mg\,ml^{-1}$ (Figs 2B and 3B). Because the HNK-1 antibody is an IgM, its large size could lead to non-specific steric hindrance. To circumvent this problem, small proteolytic fragments (>70 000 M_r) were prepared (Matthew and Reichardt, 1982). These monovalent HNK-1 fragments inhibited neural crest cell adhesion on Low-LM as efficiently as the intact antibody but had no effect on High-LM (Fig. 3B and C). The 20B4 antibody, which also binds to the surface of neural crest cells, had no effect on adhesion to high or low coating concentrations of laminin (Fig. 3B and C). The results suggest that there are at least two mechanisms by which neural crest cells attach to laminin: one that is HNK-1 insensitive and evident at

Table 1. *Comparison of neural crest cell attachment to laminin from different sources*

[LM] $\mu g\,ml^{-1}$	[HNK-1] $\mu g\,ml^{-1}$	[Ca] mM	Laminin source: Mouse LM (EHS) TL-MBF	Mouse LM (EHS) CR	Mouse LM (EHS) MP	Rat Telios
10	0	1*	82%±5%	73%±3%	85%±2%	81%±5%
	50	1*	83%±4%	74%±7%	82%±2%	82%±6%
	0	0	6%±1%	8%±3%	22%±7%	–
	0	1	–	69%±4%	89%±2%	–
1	0	1*	46%±3%	51%±2%	76%±4%	72%±1%
	50	1*	12%±3%	12%±2%	36%±7%	32%±5%
	0	0	44%±4%	44%±7%	47%±7%	–
	0	1	–	55%±5%	48%±7%	–

TL-MBF=Mouse laminin isolated in our laboratory.
CR=Mouse laminin purchased from Collaborative Research Inc.
MP=Mouse laminin-nidogen generously donated by Mats Paulsson.
Telios=Rat laminin purchased from Telios Inc.
*=MEM containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+}.

high substratum concentrations, and one that is HNK-1 sensitive and active at lower substratum concentrations.

The differential binding of neural crest cells to low *versus* high coating concentrations of laminin might be related to impurities in the mouse laminin. To test this possibility, we assayed neural crest cell adhesion to laminin purified in different ways from different sources. No significant differences were noted between mouse EHS laminin and rat laminin with respect to neural crest cell binding efficiency or the degree of inhibition by CSAT or HNK-1 antibodies at various substratum coating concentrations (Table 1). Furthermore, a mouse laminin–nidogen complex tested for purity by HPLC analysis (Paulsson *et al.* 1987) supported comparable attachment. Since neural crest cells do not attach to nidogen (Perris *et al.* 1989), this suggests that the observed binding is to laminin. An additional argument that impurities are unlikely to contribute to the binding characteristics of neural crest cells to laminin is that we observe 'typical' cell–laminin interactions at high coating concentrations of laminin, which would be expected to contain the highest amount of impurities. At lower concentrations, in which 'unusual' cell–laminin interactions are observed, the concentrations of impurities would be decreased by one or more orders of magnitude. We also tested for the possibility that the HNK-1 antibody was blocking adhesion of neural crest cells to laminin by directly binding to carbohydrate chains on the laminin molecule. In studies using biotinylated HNK-1 antibody, the HNK-1 antibody did not bind significantly to laminin substrata at either the high or low coating concentrations (data not shown).

Divalent cations dependence of neural crest cell attachment to laminin

It has been suggested that integrin receptor heterodimers require divalent cations (Ca^{2+}, Mg^{2+}, or Mn^{2+}) for binding to their ligands (Hynes, 1987). We examined the divalent cation requirements of neural crest cell attachment to both High-LM and Low-LM coating concentrations (10 and 1 $\mu g\,ml^{-1}$ respectively). Attachment to High-LM required >100 mM of either Ca^{2+} or Mn^{2+}, both of which were equally efficient at promoting attachment, while concentrations of up to 1 mM Mg^{2+} did not promote adhesion (Fig. 4). In the presence of divalent cations, neural crest cells attached to both High-LM and Low-LM, though binding to High-LM was significantly greater ($P<0.001$) than to Low-LM (~80% versus ~50% of total cells; Fig. 8A and B). In contrast, with divalent cation concentrations below 100 μM, neural crest cells exhibited significant attachment to Low-LM (~50%; Fig. 4 and 8B) but not to High-LM (Fig. 4 and 8A; $P<0.01$). Attachment of neural crest cells to Low-LM in the absence of divalent cations was inhibited significantly by both the HNK-1 and CSAT antibodies ($P<0.01$; Fig. 5), suggesting that the adhesion is mediated by β_1-integrin receptors. We define the absence of divalent cations as incubation conditions containing 1.5 mM EDTA, with no supplementation of divalent cations.

Effects of antibody and divalent cation concentration on neural crest cell attachment to polyanionic and polycationic substrata

Neural crest cells can adhere to some substrata by means of charge interactions that are not mediated by specific receptor–ligand recognition. To test the specificity of the antibodies used in this study, neural crest cells were grown on four charged substratum molecules: heparin, dextran sulfate, polylysine and ovalbumin. These were chosen because they represent both polyanionic and polycationic substrata, as well as the blocking protein used in the present experiments. Neural crest cells were allowed to attach to these substrata in the presence (Fig. 6A) and absence (Fig. 6B) of divalent cations. The HNK-1 and CSAT antibodies failed to inhibit neural crest cell attachment to any of these substrata ($P>0.1$). The ability of these antibodies to block attachment to laminin but not to

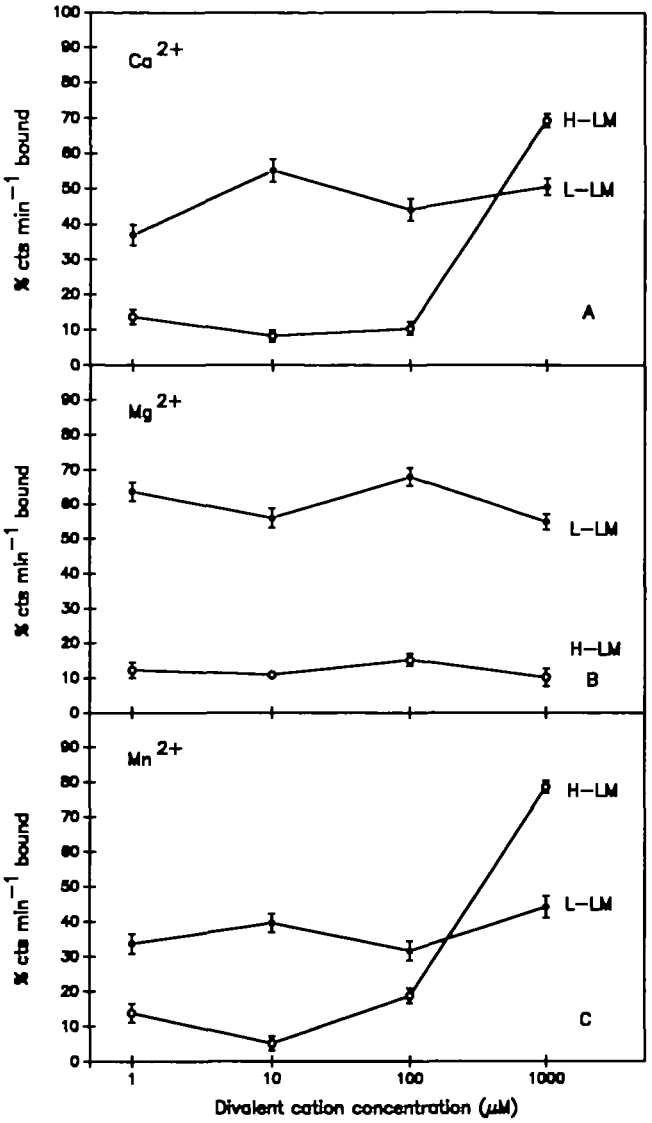

Fig. 4. The dependence of neural crest cell attachment to laminin on divalent cations. Two coating concentrations of laminin, $10\,\mu g\,ml^{-1}$ (H-LM) and $1\,\mu g\,ml^{-1}$ (L-LM), were tested. Divalent cations, (A) Ca^{2+}, (B) Mg^{2+}, and (C) Mn^{2+}, were tested over a range of concentrations from $1\,\mu M$ to 1 mM. Points represent the mean of at least six experiments and the error bars represent the S.E.M.

polyionic substrata suggests that attachment to laminin is mediated by specific cell surface receptors.

Neural crest cell attachment to fragments of laminin

To determine the region of laminin to which neural crest cells bind, we tested proteolytic fragments of the intact laminin molecule for their ability to promote attachment in the presence or absence of the HNK-1 and CSAT antibodies ($50\,\mu g\,ml^{-1}$; Fig. 7). Three fragments were utilized: the E8 fragment, corresponding to the C-terminal 'long arm' of laminin including the heparin-binding domain; the E1′ fragment, corresponding to the N-terminal three 'short arms' of laminin; and the P1 fragment, corresponding to the

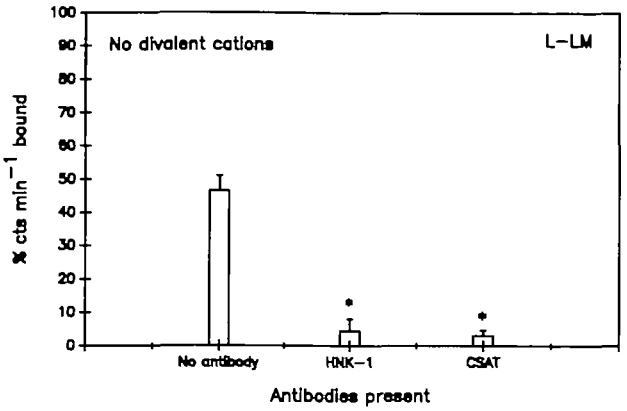

Fig. 5. Neural crest cells were assayed for their ability to attach to $1\,\mu g\,ml^{-1}$ of laminin (L-LM) in the absence of the divalent cations. The CSAT and HNK-1 antibodies ($50\,\mu g\,ml^{-1}$) were tested for their ability to inhibit neural crest cell attachment to L-LM in the absence of divalent cations. Asterisks (*) indicate significant ($P<0.05$) differences from adhesion in the absence of divalent cations. Points represent the mean of at least six experiments and the error bars represent the S.E.M.

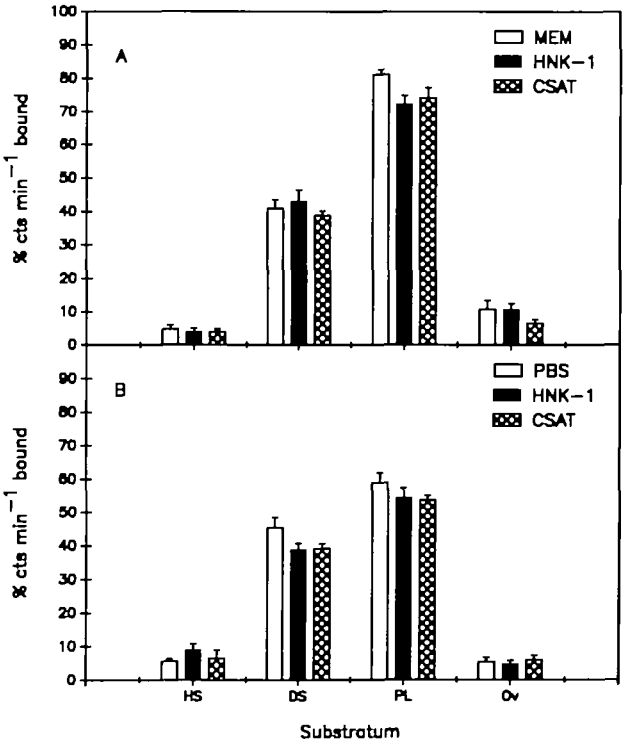

Fig. 6. Neural crest cells were assayed for their ability to attach to heparin (HS), dextran sulfate (M_r 500 000) (DS), poly-D-lysine (PL), and ovalbumin (Ov) with and without the CSAT or HNK-1 antibodies ($50\,\mu g\,ml^{-1}$). Adhesion was measured in the presence (A) and absence (B) of divalent cations (1 mM Ca^{2+} and 0.5 mM Mg^{2+}). Asterisks (*) indicate significant ($P<0.05$) differences from adhesion in the absence of antibodies. Points represent the mean of at least six experiments and the error bars represent the S.E.M.

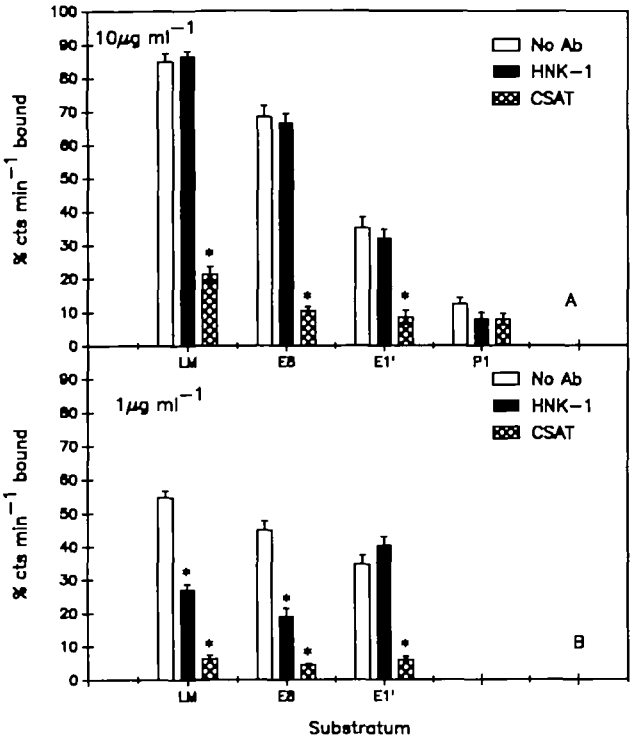

Fig. 7. Proteolytic fragments of laminin were compared to intact laminin for their ability to promote neural crest cell adhesion in the presence and absence of antibodies. Laminin, the E8 (constituting the long arm of laminin), the E1′ (constituting the three short arms of laminin) and the P1 (constituting the cross region of laminin) fragments were coated onto the substratum at concentrations of equivalent to 10 (A) and 1 $\mu g\,ml^{-1}$ (B) of laminin. The CSAT and HNK-1 antibodies (50 $\mu g\,ml^{-1}$) were tested for their ability to inhibit neural crest cell adhesion to laminin and its proteolytic fragments. Asterisks (*) indicate significant ($P<0.05$) differences from controls. Points represent the mean of at least six experiments and the error bars represent the S.E.M.

'cross' region of laminin. The purity of these fragments was determined using HPLC analysis (Paulsson *et al.* 1987). Coating concentrations of laminin fragments were chosen at molar equivalents to those used for intact laminin; for simplicity, we refer to the concentrations used for the *intact* molecule. The E8 fragment of laminin coated at 1 or 10 $\mu g\,ml^{-1}$ equivalents promoted attachment nearly as well as the intact molecule (~45% and ~70%, respectively; $P>0.1$). The E1′ fragment promoted attachment of ~35% of the cells at 1 or 10 $\mu g\,ml^{-1}$. The P1 fragment promoted no significant attachment at 10 $\mu g\,ml^{-1}$. CSAT antibodies inhibited neural crest cell adhesion to the E8 and E1′ fragments by ~85% and ~75% ($P<0.001$ and <0.01), respectively, at a substratum coating concentration of 10 $\mu g\,ml^{-1}$. At this substratum concentration, HNK-1 antibodies caused no significant inhibition of adhesion ($P>0.1$). In contrast at 1 $\mu g\,ml^{-1}$ coating concentration (Fig. 7B), neural crest cell binding to the E8 fragment of laminin was inhibited by both HNK-1 and CSAT antibodies (~60% and ~90%, respectively; $P<0.01$); adhesion to E1′ fragment was inhibited only by the CSAT antibody ($P<0.01$). Table 2A summarizes a statistical analysis of data in Fig. 7. In summary, molar equivalents of the E8 fragment and intact laminin demonstrated similar neural crest cell attachment and antibody inhibition. In contrast, attachment to the E1′ fragment of laminin was significantly different from that observed for both the E8 fragment and intact laminin with respect to attachment and antibody sensitivity.

The divalent cation dependence of neural crest cell attachment to the E8 fragment of laminin was similar to that observed for the intact molecule (Fig. 8). At high coating concentrations (10 $\mu g\,ml^{-1}$ equivalents; Fig. 8A), neural crest cells bound avidly in the presence of 1 mM Ca^{2+} or Mn^{2+}. This attachment was signifi-

Table 2. *Summary of statistically significant differences of neural crest cell attachment to various substrata and conditions*

(A) Laminin and laminin fragments *vs.* antibodies

Substratum one	Substratum two	Antibody: None	HNK-1	CSAT
High-LM*	High-LM		0	+
High-E8*	High-E8		0	+
High-E1′*	High-E1′		0	+
Low-LM*	Low-LM		+	+
Low-E8*	Low-E8		+	+
Low-E1′*	Low-E1′		0	+
High-LM	Low-LM	+		
High-E8	Low-E8	+		
High-E1′	Low-E1′	0		
High-LM	High-E8	+		
High-LM	High-E1′	+		
Low-LM	Low-E8	0		
Low-LM	Low-E1′	+		

+=Significant differences between adhesion of cell populations by Students *t*-test $P<0.05$.
0=No significant differences.
*=In the absence of antibodies.

(B) Laminin and laminin fragments *vs.* divalent cations

Substratum one	Substratum two	Cation: None	Ca^{2+}	Mg^{2+}	Mn^{2+}
High-LM*	High-LM		+	0	+
High-E8*	High-E8		+	0	+
High-E1′*	High-E1′		0	+	+
Low-LM*	Low-LM		0	0	0
Low-E8*	Low-E8		0	0	0
Low-E1′*	Low-E1′		0	0	0
High-LM	Low-LM	+	+	+	0
High-E8	Low-E8	+	+	+	0
High-E1′	Low-E1′	0	0	0	0
High-LM	High-E8	0	+	0	+
High-LM	High-E1′	0	+	+	+
Low-LM	Low-E8	0	0	0	0
Low-LM	Low-E1′	+	+	+	0

+=Significant differences between adhesion of cell populations by Students *t*-test $P<0.05$.
0=No significant differences.
*=In the absence of divalent cations.

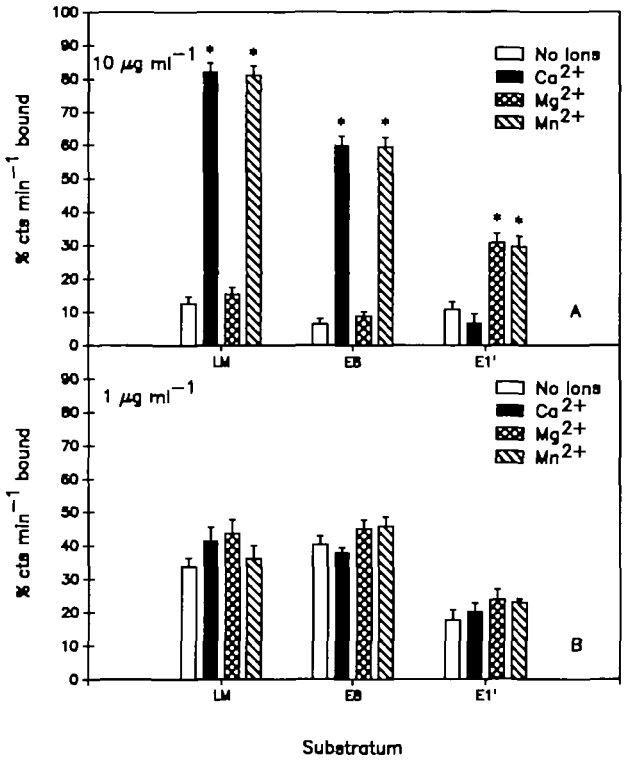

Fig. 8. Proteolytic fragments of laminin were compared to intact laminin for their ability to promote neural crest cell adhesion in the presence and absence of divalent cations. Laminin, the E8 (constituting the long arm of laminin), and the E1′ (constituting the three short arms of laminin) fragments were coated onto the substratum at concentrations of 10 (A) and 1 $\mu g\,ml^{-1}$ (B). Neural crest cell attachment to the E8 and E1′ fragments was also tested for dependency on the divalent cations Ca^{2+}, Mg^{2+} and Mn^{2+}. Asterisks (*) indicate significant ($P<0.05$) differences from controls. Points represent the mean of at least six experiments and the error bars represent the S.E.M.

cantly better than that observed in the absence of divalent cations or in the presence of 1 mM Mg^{2+} ($P<0.001$). On low coating concentrations of the E8 fragment of laminin (Fig. 8B), neural crest cell attachment was not significantly affected by the presence or absence of divalent cations ($P>0.1$). In contrast, neural crest cell attachment to high (10 $\mu g\,ml^{-1}$ equivalent) concentrations of the E1′ fragment required the divalent cations Mg^{2+} or Mn^{2+}, but not Ca^{2+}. Table 2B summarizes a statistical analysis of data in Fig. 8. In summary, neural crest cell attachment to the E8 fragment was comparable to that on intact laminin, but was significantly different from that to the E1′ fragment.

Effects of heparin on neural crest cell attachment to laminin

The adhesion of many cell types to laminin has been shown to involve heparan sulfate proteoglycans (Charonis *et al.* 1988). Exogenous heparin (1–500 $\mu g\,ml^{-1}$) was added to the assay wells as a competitive inhibitor of heparan sulfate proteoglycans. Neural crest cell binding to High-LM and Low-LM was not significantly altered by exogenous heparin ($P>0.1$; Fig. 9).

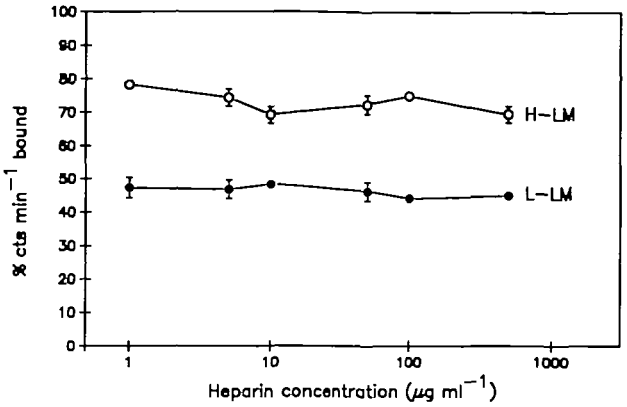

Fig. 9. Addition of exogenous heparin (1 to 500 $\mu g\,ml^{-1}$) to neural crest cell adhering to 10 $\mu g\,ml^{-1}$ of laminin (H-LM) and 1 $\mu g\,ml^{-1}$ of laminin (L-LM). Points represent the mean of at least six experiments and the error bars represent the S.E.M.

Neural crest cell morphology on fibronectin and laminin

Neural crest cell morphology was investigated on three substrata; fibronectin, High-LM and Low-LM. The morphology of the cells was visualized by staining with FITC–phalloidin, which binds to filamentous actin. On fibronectin, neural crest cells possessed multiple long thin processes and appeared to be sessile (Fig. 10A). On both High-LM and Low-LM (Fig. 10B and C), neural crest cells possessed multiple broad lamellapodia characteristic of migratory cells, but lacked long thin processes. The distal portion of these lamellapodia contained abundant microfilaments, adjacent to an actin-free zone. Neural crest cells appeared morphologically similar on both types of laminin but different of fibronectin.

Identification of HNK-1- and CSAT-bearing proteins on neural crest cells

The HNK-1, CSAT and 20B4 antigens were isolated by immunoprecipitation of extracts from surface biotinylated neural crest cells. The CSAT antibody immunoprecipitated three bands major protein bands of 180 000, 165 000 and 130 000 M_r, as well as a minor 65 000 M_r protein separated by SDS–polyacrylamide gel electrophoresis (PAGE) under non-reducing conditions (Fig. 11; Lane 2). The 180 000 and 165 000 M_r bands probably represents α subunits while the 130 000 M_r band probably represents the β_1 subunit. HNK-1 immunoprecipitations revealed three broad bands with M_r of 180 000, 165 000 and 130 000 M_r (Fig. 11; Lane 3); the level of the 130 000 M_r protein was reduced in the presence of EDTA (Fig. 11; Lane 4). With the addition of 1% SDS (which causes integrin complexes to dissociate), a single band at 165 000 M_r was detected with the HNK-1 antibody (Fig. 11; Lane 1). Immunoprecipitations using the 20B4, which recognizes another

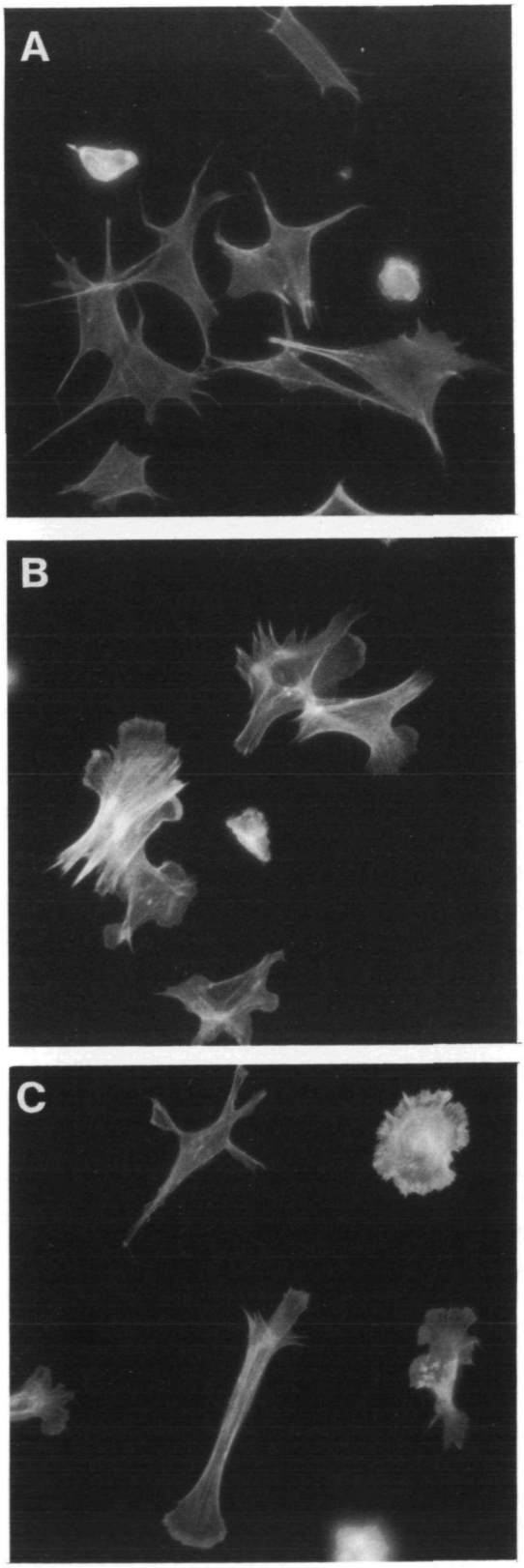

Fig. 10. Neural crest cell morphology was examined after adhesion to fibronectin and laminin substrata. Neural crest cells were labelled with phalloidin to visualize filamentous actin. Neural crest cells adhering to fibronectin (A) possessed multiple focal contacts. Neural crest cells adhering to either High-LM (B) and Low-LM (C) possessed multiple focal lamellapodia.

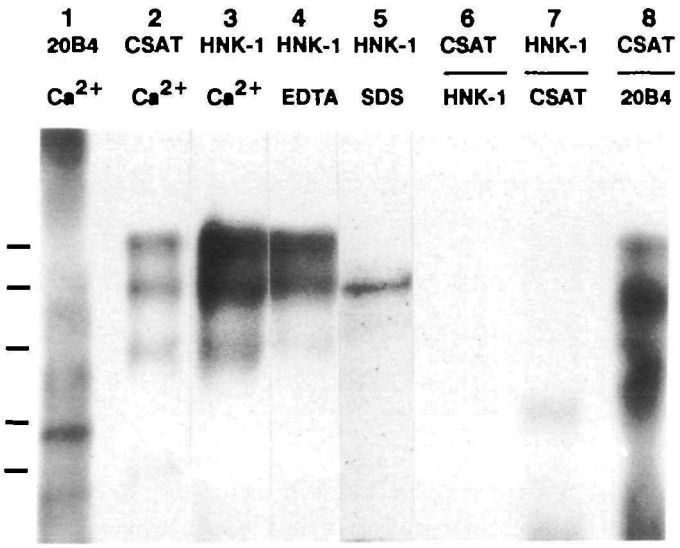

Fig. 11. Neural crest cells were surface biotinylated and the antigens for the 20B4 (Lane 1), CSAT (lane 2) and HNK-1 antibodies (Lane 3) were immunoprecipitated in a buffer containing Ca^{2+} and 1% Triton X-100. The HNK-1 antigen was also immunoprecipitated in the presence of EDTA (Lane 4) and 1% SDS in order to dissociate it from any proteins with which it might aggregate (Lane 5). Lanes 6–8 show the results of immunodepletion experiments, in which the HNK-1, CSAT and 20B4 antibodies were used to deplete their respective antigens prior to immunoprecipitations with the HNK-1 or CSAT antibodies. Dashes indicate calculated molecular masses of the identified bands, from top to bottom, of 180 000, 165 000, 130 000, 90 000 and 65 000 M_r.

epitope on neural crest cells, revealed a different pattern of bands (Fig. 11; Lane 1).

Immunodepletion experiments were performed to show that the proteins recognized by the CSAT and HNK-1 antibodies were the same. Samples were immunoprecipitated repeatedly with HNK-1, CSAT or 20B4 antibodies in order to immunodeplete them of the respective antigens: (Fig. 11; Lanes 6, 7 and 8, respectively). The samples then were immunoprecipitated with a different antibody: the CSAT antibody after HNK-1 immunodepletion (Lane 6), the HNK-1 antibody after CSAT immunodepletion (Lane 7) or the CSAT antibody after 20B4 immunodepletion (Lane 8). Lanes 6 and 7 show no protein bands, indicating that the two antibodies recognize the same proteins. Lane 8 shows the 180 000, 165 000 and 130 000 M_r bands of the CSAT antigen, indicating that the 20B4 antibody does not recognize these integrin proteins. Comparable protein bands were detected by immunoprecipitation with the HNK-1 antibody, following immunodepletion by the 20B4 antibody (data not shown).

It is possible that the technique of surface labelling neural crest cells does not recognize all HNK-1 reactive

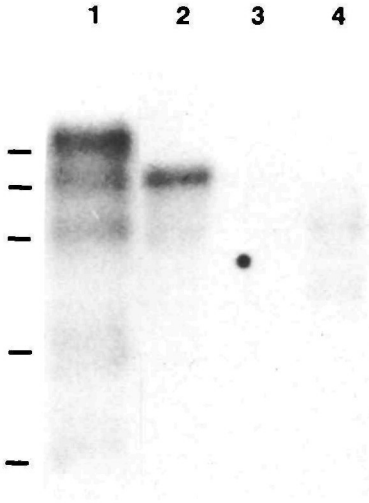

Fig. 12. The CSAT antigen was immunoprecipitated from trunk tissue extracts of 2.5 day old quail embryos, biotinylated, and detected following non-reducing SDS–PAGE by ^{125}I-streptavidin (Lane 1). Three major bands of 180 000, 165 000 and 130 000 M_r were detected, along with two minor bands of 90 000 and 65 000 M_r. Samples of unlabelled CSAT antigen (Lane 2), purified CSAT antibody (Lane 3), and whole tissue extracts (Lane 4) were immunoblotted with the HNK-1 antibody and visualized using ^{125}I-conjugated secondary antibodies. One major band (probably an integrin α subunit) was observed in the CSAT immunoprecipitate.

glycoproteins. In order to identify whether the HNK-1 epitope was associated with integrin receptors on unlabelled neural crest cells, we examined extracts from the trunk tissue derived from 2.5 day quail embryos. In embryos at this stage, neural crest cells are actively migrating and represent the vast majority of HNK-1 immunoreactive cells. Tissue extracts were immunoprecipitated with the CSAT antibody. After separation of the CSAT immunoprecipitates by non-reducing SDS–PAGE, three major bands were detected of 180 000, 165 000 and 130 000 M_r, as well as two minor bands of 90 000 and 65 000 M_r (Fig. 12; Lane 1). In immunoblots of these precipitates, the HNK-1 antibody recognized a dominant 165 000 M_r glycoprotein (Lane 2). This band was absent in immunoblots of the CSAT antibody along (Lane 3) and greatly enriched over immunoblots of whole tissue extracts (Lane 4). This confirms that the HNK-1 antibody recognizes a 165 000 M_r glycoprotein which is immunoprecipitated by an antibody to the β_1 subunit of integrin.

Discussion

Our results demonstrate that neural crest cells possess at least two distinct mechanisms for attachment to laminin, as revealed by experiments analyzing cell adhesion to various laminin-coating concentrations (summarized in Table 3). Both attachment mechanisms are inhibited by the CSAT antibody, which recognizes the avian β_1 subunit of integrin. Cell attachment to High-LM is unaffected by the HNK-1 antibody and is divalent cation dependent. In contrast, cell attachment to Low-LM is inhibited by the HNK-1 antibody and does not require divalent cations. The HNK-1 antibody recognizes a dominant 165 000 M_r glycoprotein on the surface of neural crest cells which appears to be on or associated with an integrin. The 20B4 antibody, which binds to the surface of neural crest cells, does not block adhesion to laminin at any coating concentration.

One possible explanation for the distinct binding mechanisms observed at different coating concentrations of laminin is that the conformation of laminin may be altered as a function of coating concentration. For example, physiological concentrations of Ca^{2+} may alter the configuration of laminin at high versus low coating concentrations. In fact, preliminary results indicate that the ratio of laminin to Ca^{2+} in the substratum solution may determine the conformation of the molecule (unpublished observation). Given the measured concentrations of laminin adsorbed onto the PVC plates, one can perform calculations that suggest that Low-LM may be monolayered whereas High-LM must be multilayered or aggregated (as depicted in Fig. 13). This suggests that different receptor sites and/or conformations may be exposed at the different coating concentrations.

An alternate explanation is that there may be different isoforms of laminin that are preferentially exposed at different coating concentrations. Recently, evidence for a laminin gene family has emerged. S-laminin, an analog of the B1 chain of laminin, has been identified in the synaptic cleft (Hunter *et al.* 1989) and merosin, an A chain variant, is located in the basement membrane of numerous human tissues (Ehrig *et al.* 1990). Independent of whether different coating concentrations result in different conformations or

Table 3. *Summary of results for neural crest cell attachment to laminin*

Coating concentration of laminin	Antibody			Divalent cations			
	HNK-1	CSAT	20B4	Ca^{2+}	Mg^{2+}	Mn^{2+}	Heparin
10 μg ml^{-1} (High-LM)	0	–	0	+	0	+	0
1 μg ml^{-1} (Low-LM)	–	–	0	0	0	0	0

+ indicates a requirement for mM concentrations of the molecule.
– indicates inhibition by the antibody at 50 μg ml^{-1}.
0 indicates no effect by the molecule over the range of concentrations tested.

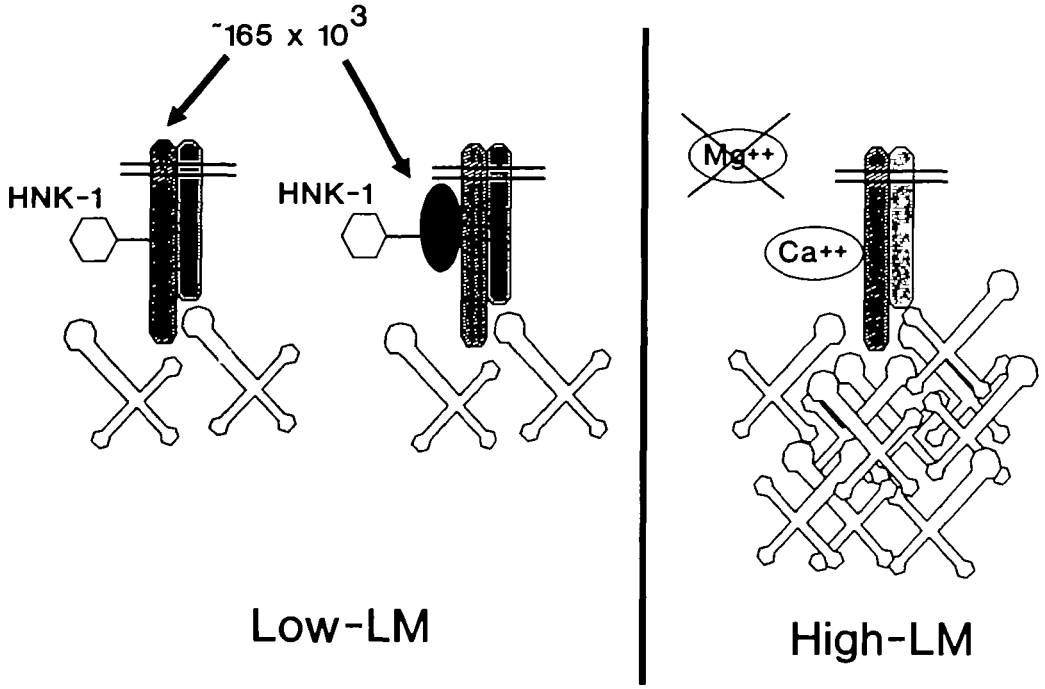

Fig. 13. Schematic diagram illustrating possible mechanisms of neural crest attachment to laminin mediated by β_1-integrins. At low coating concentrations of laminin (Low-LM), the laminin is likely to be monolayered on the substratum. Neural crest cells adhere to Low-LM by means of an integrin which has an HNK-1 epitope either directly attached to it or on an associated molecule which is involved in receptor binding to laminin; this integrin receptor complex functions in the absence of divalent cations. At high coating concentrations of laminin (High-LM), the laminin is multilayered and aggregated. This may confer a different conformation to the receptor binding site or may expose a different site on the E8 fragment of laminin. Neural crest cell binding at this concentration depends on a β_1-integrin that requires Ca^{2+} or Mn^{2+}, but not the HNK-1 epitope, for function.

forms of laminin, either of these scenarios supports the idea that neural crest cells possess two independent mechanism for attachment to laminin(s).

Laminin preparations typically contain numerous contaminants including collagen type IV and heparan sulfate proteoglycans. Thus, it is conceivable that the differential binding of neural crest cells to low *versus* high coating concentrations of laminin might be related to impurities in the mouse laminin. However, we found that laminin derived from a variety of sources, including a purified laminin–nidogen complex (Paulsson *et al.* 1987) and purified E8 fragment of laminin were comparable to mouse tumor laminin in supporting neural crest cell attachment. Because neural crest cells do not bind to nidogen alone (Perris *et al.* 1989), these results suggest that the observed binding characteristics represent adhesion to laminin.

Our data support the idea that neural crest cells possess at least two receptors for laminin. These receptors appear to function independently, with one active at high coating concentrations of laminin and the other active at low coating concentrations. A simple avidity model in which neural crest cells possess one high and one low affinity receptor for laminin cannot explain these two receptor systems. According to such a model, the only detectable binding at low coating concentrations of laminin would be mediated by a high affinity receptor whereas both low and high affinity receptors would function at higher coating concentrations of laminin. However, our data suggest that the receptor that binds to Low-LM is inactive at high laminin concentrations, since neural crest cell attachment to High-LM is significantly less than that to Low-LM in the absence of divalent cations. It is possible that each receptor system recognizes different conformations of the laminin molecule, resulting from the various coating concentrations used in our *in vitro* system. Regardless of whether the concentration-dependent attachment observed in culture is analogous to neural crest cell attachment to laminin within the embryo, this assay can now be used to study these different receptors on neural crest cells.

Both adhesive mechanisms to laminin appear to be mediated by integrin receptors, one requiring divalent cations for binding and the other functioning in the 'absence' of divalent cations. The involvement of β_1 containing integrins (β_1-integrins) was demonstrated by the finding that the CSAT antibody inhibited neural crest cell attachment to laminin at all substratum coating concentrations. Even in the 'absence' of Ca^{2+}, Mg^{2+} and Mn^{2+}, a significant percentage of neural crest cells attached to Low-LM and their adhesion was inhibited by the CSAT antibody. These results taken together indicate that at least one integrin can recognize specific ligands without divalent cations. All previously reported integrins require physiological concentrations

of divalent cations to interact with their ligands (Hynes, 1987). Typically, millimolar concentrations of Ca^{2+} and/or Mg^{2+} are utilized (Cheresh *et al.* 1987; Edwards *et al.* 1987; Smith and Cheresh, 1988), though Mn^{2+} can be employed by some heterodimeric receptors (Edwards *et al.* 1988; Ignatius and Reichardt, 1988). Consistent with these characteristics, we observed that neural crest cell attachment to high coating concentrations of laminin ($>10\,\mu g\,ml^{-1}$) required Ca^{2+} or Mn^{2+} at 1 mM concentrations. We cannot rule out the possibility that extremely low cation concentrations remain under our assay conditions, in the presence of EDTA and the absence of exogenous cations. If the integrin receptors functioning at Low-LM have very high binding affinities for cations, it is conceivable that residual cations may fulfill their divalent cation requirements. However, this would require the integrin receptors to bind cations in the nanomolar concentration range, whereas all reported integrins bind cations in the millimolar range.

Unlike the CSAT antibody, which blocks neural crest cell adhesion to laminin at all coating concentrations, the HNK-1 antibody only inhibits neural crest cell adhesion to laminin at low coating concentrations. Even in the absence of divalent cations, neural crest cell attachment to Low-LM was significantly inhibited (70%) by the HNK-1 antibody, whereas no detectable inhibition was noted on High-LM. In contrast, neither the CSAT nor the HNK-1 antibodies blocked neural crest cell attachment to charged substrata. These findings, in conjunction with the divalent cation sensitivity, suggest that neural crest cells possess at least two mechanisms for attachment to laminin: (*i*) a divalent cation-dependent integrin(s), which mediates binding at high coating concentrations; and (*ii*) an HNK-1 bearing integrin(s), which mediates binding at low coating concentrations and functions with or without exogenous cations (Fig. 13). The presence of the latter type of integrin is further supported by the finding that HNK-1 antibody recognizes bands with characteristics of both integrin α and β subunits in immunoblots of the CSAT antigen. Because we cannot determine functionality from our biochemical analyses, we cannot determine whether the HNK-1 epitope on integrins is functionally active. Therefore, we cannot distinguish whether the receptor used for binding on Low-LM has an HNK-1 epitope on the β_1-integrin itself or on an associated molecule involved in attachment to laminin (Fig. 13). Furthermore, we cannot rule out the possibility that there are other non-integrin HNK-1 immunoreactive proteins that are involved in neural crest cell adhesion to laminin. The HNK-1 bearing β_1-integrin receptor system is distinct in cation dependency from that operating at High-LM and is likely to recognize a different binding site or configuration on laminin, that may be determined by the concentration/state of aggregation of laminin on the substratum.

Several integrin receptors that bind laminin have been described. All reported laminin-binding integrins, however, can also bind to other ligands including fibronectin and collagens. For example, the CSAT antibody blocks chick skeletal myoblast and fibroblast adhesion to fibronectin and laminin (Horwitz *et al.* 1985; Buck *et al.* 1986), as well as adhesion and neurite outgrowth of chick retinal cells on fibronectin, laminin and collagen type IV (Hall *et al.* 1987). Rat neuronal cell lines possess integrins involved in adhesion to laminin (Ignatius and Reichardt, 1988) and collagen type IV (Tomaselli *et al.* 1988). Recently, a number of laminin-binding integrin receptors have been identified in mammals. These integrins have been classified as $\alpha_1\beta_1$ (Forsberg *et al.* 1990; Hall *et al.* 1990), $\alpha_2\beta_1$ (Languino *et al.* 1989; Elices and Hemler, 1989; Carter *et al.* 1990; Lotz *et al.* 1990), $\alpha_3\beta_1$ (Gehlsen *et al.* 1989; Carter *et al.* 1990), $\alpha_6\beta_1$ (Sonnenberg *et al.* 1988, 1990; Hall *et al.* 1990; Shaw *et al.* 1990), and $\alpha_6\beta_4$ (Lotz *et al.* 1990) integrins. The $\alpha_3\beta_1$ apparently requires Ca^{2+} to function, while $\alpha_6\beta_1$ functions in the presence of Mg^{2+} and Mn^{2+}, but not in the presence of Ca^{2+} (Sonnenberg *et al.* 1988). Another laminin/collagen receptor that has been reported to be a β_1-integrin is a receptor on PC12 cells that requires Mg^{2+} rather than Ca^{2+} for function (Turner *et al.* 1987), similar to the divalent cation dependency for human platelet $\alpha_6\beta_1$ integrin and neural crest cell adhesion to the E1′ fragment of laminin. The rat hepatocyte $\alpha_1\beta_1$ integrin has been shown to adhere to the E8, E1 and P1 fragments of laminin (Forsberg *et al.* 1990; Hall *et al.* 1990), while the human $\alpha_3\beta_1$ (Gehlsen *et al.* 1989) and $\alpha_6\beta_1$ (Hall *et al.* 1990; Sonnenberg *et al.* 1990) integrins adhere to the E8 fragment alone. In addition, two as yet unidentified mammalian β_1-integrins for laminin have been identified. A bovine β_1-integrin appears to adhere to laminin in an Arg-Gly-Asp (RGD) dependent manner (Basson *et al.* 1990), while a human β_1-integrin adheres to laminin in an RGD-independent manner and does not contain the known α_1-α_6 subunits (Kramer *et al.* 1989). From these studies, laminin-binding integrins are relatively common and diverse in their subunit composition, ligand specificity and divalent cation requirements.

Although the integrin heterodimers present on neural crest cells have not yet been characterized, it is possible to rule out some integrins on the basis of recent immunohistochemical data. Using antibodies to the avian α_5 and α_6 integrin subunits, it has been shown that these proteins are absent from the surface of migrating neural crest cells (Bronner-Fraser *et al.* 1992). In addition, it seems unlikely that $\alpha_4\beta_1$ or $\alpha_5\beta_1$ is a laminin receptor on neural crest cells, since these integrin binds only to fibronectin in other species. Other heterodimers, including $\alpha_1\beta_1$, $\alpha_2\beta_1$ and/or $\alpha_3\beta_1$ are reasonable candidate molecules for mediating neural crest cell attachment to laminin.

Our results and those of a previous study (Perris *et al.* 1989) suggest that neural crest cells bind to the E8 fragment of laminin, corresponding to the cell binding site on the long arm in the vicinity of the heparin binding domain (Aumailley *et al.* 1989). In our attachment assays, neural crest cells bind to the E8 fragment in a manner similar to their attachment to

intact laminin; attachment is blocked by the CSAT antibody at all coating concentrations, and by HNK-1 antibody at low coating concentrations only. The E8 fragment of laminin also mimics the intact molecule with respect to the requirements for divalent cations (Ca^{2+}/Mn^{2+}) at high substratum coating concentrations.

Some neural crest cell attachment was observed on the E1′ fragment of laminin. Although unaffected by the HNK-1 antibody, binding of neural crest cells to the E1′ fragment was inhibited by the CSAT antibody. This adhesion to the E1′ fragment of laminin utilized different divalent cations (Mg^{2+}/Mn^{2+}) than the intact molecule (Ca^{2+}/Mn^{2+}). This is consistent with previous suggestions that the E1′ fragment may possess a cryptic RGD site that is not exposed in intact laminin (Nurcombe *et al.* 1989). The RGD sequence corresponds to the attachment sequence in a number of extracellular matrix molecules, including fibronectin, vitronectin and tenascin, to which integrins bind (Ruoslahti and Pierschbacher, 1987). Laminin also contains an RGD sequence (Sakai *et al.* 1988), though RGD-peptides fail to inhibit neural crest cell attachment to laminin (Nurcombe *et al.* 1989). It is possible that an RGD-sensitive integrin, perhaps the 'fibronectin receptor', may be utilized for adhesion to the E1′ fragment of laminin. Thus, neural crest cell attachment to the E1′ fragment of laminin is likely to be mediated by a third distinct β_1-integrin.

The observation of different mechanisms of attachment to laminin substrata created at different coating concentrations highlights the importance of considering not only the presence but also the amount of available substratum molecules. Many studies have used immunocytochemical techniques to establish the distribution of ECM molecules in the regions through which neural crest cells migrate (Newgreen and Thiery, 1980; Krotoski *et al.* 1986; Rogers *et al.* 1986). Our data suggest that the local conformation, perhaps conferred by a change in concentration, of a particular ECM molecule may be important for cell recognition and attachment. Although we find that neural crest cell attachment to laminin increases with substratum coating concentration in the presence of divalent cations, the extent of migration, as assayed by the area of cell outgrowth from the neural tube (Perris *et al.* 1989), actually *decreases* at higher laminin concentrations. Thus, more avid adhesion may lead to reduced migration in this system. These results lead to the intriguing possibility that the two distinct adhesion mechanisms for laminin could be differentially involved in active cell movement (at low laminin concentrations) and cessation of movement (at high laminin concentrations). Accordingly, the conformation of laminin in the local environment could dictate the cellular response.

The two distinct mechanisms of neural crest cell attachment to laminin are probably mediated by integrins that differ in their glycosylation, divalent cation requirements and specificity. There are several possible explanations that could account for our observations. For example, there may be two β_1-integrins, each of which contains a distinct α subunit. The different α subunits could be the products of distinct genes or alternative splicing of the same gene; they may confer the different specificities for laminin conformation upon the heterodimer receptor complex. Alternatively, differential glycosylation of a single α integrin subunit could lead to the formation of functionally distinct receptor complexes. The glycosylation could dictate both the divalent cation requirements and the specificity for laminin conformation. Two pieces of evidence support the idea that the two attachment mechanisms may differ in glycosylation: (1) the finding that the HNK-1 antibody (which recognizes a carbohydrate epitope) only blocks the function of one mechanism of adhesion to laminin; and (2) the biochemical evidence demonstrating the HNK-1 epitope is present on a molecule which co-immunoprecipitates with the β_1-subunit. There is some evidence that glycosylation alters integrin function. Murine integrins synthesized by a mutant B16 cell line deficient in glycosylation show reduced affinity for fibronectin and laminin (Oz *et al.* 1989). Furthermore, the vitronectin receptor has an associated ganglioside (Cheresh *et al.* 1987), which is required to function in adhesion. We cannot rule out the possibility that the two β_1-integrin receptor systems have different β subunits, both of which are recognized by the CSAT antibody. These models, which are not necessarily mutually exclusive, are readily testable, and warrant further study. The 165 000 M_r glycoprotein detected by the HNK-1 antibody on neural crest cells may also be a integrin-associated molecule, and not a member of the α integrin family. Future experiments will be directed toward identifying the α subunits and examining the effects of glycosylation on the function of the integrins on the surface of neural crest cells.

We thank Drs Scott Fraser and Roberto Perris for helpful comments on the manuscript and invaluable discussion. We also would like to thank Dr Mats Paulsson for his generous gift of laminin fragments and the laminin–nidogen complex. This study was supported by USPHS Grant HD-15527.

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(*Accepted 28 August 1991*)

Note added in proof

While this paper was in press, we discovered that a polyclonal antiserum to the chicken α_1 subunit of integrin inhibited attachment of neural crest cells to laminin in the absence of divalent cations, paralleling the effects of the HNK-1 antibody. Chicken α_1 integrin previously has been shown to be a 165000 M_r protein, making it a good candidate for the HNK-1 bearing molecule on neural crest cells.