Cranial neural crest recycle surface integrins in a substratum-dependent manner to promote rapid motility

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Cell migration is essential for proper development of numerous structures derived from embryonic neural crest cells (NCCs). Although the migratory pathways of NCCs have been determined, the molecular mechanisms regulating NCC motility remain unclear. NCC migration is integrin dependent, and recent work has shown that surface expression levels of particular integrin α subunits are important determinants of NCC motility in vitro. Here, we provide evidence that rapid cranial NCC motility on laminin requires integrin recycling. NCCs showed both ligand- and receptor-specific integrin regulation in vitro. On laminin, NCCs accumulated internalized laminin but not fibronectin receptors over 20 min, whereas on fibronectin neither type of receptor accumulated internally beyond 2 min. Internalized laminin receptors colocalized with receptor recycling vesicles and were subsequently recycled back to the cell surface. Blocking receptor recycling with bafilomycin A inhibited NCC motility on laminin, indicating that substratum-dependent integrin recycling is essential for rapid cranial neural crest migration.

Introduction

The neural crest is a transient cell population that arises on the dorsal side of the neural tube and migrates extensively throughout the developing vertebrate embryo. These cells generate a diverse array of derivatives, including the neurons and glia of the peripheral and autonomic nervous systems, craniofacial connective tissue and bone, pigment cells, and adrenomedullary cells, as well as the outflow tract of the heart (for review see Bronner-Fraser, 1993a; Anderson, 1997; Le Douarin and Kalcheim, 1999; Christiansen et al., 2000; Dorsky et al., 2000; Gammill and Bronner-Fraser, 2003). Numerous reports have documented severe perturbation of neural crest cell (NCC) migration after manipulations of integrin function both in vitro and in vivo (Kil et al., 1996; Testaz and Duband, 2001; Alfantari et al., 2003; Tucker, 2004), but the molecular and cellular basis of this flawed motility remain unclear.

Integrins are a major metazoan family of cell adhesion receptors and play key roles in development, immune response, and cancer metastasis (for review see De Arcangelis and Georges-Labouesse, 2000; van der Flier and Sonnenberg, 2001). These heterodimeric transmembrane receptors, composed of an α and a β subunit, bind the ECM and convey signals intracellularly. During vertebrate development, integrins are required at numerous stages for proper cell migration, proliferation, survival, and differentiation of many embryonic cell populations, including the neural crest.

To migrate long distances through diverse tissues in vivo, NCCs must be able to adapt to changing extracellular environments. We have previously shown that embryonic sensory neurons and their immediate embryonic precursors, NCCs, are able to migrate across at least a 10-fold range of ECM protein concentrations in vitro (Condic and Letourneau, 1997; Condic, 2001; Strachan and Condic, 2003). NCCs attain optimal adhesion for sustained motility over a wide range of ECM concentrations by altering surface integrin expression in order to match their adhesion receptor levels to the concentration of ligand. In contrast, many other motile cell types appear unable to modulate surface integrin levels and therefore only migrate on a limited range of ECM concentrations (Goodman et al., 1989; Buettner and Pittman, 1991; Duband et al., 1991; Arroyo et al., 1992; Dimilla et al., 1993; Palecek et al., 1997). These results suggest that rapid NCC motility over a wide range of substratum concentrations is dependent on continuous monitoring of and response to the extracellular environment.

The response of NCCs to the ECM varies along the rostro-caudal axis of the embryo. The neural crest can be divided into four subpopulations (cranial, vagal, truncal, and sacral), each of
which occupies its own segment of the neural tube and gives rise to distinct derivatives (Bronner-Fraser, 1993b). We have shown that different crest populations have distinct motility and integrin regulation in culture. For example, cranial and trunk neural crest have similar migratory properties on low concentrations of laminin. Yet, on high concentrations of laminin, cranial NCCs migrate nearly twice as fast as trunk NCCs. Correspondingly, cranial NCCs regulate surface levels of integrin α6 (a laminin receptor) to a greater extent than do trunk NCCs. When integrin α6 is overexpressed in cranial NCCs, their velocity slows to that of trunk NCCs, suggesting that low surface integrin levels are required for rapid motility (Strachan and Condic, 2003). Thus, we focused here on the mechanism cranial NCCs use to modulate their surface integrin levels, thereby promoting rapid cell migration.

One mechanism by which cells can modulate their surface integrin levels is via the clathrin-mediated receptor recycling pathway (Bretschger, 1992; Fabbri et al., 1999; Pierini et al., 2000; Long et al., 2001). Clathrin-mediated endocytosis modulates signal transduction both by controlling the levels of surface signaling receptors and by mediating the rapid clearance and down-regulation of activated signaling receptors. For motile cells, receptor recycling also provides an efficient way to transport receptors from the tailing edge, where the cell is releasing from the substratum, to the leading edge, where new adhesions are being formed (Roberts et al., 2001; Rappoport and Simon, 2003; Powelka et al., 2004). Receptors may either be returned to the surface nearby the site of internalization via fast recycling vesicles, or can be trafficked through the cell via the slower receptor recycling compartment (Sonnichsen et al., 2000). Each endocytic compartment is characterized by the expression of specific rab GTPases (Mellman, 1996; Sheff et al., 1999; Qualmann and Mellor, 2003). Because cranial NCCs down-regulate surface levels of integrin α6 in response to the same conditions under which they migrate relatively quickly (i.e., high laminin concentrations), we questioned whether laminin receptors were being transported through the receptor recycling pathway in rapidly moving NCCs.

In the present work we demonstrate that cranial NCCs regulate their surface integrin expression in a substratum-dependent manner. Using a modified, short-term, biochemical assay we followed the internalization kinetics of α5 (a fibronectin receptor) and α6 (a laminin receptor) integrins in cranial NCCs cultured on fibronectin and laminin. We find that in cells cultured on laminin, but not fibronectin, surface integrin α6 is internalized and intracellular pools of internalized receptor accumulate over a 20-min time period. Internalized laminin receptors colocalize in rab4+ and rab11+ recycling vesicles, indicating that they are transported via the receptor recycling pathway. By labeling surface receptors immunohistochemically, we also demonstrate that internalized receptors are recycled back to the cell surface. Finally, using an acute time-lapse assay we show that inhibiting receptor recycling with baflomycin A (BafA) significantly slows cranial neural crest migration on high laminin but not on low laminin, suggesting that this pathway is critical for regulating adhesion and supporting rapid motility on this substrata. We conclude that receptor recycling is an important mechanism underlying rapid cranial neural crest migration.

Results

Surface integrin expression is substratum dependent

To determine whether cranial NCCs modulate their surface integrin levels in a substratum-dependent manner, we examined the steady-state surface expression of two integrin receptors, α6 (a laminin receptor) and α5 (a fibronectin receptor), in cranial NCCs cultured on two different concentrations of laminin and fibronectin. Mesencephalic neural tubes were cultured on glass coated with low (1 μg/ml) and high (20 μg/ml) concentrations of either laminin or fibronectin. Application of laminin at 1 and 20 μg/ml resulted in a density of 45 and 430 ng/cm² bound laminin, respectively, and application of fibronectin at 1 and 20 μg/ml resulted in 35 and 700 ng/cm² bound fibronectin, respectively (Strachan and Condic, 2003). After labeling surface proteins with biotin, integrins α5 and α6 were immunoprecipitated, and the surface pools were detected with avidin on Western blots. As we previously observed (Strachan and Condic, 2003), cranial NCCs cultured on varying concentrations of laminin regulated surface levels of the laminin receptor, integrin α6, but not the fibronectin receptor, integrin α5 (Fig. 1, A and C). On low concentrations of laminin (LM1), cells expressed greater amounts of integrin α6 on their surface compared with cells cultured on high laminin concentrations (LM20). Quantification of band intensities from both laminin concentrations indicates an average twofold increase in surface α6 protein on LM1 relative to LM20 (n = 6; P < 0.002, Mann-Whitney U-test). On both concentrations of laminin, surface levels of the fibronectin receptor, integrin α5, were equivalent, suggesting that integrin regulation is receptor specific (Fig. 1, A and C). In contrast to cells cultured on laminin, cranial NCCs cultured on varying concentrations of fibronectin did not modulate surface levels of either integrin α5 or α6 (Fig. 1, B and C).

Figure 1. Cranial neural crest modulate surface integrin levels in a substratum-dependent manner. Cell surface–labeled integrins were immunoprecipitated and analyzed on Western blot. [A and B] Average band intensities (relative to the high substratum condition) are given below each lane. [A] Cranial neural crest down-regulate surface levels of the laminin receptor integrin α6, but not the fibronectin receptor integrin α5, when cultured on high (LM20) compared with low (LM1) concentrations of laminin. [B] Surface levels of both integrin α5 and α6 are equivalent in cranial neural crest cultured on low (FN1) and high (FN20) concentrations of fibronectin. [C] The average relative band intensity ratio (LM1:LM20 or FN1:FN20) for both integrins α5 and α6; y-axis is a log scale. Means and 95% confidence intervals from at least five independent experiments are as follows: LM1:LM20 1.07x [1.01–1.13], LM20:LM1 2.04x (1.79–2.34), FN20:FN1 1.02x [0.95–1.11], and FN1:FN20 0.95x [0.90–1.01]. Asterisk indicates that the average band intensity of integrin α6 on LM1 is significantly greater than on LM20 (P < 0.002; Mann-Whitney U-test). Average band intensities for all other conditions did not significantly differ. Equal protein amounts were used for immunoprecipitations.
Modulating surface integrin levels (0.17- to 3-fold) has been shown to affect motility in a substratum-dependent manner (Palecek et al., 1997; Condic et al., 1999; Condic, 2001; Strachan and Condic, 2003). Based on our previous finding that cranial neural crest on high concentrations of laminin compared with low both down-regulated surface integrin α6 levels and had greater velocities (Strachan and Condic, 2003), we speculated that integrin internalization on high substratum concentrations was related to the rapid motility of these cells. An alternative explanation is that low substratum concentrations stabilize receptor expression at the surface; however, that does not seem to be the case either for integrin α5 on low laminin, or for integrin α5 and α6 on low fibronectin concentrations. These results suggest that down-regulation of relevant (i.e., ligand-binding) surface receptors in response to high laminin concentrations on cranial neural crest may involve the rapid internalization of receptors with markers of the receptor recycling pathway. Internalized integrins could be targeted for destruction or sequestration in cellular compartments that were neither Rab 4 nor Rab11 positive, or double-positive for both (Rab 4 and Rab11). Thus, cranial NCCs on high laminin concentrations are unique to this substratum. Thus, we decided to further investigate the substratum-related differences in cranial neural crest surface integrin regulation.

**Integrin internalization kinetics are substratum dependent**

To analyze the kinetics of surface integrin regulation in cranial NCCs, we followed the internalization of labeled receptors during a short time period. We found a difference between the accumulation of internalized integrin α6 and integrin α5 (Fig. 2). On both low and high laminin concentrations, internalized integrin α6 reached steady-state levels by 20 min; i.e., there was no greater accumulation after 30 or 60 min (unpublished data). In contrast to integrin α6, low levels of integrin α5 accumulated rapidly (within 2 min) in cells cultured on both laminin concentrations, and the internalized pool did not increase over time (Fig. 2, B and D). Thus, cranial NCCs on laminin internalize and accumulate laminin, but not fibronectin.

To determine whether the lack of integrin α5 internal accumulation on laminin was due to its inability to bind laminin, we analyzed the internalization of integrin α5 and α6 on fibronectin. When cranial NCCs were cultured on high concentrations of fibronectin, they rapidly internalized both integrin α5 and integrin α6, but there was no differential accumulation of either receptor inside the cell beyond the steady-state level reached after 2 min (Fig. 2, C and D). Thus, the internal accumulation of integrins in cranial NCCs depends both on the receptor (i.e., α5 vs. α6) and on the substratum. These results indicate that cranial NCCs respond to high concentrations of laminin differently than they do to fibronectin, suggesting that differences in receptor trafficking may underlie differences in motility on dissimilar substrata.

**Internalized integrins colocalize with receptor recycling pathways**

To determine in which intracellular pathway internalized integrins were being trafficked, we investigated the colocalization of receptors with markers of the receptor recycling pathway. Internalized integrins could be targeted for destruction or sequestrated in receptor recycling compartments; however, the latter possibility is more likely because synthesis of new receptor molecules is generally incompatible with rapid motility (Breitscher and Aguado-Velasco, 1998). Cranial NCCs were cultured on high concentrations of laminin or fibronectin and colabeled for either integrin α5 or integrin α6, and Rab4 and Rab11, markers of both the fast and slow recycling pathways. To quantify the colocalization events we used Volocity (Improvision), a program that reconstructs confocal z-series of cells three dimensionally to allow XZ and YZ reslicing in order to verify true colocalization events.

On high laminin concentrations, both integrin α5 and integrin α6 colocalized with vesicles positive for either Rab4 or Rab11, or double-positive for both (Rab+ vesicles), but a significantly greater percentage of Rab+ vesicles contained integrin α6 (40%) compared with integrin α5 (21%) (Fig. 3, A and B; Table I). Integrin α5 appeared to be largely localized in intracellular compartments that were neither Rab 4 nor Rab11 positive.
Interestingly, in the conditions in which we saw less colocalization (i.e., α5 and α6 on fibronectin; α5 on laminin), the observed colocalization was similar for all receptors (~20%), suggesting that one fifth of the rab+ vesicles are occupied by internalized integrins regardless of their substratum relevance (Fig. 3 B, Table I). The fact that integrin α5 does not colocalize with rab+ vesicles to any greater degree on fibronectin compared with laminin indicates that unlike integrin α6, this receptor does not colocalize with the recycling pathway in a substratum-dependent manner. Thus, in cranial neural crest the intracellular trafficking of surface receptors is different when cells are cultured on laminin compared with fibronectin, suggesting cranial NCCs use different mechanisms to support efficient motility on these two ECM molecules.

**Internalized integrins are recycled back to the cell surface**

Next, we investigated the fate of internalized surface integrin α6 in cranial NCCs cultured on high laminin concentrations. In other fast-moving cells and growth cones, internalized receptors are recycled back to the leading edge rather than being degraded (Lawson and Maxfield, 1995; Kamiguchi and Lemmon, 2000). Using an adapted immunohistochemical technique based on that of Kamiguchi and Lemmon (2000) we examined the recycling of integrin α6 in cranial NCCs. Surface α6 was labeled with anti-α6-Fab while cells were allowed to endocytose and traffic receptors for 30 min at 37°C. Cells were cooled to room temperature to prevent further trafficking and Fab remaining at the surface was blocked with unconjugated secondary antibodies. Subsequently, cells were allowed to traffic internalized receptors at 37°C for 20 or 90 min, and any integrin α6-Fab complexes that returned to the surface were detected with labeled secondary antibodies. Intentionally permeabilized cells (Fig. 4 A, first column; Fig. 4 B) could be distinguished from unpermeabilized cells by significantly higher integrin α6 and actin fluorescence. In the 0-min condition, cells fixed at the beginning of the recycling period and reincubated for 90 min had low fluorescence of both integrin α6 and actin (Fig. 4 C).

Our results demonstrate that internalized integrin α6 is returned to the surface (Fig. 4). After 20 min, a small amount of recycled receptors could be detected on the cell surface, and by 90 min the levels of recycled receptors on the surface had further increased (Fig. 4 C). Levels of integrin α6 fluorescence at both 20 min (988 ± 71 average intensity units/μm²) and 90 min (1,627 ± 87) were significantly different from 0-min (608 ± 25) levels (P < 0.0001; t test), reflecting a 39 and 63% increase from the 0-min time point, respectively (Fig. 4 C). Recycled receptors were observed near the cell center and the leading edges of filopodia and lamellipodia, which is consistent with the observations of recycled transferrin receptors in migrating fibroblasts (Hopkins et al., 1994), and recycled L1 protein in neuronal growth cones (Kamiguchi and Lemmon, 2000). Thus, in cranial NCCs cultured on high concentrations of laminin, integrin α6 receptors are rapidly internalized, transported intracellularly, and then recycled back to the cell surface.
Inhibiting receptor trafficking slows cranial neural crest motility

Because cranial NCCs down-regulate surface levels of integrin α6 (Fig. 1), internalize this receptor only on laminin (Fig. 2 and Fig. 3), and recycle it back to the cell surface when cultured on high concentrations of laminin (Fig. 4), we postulated that rapidly moving cranial NCCs cultured on high concentrations of laminin would be more dependent on receptor trafficking than the same cells on low levels of laminin where they migrate more slowly. To determine whether substratum-dependent receptor recycling affects cranial neural crest motility, we inhibited receptor trafficking using a pharmacologic approach.

Bafilomycin A is a specific inhibitor of the vacuolar type H⁺-ATPase found in all animal cells, plant cells, and microorganisms. BafA does not prevent endocytosis, yet it prevents the acidification of endocytic structures, which impairs the trans-

Table I. Distribution of rab+ and integrin+ vesicles

| Condition (# of cells) | Labeled vesicles | Double labeled | Percentage of total labeled vesicles | Percentage of Rab+ vesicles | Percentage of Int+ vesicles |
|------------------------|------------------|----------------|-------------------------------------|-----------------------------|-----------------------------|
|                        | Rab+ Int+ | (Rab/Int)+ Rab+ Int+ | (Rab/Int)+ | Rab+ Int+ | (Rab/Int)+ Rab+ Int+ | (Rab/Int)+ |
| CL20 α5 (n = 12)       | 215 ± 25 | 147 ± 16 | 42 ± 4 | 58 ± 4 | 42 ± 4 | 12 ± 1 | 31 ± 4 | 21 ± 2 |
| CL20 α6 (n = 11)       | 273 ± 31 | 171 ± 18 | 102 ± 10\* | 60 ± 4 | 40 ± 4 | 23 ± 1\* | 66 ± 11\* | 40 ± 3\* |
| CF20 α5 (n = 10)       | 288 ± 37 | 184 ± 34 | 53 ± 10 | 62 ± 6 | 38 ± 6 | 11 ± 2 | 36 ± 7 | 18 ± 2 |
| CF20 α6 (n = 10)       | 222 ± 25 | 202 ± 42 | 41 ± 9 | 55 ± 5 | 45 ± 5 | 10 ± 1 | 27 ± 7 | 17 ± 2 |

Average number of labeled vesicles per cell (± SEM) for each condition from at least three independent experiments.

*Conditions that are statistically different from all others (P < 0.001; t test).

Figure 4. Cranial NCCs recycle internalized integrin α6 back to the cell surface. Cells cultured on high laminin concentrations were allowed to internalize anti-integrin α6 Fab bound to surface receptors for 30 min. The remaining cell surface Fab was blocked and cells were reincubated at 37°C for 20 or 90 min to allow for exocytosis of the integrin α6-Fab complex. (A) Recycled integrin α6 was detected by labeling the unblocked Fab that had reap- peared on the surface (red). Cells were also stained for actin (green) as a permeabilization control. Control cells were intentionally permeabilized to show total integrin α6 and actin staining. Bars, 20 μm. (B and C) Average intensities per unit area ± SEM (arbitrary units) of integrin α6 and actin fluorescence were determined for at least 20 cells from three independent experiments for each condition. [B] Measurements from permeabilized control cells, as shown in the first column in A, have significantly higher integrin α6 and actin fluorescence than unpermeabilized 0-min controls, as shown in C (P < 0.001; t test). In unpermeabilized cells, actin fluorescence is con- stant, whereas integrin α6 fluorescence increases at both 20 and 90 min. Asterisk indicates significant difference from 0-min levels (P < 0.0001; t test).
Figure 5. Inhibiting receptor trafficking slows cranial NCC motility on high concentrations of laminin. (A) Internalized cell surface receptors are endocytosed via clathrin-dependent mechanisms to the early endosome. Cargo is then transported either to the late endosome, to the receptor recycling compartments, or rapidly recycled back to the cell surface through a fast recycling pathway. Distinct areas of the pathway are characterized by specific Rab GTPases. The vacuolar proton pump inhibitor bafilomycin A prevents acidification of endocytic structures, thereby impairing transport out of the early endosome. (B) Cumulative distribution plots of cell velocity before drug treatment are shown where each point represents the pre-treatment velocity of a single cell. Cumulative percent on the y-axis refers to the percentage of the population traveling that speed or faster. The average velocity of cranial NCCs before treatment is 232 μm/h ± 11 (SEM; n = 22) on LM20 (red squares), compared with 196 μm/h ± 8 (n = 24) on LM1 (black diamonds). The difference between the two conditions is statistically significant (P < 0.01; t-test). (C) Cumulative distribution plots of cell velocity after drug treatment are shown where each point represents the post-treatment velocity of a single cell. Cumulative percent on the y-axis refers to the percentage of the population traveling that speed or faster. The average velocity of cranial NCCs post-treatment is 174 μm/h ± 10 (SEM; n = 22) on LM20 (red squares), compared with 199 μm/h ± 8 (n = 24) on LM1 (black diamonds). The difference between the two conditions is statistically significant (P < 0.05; t-test). (D) Cumulative distribution plots of the change in cell velocity after BafA (100 nM) treatment. Each point represents the change in velocity of a single cell after BafA treatment. Cells cultured on LM20 (red squares) and treated with BafA slowed an average of 58 μm/h ± 14 (n = 22) (post-treatment velocities were statistically different from pre-treatment velocities; P < 0.001; paired t-test). In contrast, cells cultured on LM1 and treated with BafA (black diamonds; n = 24), and controls, cells cultured on either laminin concentration and treated with vehicle (green triangles; n = 31), did not slow significantly (pre- and post-treatment velocities were not statistically different; P > 0.05, paired t-test). Post-treatment velocities of control cells were significantly different from post-treatment velocities of BafA-treated cells on high laminin (P < 0.05; t-test). Specific points in each graph are not directly comparable to corresponding points in other graphs, as each point merely reflects a cell’s velocity, or change in velocity, in relation to the whole population.
Integrin recycling and receptor specificity

The first observation that integrins differentially participate in the endocytic pathway was published over a decade ago (Bretschger, 1992). In this paper, Bretschger showed that in CHO cells grown in suspension, integrins α5β1 and α6β4 circulate, but α3β1, α4β1, and αLβ2 do not. Although it was initially postulated that the ability to participate in endocytic trafficking may be a property of the receptor itself, other groups have since shown that the substratum onto which the cells are adhered also greatly influences this process. When neutrophils are cultured on vitronectin, the vitronectin receptor mediating their migration (integrin αVβ3) is recycled in a polarized manner (Lawson and Maxfield, 1995). In contrast, when the same cells are cultured on fibronectin, integrin αVβ3 is no longer distributed in a polarized manner, and instead integrin α5β1 (a fibronectin receptor) is internalized into endocytic recycling compartments (Pierini et al., 2000). These studies suggest that cells may preferentially recycle the receptors that are mediating their migration, and this is consistent with our current demonstration of substratum-dependent integrin recycling in cranial NCCs.

Our finding that, in contrast to neutrophils, integrin α5 participates predominantly in short time-frame recycling in cranial NCCs cultured on fibronectin suggests that distinct cell types can regulate the same receptor in different ways. How NCCs modulate adhesion to maintain motility on fibronectin is unknown, but could involve functional modulation of integrin receptors; a possibility we are currently investigating.

Substratum-specific receptor internalization has been shown for nonintegrin receptors as well. For example, insulin receptor internalization was inhibited 40–60% in CHO cells adhered to galectin-8 (an ECM protein and an integrin ligand) when compared with the same cells adhered to fibronectin, collagene, or laminin (Boura-Halfon et al., 2003). Other studies have shown that in embryonic dorsal root ganglia neurons, L1, a homophilic cell adhesion receptor that promotes axon growth along preexisting axon bundles, is recycled in a spatially regulated manner within growth cones cultured on L1 but not laminin (Kamiguchi and Lemmon, 2000). It is likely that the internalization of many receptors may depend on the extracellular environment of the cell. In this way, intracellular signaling of downstream events such as migration, differentiation, and apoptosis could be regulated in a region specific manner throughout the embryo.

Recycling kinetics and substratum density

Receptor recycling has not been previously investigated in a single cell population cultured on different concentrations of the same substrata. Our findings suggest that not only is substratum composition a key regulator of receptor recycling, but also that the density of ligands on the substratum can affect the importance of receptor internalization for motility. BafA treatment has a greater effect on cranial neural crest motility when the cells are cultured on high concentrations of laminin compared with low. Thus, when cranial NCCs are cultured on low concentrations of laminin, their dependence on receptor recy-
Efficient motility and cell fate

Although the lessons learned from investigating cell motility in vitro have established a strong model for integrin-mediated migration, it is the application of these lessons that provides insight into the importance of efficient motility in a specific biological context (in this case, the developing embryo). During development, NCCs migrate along defined pathways in the embryo and differentiate into distinct cell types. For example, in the trunk, NCCs initially migrate either dorsolaterally along a laminin-rich basal lamina, or ventromedially through tissue rich in fibronectin. Cells that follow the dorsolateral pathway are destined to become melanoblasts, whereas those which migrate ventromedially will largely become neurons and glia (Bronner-Fraser, 1993a). In comparison, in the head, NCC migration proceeds through the mesoderm lateral to the neural tube and then ventrally toward the branchial arches, areas rich in ECM proteins including both laminin and fibronectin. Although the relative amounts of ECM proteins that these cells encounter while migrating have not been determined, our results suggest the intriguing possibility that differences in migratory speed may influence NCC differentiation. The ability of cranial NCCs to migrate so quickly on high laminin concentrations may reflect a difference in the capability of these cells to reach certain targets within a critical time period and thereby be available to receive short-lived, target-derived differentiation cues. Alternatively, if NCC differentiation affects cell motility, the high speed of cranial NCCs on high laminin may reflect early cell fate specification.

In summary, we have shown that substratum-dependent receptor recycling is an important molecular mechanism used by cranial NCCs to promote rapid motility on laminin. We have also demonstrated that the same cell population can respond differently to the same substratum, depending on its concentration. Our data suggest that cranial NCCs tightly regulate the cellular mechanisms that support motility in response to the specific conditions the cells encounter. The investigation of receptor recycling in a primary embryonic cell population is both novel and fundamental to the understanding of cell migration during development.

Materials and methods

Substratum preparation and cell culture

Glass coverslips (Goldseal; Fisher Scientific) were acid washed, rinsed in dH2O, and baked at 350°C for 12 h. 13-mm holes were drilled in the bottom of 35-mm tissue culture dishes (Fisher Scientific) and plasma fibronectin (FN; GIBCO BRL) and natural mouse laminin (LM; GIBCO BRL) were diluted to high (200 ng/ml) and low (100 ng/ml) concentrations in PBS, and 300 μl of the protein solution was added to the coverslips, incubated for 3 h at RT, and then rinsed once with PBS. As previously published (Strachan and Condic, 2003), absolute protein concentration on the coverslips was determined by measuring the amount of tritiated laminin and fibronectin bound to glass as described in de Curtis et al. (1991). Application of laminin at 1 and 20 μg/ml resulted in a density of 45 and 430 ng/cm² bound laminin, respectively. Application of fibronectin at 1 and 20 μg/ml resulted in 35 and 700 ng/cm² bound fibronectin, respectively.

White Leghorn chicken eggs (supplied by Utah State University, Logan, UT) were incubated at 38°C until the embryos reached stage 8 (Hamburger, 1992). All animal studies were approved by the University of Utah Institutional Review Board. Neural tubes from the mesencephalon were dissected away from the surrounding tissue with tungsten needles. Neural tubes were cultured at 37°C with 5% CO2 for 48–72 h in 250 μl neurobasal medium (GIBCO BRL) supplemented with 25 μM glutamic acid (Sigma-Aldrich), 300 μM γ-glutamylamine (Sigma-Aldrich), 1× B-27 and H-2 (GIBCO BRL), 100 ng/ml NT3 (CHEMICON International), 100 ng/ml EGF (Upstate Biotechnology), 10 ng/ml FGF (Upstate Biotech-
Antibodies against the following proteins were used in these experiments: integrin α5 (D71E2; DSHB, and AB1928; CHEMICON International), integrin α6 (P2C62C4, DSHB, and SC10370; Santa Cruz Biotechnology, Inc.), rab4 (610888; BD Biosciences), rab11 (610656; BD Biosciences), actin (AAN01; Cytoskeleton, Inc.), and α-tubulin (T5168; Sigma-Aldrich). For the integrin recycling experiments a Fab fragment of a monoclonal integrin α6 antibody (MAB13444; CHEMICON International) was generated. Bafilomycin A1 (B1793; Sigma-Aldrich) was dissolved in DMSO (DB418; Sigma-Aldrich). Sodium 2-mercaptoethanesulfonate (MesNa) was purchased from Sigma-Aldrich (M1511) and dissolved immediately before use in Hepes-buffered neurobasal medium.

Immunoprecipitations

Cell surface receptors were labeled with NHS-SS-biotin (Pierce Chemical Co.) on ice and the cells were then lysed on ice in a solution containing 0.1% SDS, 1% Triton X-100, and protease inhibitors. Protein concentration in each cell lysate was determined with a BCA protein assay (Pierce Chemical Co.) and equalized across conditions. Integrins were immunoprecipitated by using standard protocols (de Curtis et al., 1991), and immunoprecipitated proteins were size fractionated under nonreducing conditions on acrylamide gels and electrophoretically transferred to nitrocellulose membranes. Biotinylated proteins were detected by using Streptavidin conjugated to HRP and a chemoluminescent reagent (Pierce Chemical Co.) followed by exposure to film. For the immunoprecipitations assay (Fig. 2), where reducing agents interfere with protein concentration assays, 15 μl of lysate was removed before immunoprecipitation as a protein loading normalization control, size fractionated on an acrylamide gel, transferred to nitrocellulose, and revealed with an α-tubulin antibody (T5168; Sigma-Aldrich). Using nonreduced lysates, BCA assays were performed alongside α-tubulin Western blots to ensure that α-tubulin expression was not different in cells cultured on LM1, LM20, or FN20. Comparisons of protein concentrations (BCA) and α-tubulin band intensities (as a measure of protein loading) give identical results. Exposures of blots that were in the linear range were quantified using GelDoc and QuantityOne (Bio-Rad Laboratories).

Internalization assay

For the analysis of receptor internalization, we adapted a biochemical assay (Fabbri et al., 1999) for NCCs. Cranial neural crest cultures were grown for 48 h, the neural tubes were removed, and the remaining crest cells were scraped off the coverslip (Bronner-Fraser, 1996). This procedure yields relatively pure NCC populations as determined by HNK-1 staining (Bronner-Fraser, 1986). The cells were spun, resuspended in neuronal medium, supplemented, and plated (equal number of cells per dish) as dissociated cells overnight or until the cultures reached ~70% confluency. Cell surface receptors were labeled with biotin at 37°C for 0, 2, 5, 10, or 20 min. For the 0-min controls, the cells were gently rinsed once with biotin. After biotinylation, the cells were placed on ice, rinsed gently, and treated with a non-cellassociating reducing agent (MesNa) to remove the biotin from the receptors that remained on the cell surface. After MesNa treatment, cells were lysed on ice and immunoprecipitations and analysis were then performed as detailed above.

Recycling assay

For the analysis of recycling we adapted an immunohistochemical assay based on that of Dimes to M.L. Condic and by a National Institutes of Health grant (NIH-F31-NS43289-01) to L.R. Strachan.

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