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Cranial and trunk neural crest cells use different mechanisms for attachment to extracellular matrices

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Summary

We have used a quantitative cell attachment assay to compare the interactions of cranial and trunk neural crest cells with the extracellular matrix (ECM) molecules fibronectin, laminin and collagen types I and IV. Antibodies to the β_1 subunit of integrin inhibited attachment under all conditions tested, suggesting that integrins mediate neural crest cell interactions with these ECM molecules. The HNK-1 antibody against a surface carbohydrate epitope under certain conditions inhibited both cranial and trunk neural crest cell attachment to laminin, but not to fibronectin. An antiserum to α_1 integrin inhibited attachment of trunk, but not cranial, neural crest cells to laminin and collagen type I, though interactions with fibronectin or collagen type IV were unaffected.

The surface properties of trunk and cranial neural crest cells differed in several ways. First, trunk neural crest cells attached to collagen types I and IV, but cranial neural crest cells did not. Second, their divalent cation requirements for attachment to ECM molecules differed. For fibronectin substrata, trunk neural crest cells required divalent cations for attachment, whereas cranial neural crest cells bound in the absence of divalent cations. However, cranial neural crest cells lost this cation-independent attachment after a few days of culture. For laminin substrata, trunk cells used two integrins, one divalent cation-dependent and the other divalent cation-independent (Lallier, T. E. and Bronner-Fraser, M. (1991) *Development* 113, 1069-1081). In contrast, cranial neural crest cells attached to laminin using a single, divalent cation-dependent receptor system. Immunoprecipitations and immunoblots of surface labelled neural crest cells with HNK-1, α_1 integrin and β_1 integrin antibodies suggest that cranial and trunk neural crest cells possess biochemically distinct integrins. Our results demonstrate that cranial and trunk cells differ in their mechanisms of adhesion to selected ECM components, suggesting that they are non-overlapping populations of cells with regard to their adhesive properties.

Key words: integrins, cell adhesion, laminin, fibronectin, collagen.

Introduction

The neural crest is a migratory cell population that arises from the dorsal portion of the neural tube in vertebrate embryos. These cells migrate extensively and give rise to a large range of derivatives. Along the neural axis, there are four main subdivisions of the neural crest: cranial, vagal, trunk and sacral (LeDouarin, 1982). These different rostrocaudal populations follow different migratory pathways and produce different sets of derivatives. Cranial neural crest cells (arising from presomitic levels) migrate through the cranial mesenchyme and produce facial cartilage and bone, and cranial parasympathetic and sensory ganglia. Vagal neural crest cells (arising at the level of somites 1-7) invade the gut, migrate caudally and populate the enteric ganglia. Trunk neural crest cells (arising at the level of somites 8-28) migrate through the somites and underneath the ectoderm, and produce sensory and sympathetic ganglia, adrenal chromaffin cells and melanocytes. Sacral neural crest cells (arising below the level of somite 28) give rise to enteric ganglia and to some sympathetic and sensory ganglion cells (Pomeranz and Gershon, 1990; Serbedzija et al., 1991).

The differing developmental fates of different rostrocaudal populations of neural crest cells could reflect (1) intrinsic differences in their developmental potentials and/or (2) differences in the environmental cues that they encounter after their migration from the neural tube. The developmental potentials of cranial and trunk neural crest cells have been compared using both in vivo transplantation and in vitro techniques. These experiments have shown that cranial and trunk neural crest cells share the ability to produce melanocytes, glia, sensory neurons and various kinds of autonomic neurons (Niu, 1947; Le Douarin and Teillet,

1974; Le Douarin et al., 1975; Kahn et al., 1980; Le Lievre et al., 1980; Fauquet et al., 1981; Leblanc et al., 1990). However, only cranial neural crest cells have the ability to produce cartilage and bone (Raven, 1931; Nakamura and Ayer-Le Lievre, 1982). In addition, some cranial neural crest cells produce fibronectin (a characteristic mesenchymal secretion product) when grown in vitro, whereas trunk neural crest cells do not (Newgreen and Thiery, 1980). Taken together, these experiments indicate that cranial and trunk neural crest cells share some properties, but also have some inherent differences, particularly in their ability to produce mesenchymal derivatives. Differences in migratory properties of cranial and trunk neural crest cells also have been noted (Le Douarin and Teillet, 1974; Le Lievre et al., 1980; Bronner-Fraser, 1985, 1986, 1987; Bronner-Fraser and Lallier, 1988).

Neural crest cell migratory pathways contain many extracellular matrix (ECM) molecules including fibronectin (Newgreen and Thiery, 1980), laminin (Krotoski et al., 1986), tenascin/cytotactin (Tan et al., 1987), collagens (Duband and Thiery, 1987; Perris et al., 1991b) and proteoglycans (Erickson, 1988; Perris et al., 1991a) which are thought to influence their migration and differentiation. Perturbation experiments using antibodies that recognize ECM components or their receptors have demonstrated the importance of the ECM for proper migration of some neural crest cell populations. For example, antibodies to fibronectin (Poole and Thiery, 1986), tenascin (Bronner-Fraser, 1988) and a laminin-heparan sulfate proteoglycan complex (Bronner-Fraser and Lallier, 1988) can disrupt cranial neural crest cell migration in vivo. Cranial neural crest cell migration also is blocked by antibodies against the β_1 subunit of integrin (Bronner-Fraser, 1985, 1986) or against the HNK-1 carbohydrate epitope (Bronner-Fraser, 1987).

Neural crest cell interactions with ECM molecules may be mediated by a class of receptors called integrins (Hynes, 1987). An integrin receptor is a heterodimer consisting of two subunits, α and β, that are non-covalently linked. These transmembrane glycoproteins mediate cell adhesion to a variety of ECM molecules including fibronectin, laminin, vitronectin and various collagens (Horwitz et al., 1985; Buck et al., 1986; Hynes, 1987; Tomaselli et al., 1988). Integrin α subunits possess binding sites for divalent cations that are thought to be required for function (Cheresh et al., 1987; Edwards et al., 1988; Ignatius and Reichardt, 1988; Smith and Cheresh, 1988). Many integrins appear to have a preference for specific divalent cations. For instance, $\alpha_2\beta_1$ integrins recognize collagens in a magnesium-dependent manner, while $\alpha_3\beta_1$ integrins recognize collagens in a calcium-dependent manner. This cation selectivity may be a useful tool for classifying different integrins. Neural crest cells have several β_1-integrins; for example, trunk neural crest cells have an $\alpha_1\beta_1$ heterodimer which bears an HNK-1 epitope (Lallier and Bronner-Fraser, 1992), as well as at least one other uncharacterized β_1 heterodimer. Cranial neural crest cells also have immunocytochemically detectable β_1-integrins (Krotoski et al., 1986), but their functional properties are not understood.

One approach for elucidating possible differences between migrating cranial and trunk neural crest cells is to characterize their cell surface properties, such as the receptors that mediate their interactions with the ECM. In the present study, we use a centrifugation assay (McClay et al., 1981) to analyze the adhesive interactions of neural crest cells with laminin, fibronectin, and collagen types I and IV. We compare the abilities of cranial and trunk neural crest cells to attach to these substrata. We utilize α_1 integrin, β_1 integrin and HNK-1 antibodies to inhibit neural crest cell-substratum interaction under various conditions and to characterize their surface properties biochemically. The results suggest that cranial and trunk neural crest cells differ in the receptors that they use for attachment to these substrata.

Materials and methods

Materials

Extracellular matrix glycoproteins (mouse laminin, human fibronectin, rat collagen type I and bovine collagen type IV) were purchased from Collaborative Research Inc. (Bedford, MA). JG22 hybridoma cells were purchased from Developmental Studies Hybridoma Bank (University of Iowa). The HNK-1 antibody producing hybridoma cell line was purchased from ATCC (Rockville, MD). The anti-α_1 integrin antiserum was the generous gift of Dr Mats Paulsson.

Cranial neural crest cell primary cultures

Primary cranial neural crest cultures were prepared from the neural tubes of Japanese quail embryos (*Coturnix coturnix japonica*) as previously described (Leblanc et al., 1990). Briefly, embryos were incubated for 30-34 hours, at which time their developmental age was comparable to that of chick stages 8-9. Embryos were dissected in Howard Ringer's solution using electrolytically sharpened tungsten needles. The neural folds were excised from the mesencephalic region, and the explants were placed in a few drops of culture medium on 35 mm plastic dishes coated with human fibronectin (25 μg/ml). After 1 hour, 1.5 ml of culture medium was added to the plates. The culture medium used was MEM (Grand Island Biological Co., GIBCO) supplemented with 15% horse serum (GIBCO) and 10% 11-day chick embryo extract, which was prepared as described previously (Howard and Bronner-Fraser, 1985). Within a few hours after explantation, numerous neural crest cells migrated from the explant to form a monolayer on the plate.

Trunk neural crest cell primary cultures

Primary trunk neural crest cultures were prepared from the neural tubes of Japanese quail embryos (*Coturnix coturnix japonica*; Bronner-Fraser, 1985). Embryos were incubated for 48 hours, at which time their developmental age was comparable to that of chick stages 13-15 (Hamburger and Hamilton, 1950). 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 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. During the next several hours, the neural crest cells migrated away from the explant and onto the culture dish.

Cell preparation for attachment assays

Neural crest cultures for use in quantitative attachment assays were labelled by the addition of [^{3}H]leucine (10-50 μCi/ml) to their culture media 4 hours after explantation. The cells were

allowed to incorporate labelled leucine for 16 hours 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 ovalbumin (Sigma)]. Cells were removed by incubation in 5 mM EDTA in bMEM for 10 minutes at 37°C. This procedure removed ~95-98% of the cranial neural crest cells and 98% of trunk neural crest cells.

Cell attachment assay

Cell-substratum adhesion was measured as described by McClay et al. (1981) and modified for assaying neural crest cells (Lallier and Bronner-Fraser, 1991). Briefly, fibronectin, laminin or collagens 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 hours at 25°C. Protein substrata were plated at concentrations of 20 μg/ml (fibronectin and collagen type IV) and 100 μg/ml (collagen type I). *LN-*Ca^{2+} and *LN-EDTA* were prepared by coating plates with 20 μg/ml of laminin in a solution containing 5 mM Ca^{2+} or EDTA, respectively. Wells were washed extensively with PBS (5 times) and incubated with bMEM for 2 hours 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 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. Individual wells were seeded with 500 to 1000 cells, which were deemed to be ~90% pure neural crest cells based on morphological criteria. In those cases where cell adhesion was measured in the absence of divalent cations, cells were resuspended in Ca^{2+}/Mg^{2+}-free PBS containing 0.5 mg/ml ovalbumin and 1.5 mM EDTA. Neural crest cells were brought into contact with the substratum molecules by a centrifugal force of 150 ***g*** for 5 minutes. 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 minutes. The chambers were quickly frozen in a methanol/dry ice bath. The uncoated wells (unbound cells) and substratum-coated wells (bound cells) were removed and placed separately into scintillation vials. Samples were analyzed for ^{3}H counts per minute (c.p.m.) using a scintillation counter (Beckman LS 5801) in order to determine the percentage of counts bound to the substratum. Six wells were counted per experiment and three experiments were compared for consistency. Within these experiments, non-specific binding to ovalbumin ranged from 5 to 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. Two values for percentage of neural crest cell attachment were determined as significantly different when the P value, by Student's one-sided t-test, was <0.05.

Immunoprecipitation and immunoblots of HNK-1 antigen from surface-labelled cranial neural crest cells

Immunoprecipitations were performed on cultures of surface-labelled neural crest cells as described in Lallier and Bronner-Fraser (1991). Briefly, cells were isolated as described above (*Cranial neural crest cell primary cultures*) and removed from their substratum by incubation in 5 mM EDTA for 5 minutes. 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 according to the protocol of von Boxberg et al. (1990). A solution of 50 μg/ml biotin-X-NHS (biotin-ε-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 minutes 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-100, 1 mM PMSF) for 1 hour at 4°C. Extracts were cleared twice by incubation with Sepharose 4B for 1 hour at 4°C. Antibodies were added to the extracts, incubated for 12 hours at 4°C, followed by incubation with protein A-Sepharose for 1 hour 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.

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×0.75 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. Proteins were electrophoretically transferred to nitrocellulose membranes using a Tris-glycine buffer system (Burnette, 1981). Apparent relative molecular masses were estimated by comparison to prestained high range molecular weight standards (BRL). Membranes were blocked with 5% BSA prior to incubation with ^{125}I-streptavidin (Amersham) for 1 hour 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).

Immunoprecipitates of neural crest cells with the HNK-1, anti-α_1-integrin and anti-β_1-integrin (JG22) antibodies were probed with the anti-α_1-integrin antiserum and HNK-1 antibodies in immunoblots as described previously (Lallier and Bronner-Fraser, 1992). Bands were visualized using ^{125}I-protein A.

Microinjection of α_1 antiserum onto avian cranial neural crest pathways

White Leghorn chicken embryos, having 3 to 9 somites, were injected with antibodies against the chick α_1 subunit of integrin. The eggs were washed with 70% ethanol and a window was made over the embryo. India ink (Pelikan, Hanover, FRG) was injected under the blastoderm to aid in visualization. After removing the vitelline membrane over the injection site, the antiserum (~1 mg/ml) was backfilled into glass micropipettes with openings of 20 to 30 μm diameter. A micromanipulator was used to position the pipette tip lateral to the midbrain neural tube and approximately 4 nl of antiserum was expelled with a pulse of pressure prior to withdrawal of the injection micropipette. After injection, the window over the embryo was closed with adhesive tape and the embryo was returned to the incubator for approximately 24 hours prior to fixation in Zenker's fixative for 1.5 hours. Embryos were dehydrated, embedded in paraplast and serially sectioned on a Leitz microtome at a thickness of 10 μm. Slides were stained with the HNK-1 antibody as described previously (Bronner-Fraser, 1985) in order to recognize neural crest cells.

Results

To analyze neural crest cell attachment to specific ECM molecules, we employed a centrifugal cell adhesion assay

(McClay, 1980; Lallier and Bronner-Fraser, 1991). This assay allowed us to quantitate reproducibly neural crest cell attachment to fibronectin, laminin and collagens using relatively small numbers of cells.

Divalent cation dependence of neural crest cell attachment

Because different integrins have different requirements for divalent cations, the divalent cation dependency of cell attachment can be used to distinguish different receptor mechanisms. We tested the cation dependence of cranial and trunk neural crest cell attachment to various substrata by changing the divalent cations within the cell attachment buffer. Cells were added to wells coated with different substrata either in the presence of 1.5 mM EDTA, or in 1 mM Ca^{2+}, Mg^{2+} or Mn^{2+}.

Fibronectin

Cranial neural crest cells attached to fibronectin equally well either in the absence of divalent cations, or in the presence of Ca^{2+} or Mg^{2+} (P>0.05), with approximately 65% of the cells binding under each of these conditions (Fig. 1). The addition of Mn^{2+}, however, significantly inhibited cranial neural crest cell attachment to fibronectin (P<0.05). In contrast to cranial neural crest cells, trunk neural crest cell attachment to fibronectin occurred only in the presence of divalent cations (P<0.05), with no preference for a particular divalent cation (P>0.05).

The divalent cation-independent mechanism of cranial neural crest cell attachment to fibronectin was seen on cells cultured for 24 hours or less, but was subsequently lost with time in culture (Fig. 2). Concomitantly, cranial neural crest cells acquired a divalent cation-dependent mechanism for attachment to fibronectin. By three days in culture, their attachment was entirely dependent on divalent cations.

Laminin

Our previous data demonstrate that trunk neural crest cells possess at least two distinct mechanisms of attachment to laminin: one mediated by a Ca^{2+}-dependent integrin that recognizes *LN-EDTA* and the other through a divalent cation-independent integrin that recognizes *LN-*Ca^{2+} (Lallier and Bronner-Fraser, 1992). The divalent cation dependence of cranial neural crest cell attachment to laminin was examined to determine if cranial neural crest cells also use different mechanisms for attachment to the two types of laminin (Fig. 3). Cranial neural crest cells bound equally well to *LN-EDTA* and *LN-*Ca^{2+}, with approximately 65% of the cells attaching to either substratum. In parallel experiments, *maximal* levels of cranial and trunk neural crest cell attachment to *LN-EDTA* and *LN-*Ca^{2+} were comparable. However, cranial and trunk neural crest cells differed in their divalent cation requirements. Cranial neural crest cell attachment to both *LN-EDTA* and *LN-*Ca^{2+} required divalent cations, with no preference for Ca^{2+}, Mg^{2+} or Mn^{2+} (P>0.05; Fig. 3). In contrast, trunk neural crest cell attachment to *LN-*Ca^{2+} did not require divalent cations, while attachment to *LN-EDTA* was divalent cation dependent with a preference for Ca^{2+} and Mn^{2+} over Mg^{2+} (Lallier and Bronner-Fraser, 1992).

Collagens

In parallel experiments, we tested the divalent cation dependence of cranial and trunk neural crest cell attachment to collagens (Fig. 4). We were unable to detect significant attachment of cranial neural crest cells to collagen types I or IV in either the presence or absence of divalent cations (P>0.05). Trunk neural crest cells attached to both collagens I and IV (approximately 45% and 35% bound to each substratum, respectively) in a divalent cation-dependent manner. The addition of either Ca^{2+} or Mn^{2+} resulted in comparable levels of attachment to collagen type I (approximately 25%; P<0.05 above background), whereas cells failed to attach in the presence of Mg^{2+} alone. Interestingly, the highest levels of trunk neural crest cell binding to collagen type I (approximately 45%) were observed when both Ca^{2+} and Mg^{2+} were added in the attachment buffer

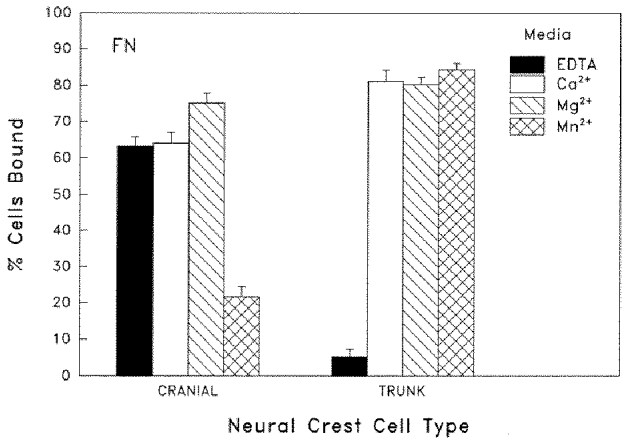

Fig. 1. Divalent cation dependence of cranial and trunk neural crest cell attachment to fibronectin substrata. Cell attachment was assayed in the presence of 1 mM Ca^{2+}, Mg^{2+} or Mn^{2+}, or 1.5 mM EDTA. Bars represent the mean of at least six wells and the error represent the s.e.m.

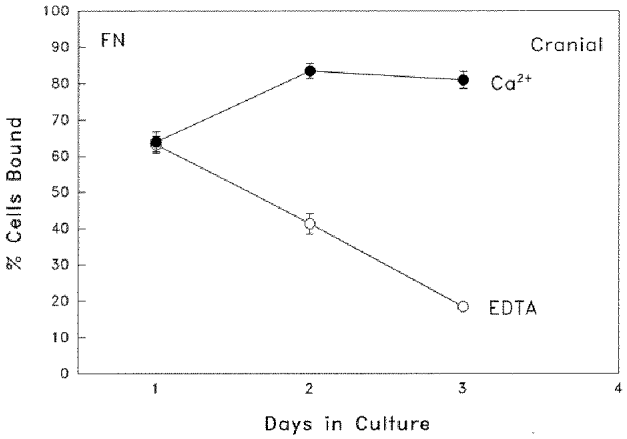

Fig. 2. Loss of divalent-cation-independent attachment of cranial neural crest cells to fibronectin with time. Cranial neural crest cell attachment to fibronectin substrata in the presence of 1 mM Ca^{2+} or 1.5 mM EDTA. Cranial neural crest cells were grown in culture for 1, 2 and 3 days prior to the assay. Points represent the mean of at least six wells and the error represent the s.e.m.

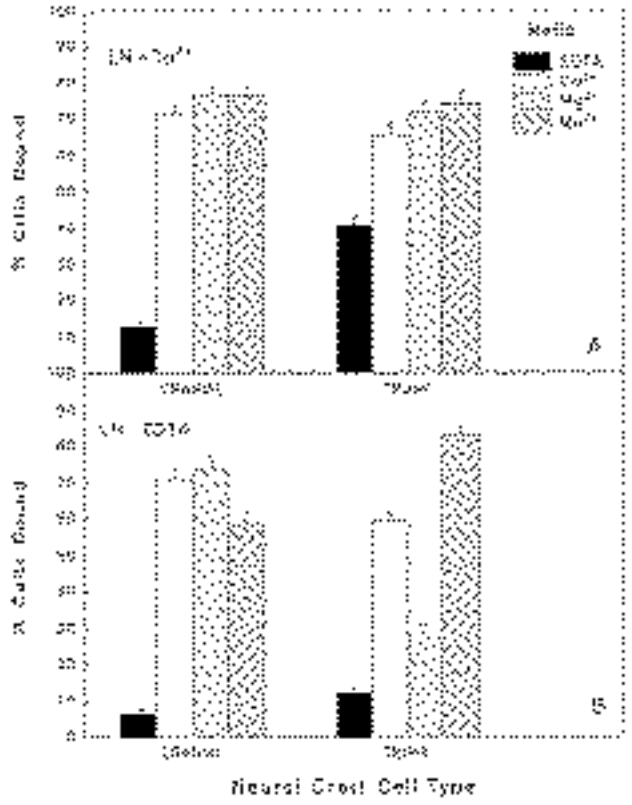

Fig. 3. Divalent cation dependence of cranial and trunk neural crest cell attachment to laminin substrata. Laminin substrata were plated in the presence of (A) 5 mM Ca^{2+}, or (B) 5 mM EDTA. Cell attachment was assayed in the presence of 1 mM Ca^{2+}, Mg^{2+} or Mn^{2+}, or 1.5 mM EDTA. Bars represent the mean of at least six wells and the error represent the s.e.m.

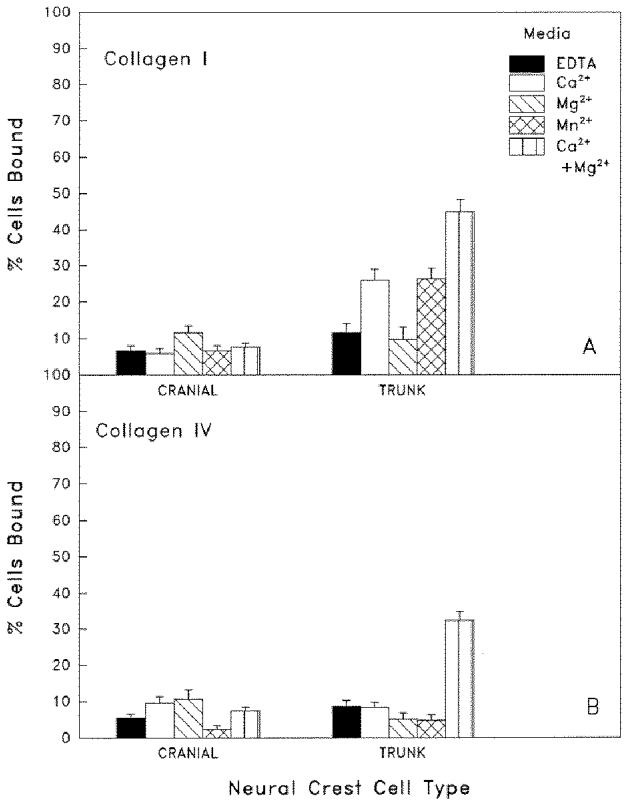

Fig. 4. Divalent cation dependence of cranial and trunk neural crest cell attachment to collagen substrata. Cell attachment was measure for (A) collagen type I, or (B) collagen type IV substrata. Cell attachment was assayed in the presence of 1 mM Ca^{2+}, Mg^{2+} or Mn^{2+}, or 1.5 mM EDTA. Bars represent the mean of at least six wells and the error represent the s.e.m.

(P<0.05). For attachment to collagen type IV, trunk neural crest cells required both Ca^{2+} and Mg^{2+} to bind significantly (approximately 30%) above background levels (P<0.05).

Effects of JG22, HNK-1 and α_1 antibodies on neural crest cell attachment

Fibronectin

In parallel experiments, cranial and trunk neural crest cells exhibited comparable levels of adhesion to fibronectin substrata in the presence of divalent cations (Fig. 5). Approximately 75-90% of the cells bound to substratum. The nature of the receptors used for adhesion was tested by adding antibodies to the cells during the course of their attachment. The JG22 antibody, which recognizes the β_1 subunit of the integrin $\alpha\beta$ heterodimer, inhibited both cranial and trunk neural crest cell attachment to fibronectin. Attachment to fibronectin substrata was significantly reduced (60%) by antibody concentrations of 50 μg/ml (P<0.05). In contrast, the HNK-1 antibody, which recognizes a carbohydrate on the surface of neural crest and other cell types, had no effect on either cranial or trunk neural crest cell attachment to fibronectin substrata (P>0.05). Similarly, the α_1 integrin antiserum had no effect on either cranial or trunk neural crest cell attachment to fibronectin substrata (P>0.05).

Laminin

We have previously shown that trunk neural crest cells use different mechanisms of adhesion to laminin depending on the molecule's conformation in the substratum. Laminin plated in the presence of Ca^{2+} (*LN-Ca*$^{2+}$) is recognized by a divalent cation-independent receptor mechanism, which is inhibited by HNK-1, β_1 and α_1 antibodies; laminin plated in the presence of EDTA and the absence of Ca^{2+} (*LN-EDTA*) is recognized by a divalent cation-dependent receptor mechanism, which is inhibited by β_1 but not by HNK-1 (Lallier and Bronner-Fraser, 1992). The attachment of both cranial and trunk neural crest cells to laminin was inhibited completely by the JG22 antibody at concentrations of 50 μg/ml (Fig. 6), with no significant cell attachment above background (P>0.05).

Cranial and trunk cell attachment to laminin substrata exhibited different sensitivities to the HNK-1 antibody and the α_1 integrin antiserum. As shown previously (Lallier and Bronner-Fraser, 1992), trunk neural crest cell attachment to *LN-Ca*$^{2+}$ was inhibited partially (about 50%) by both the HNK-1 antibody and the α_1 antiserum, while attachment to *LN-EDTA* was unaffected by either (P>0.05). In contrast, cranial neural crest cell attachment to *both LN-EDTA* and *LN-Ca*$^{2+}$ was partially (about 50%) inhibited by the HNK-1 antibody, but was unaffected by the α_1 antiserum (P>0.05).

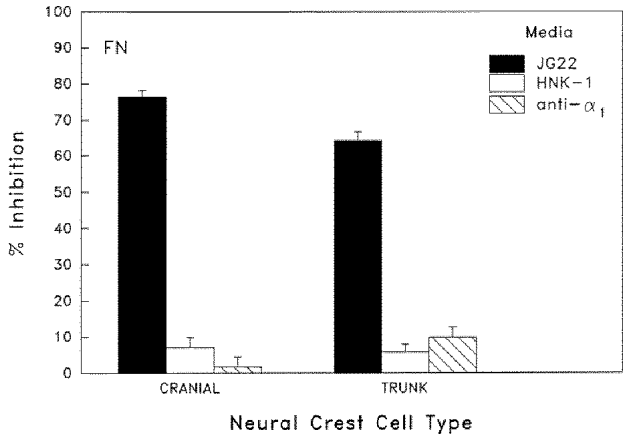

Fig. 5. Antibody inhibition of cranial and trunk neural crest cell attachment to fibronectin substrata. Cell attachment was assayed in the presence of 50 μg/ml HNK-1 antibody, 50 μg/ml JG22 antibody or ~10 μg/ml of anti-α_1 antiserum. Bars represent the mean of at least six wells and the error represent the s.e.m.

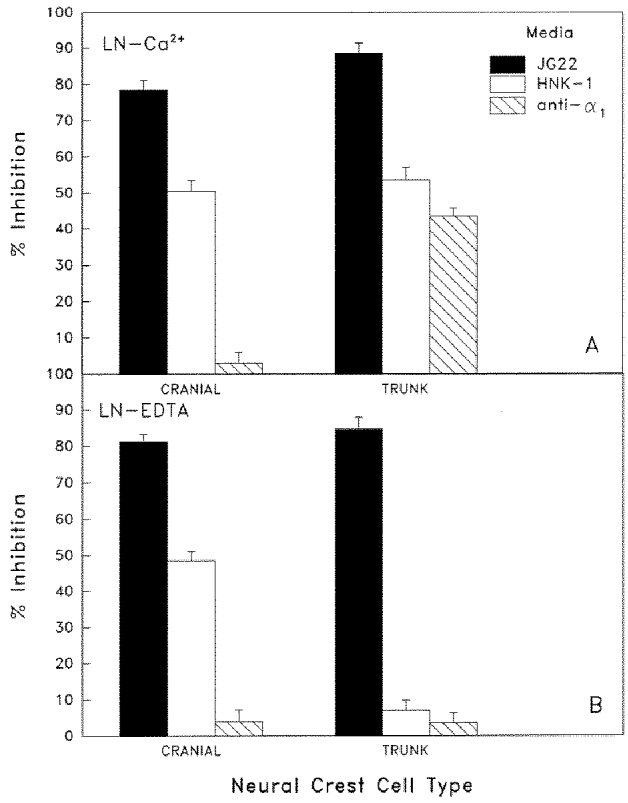

Fig. 6. Antibody inhibition of cranial and trunk neural crest cell attachment to laminin substrata. Laminin substrata were plated in the presence of (A) 5 mM Ca^{2+}, or (B) 5 mM EDTA. Cell attachment was assayed in the presence of 50 μg/ml HNK-1 antibody, 50 μg/ml JG22 antibody or ~10 μg/ml of anti-α_1 antiserum. Bars represent the mean of at least six wells and the error represent the s.e.m.

Collagens

Trunk neural crest cells were tested for the effects of blocking antibodies on their attachment to collagen types I and IV. The JG22 antibody abolished attachment of trunk neural crest cells to both collagen types I and IV at antibody concentrations of 50 μg/ml (Fig. 7; $P<0.05$). The HNK-1 antibody at concentrations ranging from 50 μg/ml to 1 mg/ml caused a partial (~50%) inhibition of trunk neural crest cell attachment to collagen type I, but had no affect on attachment to collagen type IV ($P>0.05$). The α_1 antiserum completely blocked attachment to collagen type I, but had no effect on attachment to collagen type IV.

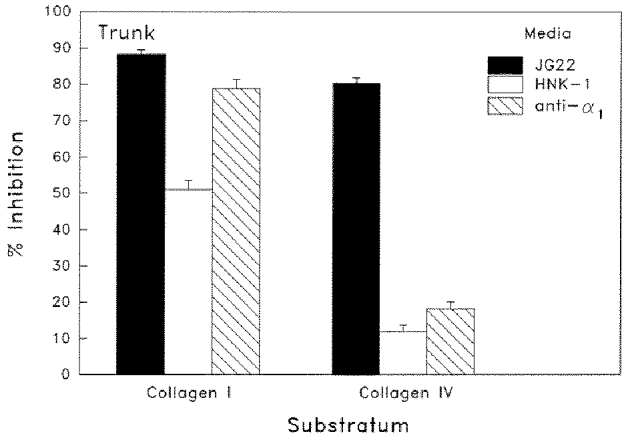

Fig. 7. Antibody inhibition of trunk neural crest cell attachment to collagen type I and IV substrata. Cell attachment was assayed in the presence of 50 μg/ml HNK-1 antibody, 50 μg/ml JG22 antibody or ~10 μg/ml of anti-α_1 antiserum. Bars represent the mean of at least six wells and the error represent the s.e.m.

Identification of the HNK-1-positive protein on cranial neural crest cells

Surface biotinylated cranial neural crest cells were immunoprecipitated using the HNK-1 antibody and then transferred to nitrocellulose membranes for detection using ^{125}I-streptavidin (Fig. 8A). A major band at 180×10^3 M_r and two minor bands of 165×10^3 M_r and 120×10^3 M_r were detected. In previous studies of trunk neural crest cells (Lallier and Bronner-Fraser, 1991, 1992), the major HNK-1 immunoreactive protein was determined to be a 165×10^3 M_r α_1 integrin subunit. The 120×10^3 M_r probably corresponds to the chick β_1 subunit of integrin.

To examine further the relationship between the HNK-1 epitope and integrins, surface labelled cranial neural crest cells were immunoprecipitated with JG22 (anti-β_1 integrin), anti-α_1 integrin or HNK-1 antibodies. The immunoprecipitates then were immunoblotted with anti-α_1 or HNK-1 antibodies (Fig. 8B). On the JG22 lane, the anti-α_1 antiserum recognizes a 165×10^3 M_r and a 120×10^3 M_r band, whereas the HNK-1 antibody recognizes a 180×10^3 M_r band. Because the JG22 antibody precipitates both the β_1 integrin subunit and associated α subunits, the 165×10^3 M_r band probably corresponds to the α_1 subunit whereas the 180×10^3 M_r band corresponds to an unidentified α integrin subunit. The 120×10^3 M_r band corresponds to the β_1 subunit, for which the antiserum has some reactivity. The HNK-1 antibody does not recognize any bands on anti-α_1 antiserum immunprecipitates or vice versa. In contrast, in

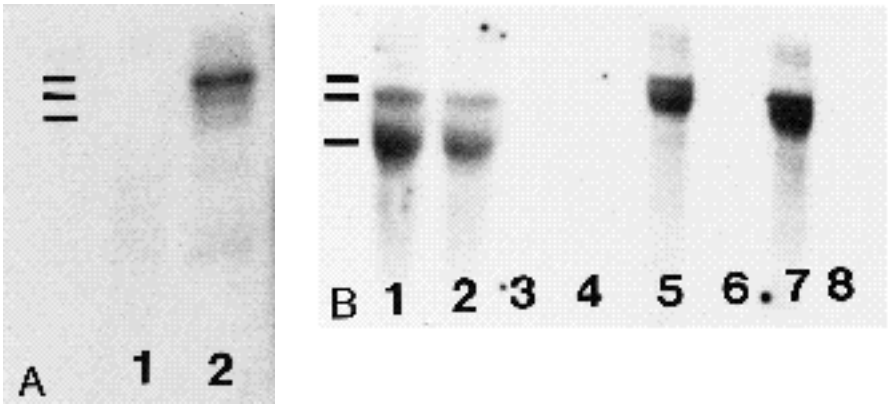

Fig. 8. (A) Immunoprecipitation of cranial neural crest cells with the HNK-1 antibody. Lane 1 represents control immunoprecipitates using BSA-coupled Sepharose 4B, while lane 2 represents the immunoprecipitation using the HNK-1 antibody coupled to Sepharose 4B. The HNK-1 antibody immunoprecipitates a major protein band of 180×10^3 M_r from surface labelled neural crest cells. In addition, two minor protein bands were detected, with M_r of 165×10^3 and 120×10^3. The lines correspond to relative molecular masses of 180, 165 and 120 $\times10^3$, respectively. (B) Immunoprecipitations of surface-labelled cranial neural crest cells with JG22 (anti-β_1 integrin), anti-α_1 integrin or HNK-1 antibodies followed by immunoblots with anti-α_1 integrin antibodies (lanes 1 to 4) or HNK-1 antibodies (lanes 5 to 8). Lanes 1 and 5 = JG22 immunoprecipitate; lanes 2 and 6 = α_1 immunoprecipitates; lanes 3 and 7 = HNK-1 immunoprecipitates; lanes 4 and 8 = control lanes with no primary antibody. The α_1 antibody immunoblots a 165×10^3 M_r band in both the JG22 and α_1 immunoprecipitates, corresponding to the α_1 subunit; it also recognizes a 120×10^3 M_r band corresponding to the β_1 subunit. The HNK-1 antibody immunoblots a 180×10^3 M_r band on the JG22 lane on and on itself; because this band co-precipitates with β_1-integrin, it corresponds to an unidentified α integrin subunit. HNK-1 antibody does not recognize any bands on α_1 antibody immunprecipitates or vice versa. The lines correspond to relative molecular masses of 180, 165 and 120 $\times10^3$, respectively.

immunoprecipitates of trunk neural crest cells, HNK-1 and anti-α_1 integrin antibodies recognize the same 165×10^3 M_r band (Lallier and Bronner-Fraser, 1992). This biochemical evidence suggests that the predominant 180×10^3 M_r HNK-1 epitope on cranial neural crest cells is an α integrin subunit other than α_1, in agreement with the functional studies.

Microinjection of anti-α_1 integrin antiserum onto cranial neural crest pathways in vivo

Our biochemical data suggest that cranial neural crest cells possess α_1 integrin subunits. However, in the cell adhesion assay, antibodies to this receptor do not block the attachment of cranial neural crest cells, suggesting it may be nonfunctional. We examined further the functional properties of this receptor by introducing α_1 integrin antiserum onto cranial neural crest migratory pathways in situ. Previous studies have showed that antibodies against the β_1 subunit of integrin interfere with the normal migration of cranial neural crest cells when introduced into the cranial mesenchyme of 3- to 9-somite-stage embryos (Bronner-Fraser, 1985, 1986). One day after microinjection of anti-α_1 antiserum (n = 9 embryos), all embryos appeared morphologically normal. This contrasts with numerous other antibodies that block cell-matrix interactions, many of which perturb cranial neural crest migration (Bronner-Fraser, 1985, 1986; Bronner-Fraser, 1988; Bronner-Fraser and Lallier, 1988). These results are consistent with the in vitro data suggesting that cranial neural crest cells do not utilize the α_1 integrin subunit for attachment to extracellular matrices.

Discussion

We have compared the adhesion of cranial and trunk neural crest cells to various ECM molecules with respect to both divalent cation dependence and antibody sensitivity. The results are summarized in Table 1. Attachment of both cranial and trunk neural crest cells to all of the tested substrata was eliminated by anti-β_1 integrin antibodies, indicating that their interactions are mediated by integrins. However, the characteristics of cell attachment to fibronectin, laminin and collagen were different for cranial versus trunk neural crest cells. To bind fibronectin, cranial neural crest cells used a divalent cation-independent integrin, whereas trunk neural crest cells used a divalent cation-dependent integrin. To attach to laminin, cranial neural crest cells used a divalent cation-dependent integrin that was sensitive to addition of HNK-1 antibody. In contrast, trunk neural crest cell

Table 1. *Summary of results for neural crest cell attachment to laminin*

Substratum	Antibody		Divalent cations				
Cell type	HNK-1	JG22	EDTA	Ca^{2+}	Mg^{2+}	Mn^{2+}	Ca/Mg
Fibronectin							
Cranial	=	−	+	+	+	−	+
Trunk	=	−	−	+	+	+	+
Laminin-EDTA							
Cranial	−	−	−	+	+	+	+
Trunk	=	−	−	+	−	+	+
Laminin-Ca^{2+}							
Cranial	−	−	−	+	+	+	+
Trunk	−	−	+	+	+	+	+
Collagen I							
Cranial	N.A.	N.A.	−	−	−	−	−
Trunk	−	−	−	+	−	+	+
Collagen IV							
Cranial	N.A.	N.A.	−	−	−	−	−
Trunk	=	−	−	−	−	−	+

+ promotes cell attachment
= allows cell attachment
− inhibits cell attachment
N.A. not assayed

attached to laminin via two different mechanisms, both distinct from that observed on cranial cells. In the case of collagens, cranial neural crest cells failed to bind to collagen types I or IV, whereas trunk neural crest cells bound to both collagens by divalent cation-dependent integrins. The results suggest that: (1) both trunk and cranial neural crest cells possess numerous integrins which can be distinguished by their cation dependency, sensitivity to antibodies and biochemical profiles; and (2) cranial and trunk neural crest cells are non-overlapping populations with respect to their cell-surface adhesive properties.

The divalent cation dependence of cranial and trunk neural crest cell binding differs for each substratum tested. By comparing their cation requirements for different substrata with those of previously characterized integrins (summarized in Table 2), we might get some insight into the distinct types of integrins used by neural crest cells. Trunk and 'old' cranial neural crest cells attach to fibronectin by means of β_1 integrins which use Ca^{2+}, Mg^{2+} or Mn^{2+}; this is consistent with the known properties of $\alpha_2\beta_1$, $\alpha_v\beta_1$, or $\alpha_v\beta_3$ integrins (Kirchhofer et al., 1990; Dedhar and Gray, 1990; Vogel et al., 1990; Charo et al., 1990). In contrast, 'young' cranial neural crest cells attach to fibronectin through a divalent cation-independent integrin, with unique binding characteristics distinct from all previously reported integrins. Cranial neural crest cells bind to laminin via β_1 integrin(s) that show no selectivity for divalent cations, and therefore may be $\alpha_2\beta_1$ integrins (Sonnenberg et al., 1988, 1990; Hall et al., 1990; Shaw et al., 1990; Dedhar and Saulnier, 1990; Kramer et al., 1990; Shimizu et al., 1990) or $\alpha_6\beta_4$ integrins (Elices and Hemler, 1989; Lotz et al., 1990; Lee et al., 1992). Trunk neural crest cells adhere to some laminin conformations via a divalent cation-independent integrin, while attaching to other laminin conformations through an integrin that utilizes Ca^{2+} or Mn^{2+}, but not Mg^{2+}. These divalent cation requirements differ from those of any known integrin. Trunk neural crest cells adhere best to both collagen type I and IV in the presence of both Ca^{2+} and Mg^{2+}, consistent with the properties of $\alpha_1\beta_1$ integrins (Turner et al., 1987; Belkin et al., 1990). In contrast, cranial neural crest cells do not attach to either collagen type.

Table 2. *Divalent cation dependence of integrin receptors*

Beta	Alpha	Ligand	Divalent cations utilized Ca^{2+}	Mg^{2+}	Mn^{2+}	Reference
Calcium dependent						
1	1	LM	+		+	7
1	1	LM,Col 1-4	+	+		1
1	3	LM	+			2
1	3	Col 1,4	+	+	+	5
1	5	FN	+			3
1	5	FN	+	+	2+	6
3	v	VN,RGD	+	+		8
3	IIb	FN,Fb,VN	2+	2+	+	6
Magnesium dependent						
1	1	LM,Col 4		+		14
1	2	Col	?	2+		15
1	2	Col 1,2,3,4	?	2+		12
1	2	Col 1,2,3,4,6	?	2+		13
1	3	LM,FN	−	+	2+	5
1	6	LM	−	+	+	11
1	v	FN,RGD	−	+		8
3	IIb	FN,VN	−	+	+	4
Divalent cation dependent						
1	180k	LM	?	?	?	10
1	130k	LM	?	?	?	9

References
1 Belkin et al., 1990
2 Carter et al., 1990
3 Edwards et al., 1987
4 Edwards et al., 1988
5 Elices et al., 1991
6 Gailit and Ruoslahti, 1988
7 Ignatius and Reichardt, 1988
8 Kirchhofer et al., 1991
9 Kramer et al., 1989
10 Rossino et al., 1990
11 Sonnenberg et al., 1988
12 Staatz et al., 1989
13 Staatz et al., 1990
14 Turner et al., 1987
15 Wayner and Carter, 1987

An interesting and surprising observation from our functional studies is that two of the integrins on neural crest cells are divalent cation-independent, in contrast to mostly previously characterized integrins which require divalent cations for function. One cation-independent integrin is on cranial neural crest cells and recognizes fibronectin; the other is on trunk neural crest cells and recognizes laminin (Lallier and Bronner-Fraser, 1991, 1992). These integrins appear to be temporally regulated since they are gradually lost with age. Because the majority of investigators use older cell populations or cell lines for studying cell-matrix adhesion, these divalent cation-independent integrins may have been missed previously. Thus, the mechanisms of cell attachment used by embryonic cells may be distinct from those employed by stable or metastatic cell types.

The HNK-1 antibody, which recognizes a surface carbohydrate found on neural crest cells, inhibits the attachment of cranial neural crest cells to laminin and trunk neural crest cells to collagen type I and laminin. The inhibition is partial (~50%), probably reflecting the fact that not all neural crest cells express the HNK-1 epitope (Teillet et al., 1987; Maxwell and Forbes, 1988). For example, we found that approximately 25% of trunk neural crest cells and 50% of cranial neural crest cells lack detectable HNK-1 immunoreactivity after two days in vitro (unpublished observation). Previous studies have shown that injection of the HNK-1 antibody into the cranial mesenchyme during neural crest cell migration causes abnormal morphogenesis (Bronner-Fraser, 1987). Similarly, antibodies against the β_1 subunit of integrin (Bronner-Fraser, 1985, 1986) or a laminin-heparan sulfate proteoglycan complex (Bronner-Fraser and Lallier, 1988) inhibit migration of a subpopulation of cranial neural crest cells. These results are consistent with the idea that neural crest-laminin interactions may be required for their normal migration. Taken together, these studies suggest that the HNK-1 epitope appears to be important for the recognition of laminin by some cranial neural crest cells both in vivo and in vitro.

Our data suggest that the proteins bearing the HNK-1 epitope are distinct for cranial versus trunk neural crest cell populations. First, the HNK-1-sensitive adhesion mechanism to laminin is divalent cation-independent for trunk neural crest cells, but divalent cation-dependent for cranial neural crest cells. Second, biochemical analysis suggests

that the HNK-1 antibody recognizes a 180×10^3 M_r glycoprotein, corresponding to an unidentified α integrin subunit, on cranial neural crest cells. In contrast, the epitope appears to be associated with a 165×10^3 M_r α_1 integrin subunit on trunk neural crest cells (Lallier and Bronner-Fraser, 1991, 1992). Furthermore, immunoprecipitates of cranial neural crest cells using the chick α_1 integrin antibody recognize a 165×10^3 M_r that is not recognized by the HNK-1 antibody in immunoblots, and vice versa. Thus, both biochemical and functional studies indicate that the HNK-1-bearing glycoprotein is different on cranial and trunk neural crest cells.

Although both trunk and cranial neural crest cells possess a biochemically detectable α_1 integrin subunit, the data demonstrate that the anti-α_1 integrin antiserum effects trunk but not cranial neural crest cell attachment. Thus, cranial and trunk neural crest cells may express some identical integrins that differ in functional properties. Previous work suggests that the functional characteristics of a particular integrin heterodimer may differ depending upon the cell type in which it is expressed. For example, the $\alpha_2\beta_1$ integrin on human endothelial cells recognizes laminin and collagens, while the same receptor heterodimer on platelets only recognizes collagens (Kirchhofer et al., 1990).

This study demonstrates that cranial and trunk neural crest cells differ in (1) the range of ECM molecules to which they can adhere; (2) the divalent-cation requirements and antibody sensitivities of the integrins that mediate these interactions; and (3) the biochemical profile of their integrins. These results suggest that cranial and trunk neural crest cells are non-overlapping populations with respect to their cell surface adhesive properties. In situ, cranial and trunk neural crest cells give rise to different, but overlapping, sets of derivatives. Both cranial and trunk neural crest cells produce peripheral neurons and glia, but only cranial neural crest cells produce cartilage and bone. The commonalities in the developmental potentials of cranial and trunk neural crest cells are not reflected in their cell-surface adhesive properties. We observed no similarities between cranial and trunk neural crest cells in terms of their mechanisms of adhesion to specific ECM molecules. This result suggests that differences in cell-surface properties are not necessarily associated with differences in differentiative potential. Furthermore, in vivo transplantation experiments suggest that cranial and trunk neural crest cells share the capacity to translocate along heterologous neural crest migratory pathways (LeDouarin and Teillet, 1974; LeLievre et al., 1980) despite the fact that their surface properties differ. However, there are numerous differences between the migratory behavior of cranial and trunk neural crest cells. After transplantation to the trunk region, some of the cranial crest cells invade the gut and populate enteric ganglia and Remak's ganglion, sites that would *not* normally be populated by trunk neural crest cells. In addition, antibody perturbation experiments indicate that cell-matrix interactions are required for the proper migration of cranial neural crest cells, whereas they may have a lesser role in the trunk (Bronner-Fraser, 1985, 1986, 1987; Bronner-Fraser and Lallier, 1988). It is possible that the differences that we have observed in the cell-surface properties of cranial and trunk neural crest cells contribute to these differences in their migratory behavior.

In conclusion, our results, based on antibody sensitivity, divalent cation dependence and substratum specificity, indicate that cranial and trunk neural crest cells use different integrins to adhere to the extracellular matrix. The migratory pathways followed by these two cell populations during normal development in vivo are distinct, as are their sets of derivatives. The present results, together with these previous findings, sum to indicate that neural crest cells arising from different axial levels are distinct populations with respect to their cell-surface properties as well as their developmental potentials.

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