INTRODUCTION

Neural crest cells arise from the dorsal neural tube and migrate along defined pathways to specific sites in the embryo, where they give rise to many derivatives, including most of the neurons and glia of the peripheral nervous system, the craniofacial skeleton and nearly all the pigment cells (Le Douarin and Kalcheim, 1999). In the trunk of the avian embryo, neural crest cells take two distinct pathways. Initially, they migrate ventrally between the neural tube and somite and through the anterior sclerotome (Rickmann et al., 1985; Bronner-Fraser, 1986; Loring and Erickson, 1987; Teillet et al., 1987) and coalesce to form the adrenal medulla and the neurons and glia of the dorsal root ganglia and sympathetic ganglia. Cells emigrating from the neural tube 12-18 hours later invade the dorsolateral pathway, between the derrnomyotome and the overlying ectoderm (Serbedzija et al., 1989; Erickson et al., 1992), where they colonize the skin and differentiate as melanocytes. Thus, early and late migrating neural crest cells from the trunk region are distinct subpopulations with different migratory behaviours and developmental fates.

The mechanisms responsible for dictating the timing and patterning of neural crest migration are not well understood, although one aspect of pattern regulation in the ventral pathway has been clarified. Specifically, the Eph receptor tyrosine kinases and their ephrin ligands have been shown to be essential for the segmental migration of neural crest cells (Krull et al., 1997; Wang and Anderson, 1997). Studies of the expression of Eph receptors and ephrins in the somites show that in the avian embryo EphB3 is expressed by ventrally migrating neural crest cells, and Ephrin-B1 is expressed in the posterior half of the somites, whereas in rodents, ephrin-B2 is transcribed in the posterior somite and EphB2 is expressed by...
neural crest cells. The interaction between the Eph receptors expressed by ventrally migrating neural crest cells and the ephrin ligands expressed in the posterior sclerotome mediate a repulsive response that restricts the migration of the cells to the anterior half of each somite. However, as there is normal migration of trunk neural crest cells in the homozygous double mutants of EphB2 and EphB3 (Wang and Anderson, 1997), it is likely that other EphB receptors are expressed by neural crest cells that compensate for the loss of EphB2 function. In addition, mice lacking ephrin-B2 have a normal pattern of neural crest migration (Wang et al., 1998; Adams et al., 1999). This may be due to the presence of functionally redundant guidance cues in the somites. Other cases of Eph-ephrin mediated repulsive behaviour have been reported. For example, these molecules restrict cell or neuronal growth cone movement, thereby preventing cells or axons from entering inappropriate territories in the embryo (Dottori et al., 1998; Drescher et al., 1995; Helmbacher et al., 2000; Henkemeyer et al., 1994; Immedi et al., 2000; Nakamoto et al., 1996; Orioli et al., 1996; Park et al., 1997; Smith et al., 1997). The repulsive response is believed to be mediated by rearrangements in the cytoskeleton that lead to the collapse of growth cones in neuronal cells (Meima et al., 1997).

A still unresolved question is how neural crest cells are directed along the dorsolateral migratory pathway. Migration into the dorsolateral path depends upon the lineage specification of the cells. Immunolabelling experiments show that neural crest cells are specified as melanoblasts prior to entering the dorsolateral path and that they are the only neural crest cells that migrate dorsolaterally (Kitamura et al., 1992; Reedy et al., 1998). Neural crest cells from the ventral pathway do not have the ability to invade the dorsolateral path, as demonstrated by grafting non-melanoblast neural crest cells into a host embryo whose endogenous neural crest cells have begun to migrate dorsolaterally (Erickson and Goins, 1995). In contrast, when melanoblasts are grafted into a young host whose endogenous crest cells are only migrating ventrally, the melanoblasts will immediately migrate dorsolaterally. Taken together, these experiments demonstrate that only melanoblasts, and no other neural crest cells, possess the ability to migrate dorsolaterally.

We wanted to identify the cues in the dorsolateral path that initially inhibit the ventrally migrating crest from entering that space, but yet later allow melanoblasts to migrate along this pathway. The expression of two inhibitory factors in the dermomyotome, chondroitin sulphate glycoconjugates, and peanut agglutinin (PNA)-binding glycoproteins, correlate with the initial inhibition of neural crest entry into the dorsolateral pathway (Oakley et al., 1994). Loss of these two markers by ablation of the dermomyotome allows neural crest cells to enter the dorsolateral path precociously (Erickson et al., 1992; Oakley et al., 1994). Furthermore, the expression of these molecules is downregulated at the time that neural crest cells initiate migration along the dorsolateral pathway. These results suggest that neural crest cells might be transiently inhibited from migrating on the dorsolateral path because of the expression of chondroitin-6-sulfate proteoglycan and PNA-binding molecules. However, these results cannot explain how melanoblasts grafted into the early embryo can overcome the dorsolateral barrier. Transmembrane ephrins have also been reported to be present along the dorsolateral pathway at the time neural crest cells are migrating (Krull et al., 1997; Wang and Anderson, 1997), suggesting that ephrins might repel ventrally migrating neural crest cells from the dorsolateral path.

In the present study, we confirm that the dorsolateral path is filled with ephrins at the time when neural crest cells move exclusively ventrally. When we perturb Eph receptor function in neural crest cells in trunk explants, the early migrating neural crest disperse dorsolaterally, demonstrating that ephrins block early dorsolateral migration. Surprisingly, we found that transmembrane ephrins are also expressed along the dorsolateral pathway during melanoblast migration and that melanoblasts express several EphB receptors. If Eph receptor function is blocked in vivo at the time when melanoblasts are migrating, this subpopulation of neural crest cells fail to migrate into the dorsolateral pathway. Moreover, in vitro cell migration assays demonstrate that ephrins promote melanoblast migration, not inhibit it. These results show that transmembrane ephrins actively promote the migration of neural crest cells into the dorsolateral path. Adhesion and motility assays suggest that engagement of ephrin-B ligands by EphB receptors promote melanoblast motility by increasing their adhesion to fibronectin.

MATERIALS AND METHODS

Receptor and ligand fusion protein staining

EphB2-Fc receptor and ephrin-B1-Fc ligand fusion proteins were obtained from Dr Nicholas Gale at Regeneron Pharmaceuticals. Whole-mount staining of chicken embryos using IgG-Fc-tagged ligand and receptor was performed essentially as described by Gale et al. (Gale et al., 1996a). After removal of the extraembryonic membranes, stage 12-24 embryos (Hamburger and Hamilton, 1951) were blocked with 10% goat serum and 2% bovine serum albumin in phosphate-buffered saline (PBS). Embryos were then incubated overnight at 4°C with receptor-Fc (5 μg/ml in 0.5x blocking solution). Embryos were repeatedly washed with PBS and then fixed with 4% paraformaldehyde (PFA). To inactivate endogenous phosphatases, embryos were incubated at 70°C for 1 hour and again blocked in 0.5x blocking solution plus 0.1% Triton X-100 followed by incubation overnight at 4°C in an alkaline phosphatase (AP)-conjugated goat anti-human antibody (1:1000; Promega). After extensive washing in Tris-buffered saline (TBS) plus 0.1% Triton X-100, embryos were transferred to AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl2) with NBT (0.34 mg/ml) and BCIP (0.18 mg/ml; Sigma). Following color development, embryos were rinsed in PBS and fixed in 4% PFA. Stained embryos were viewed using a Leica stereomicroscope and images were captured using an Optronics Magnafire camera.

Cultured neural crest cells (see below) were incubated with Fc alone or ephrin-B1-Fc for 1 hour at room temperature. Cells were rinsed in PBS and fixed in 4% PFA for 10 minutes. Bound ephrin-Fc was detected using AP-conjugated goat anti-Fc, and AP activity was detected by incubation with NBT/BCIP (Sigma), as described above.

Quail neural crest cultures and immunofluorescence

Fertile Japanese quail eggs (Coturnix japonica) from the Animal Sciences Department (University of California, Davis) were incubated in a humidified 37°C incubator until they reached stage 13. Neural tubes at somite level I to VIII (between the last-formed somite to the eight from the last-formed somite) were dissected from embryos and separated from surrounding tissues, including ectoderm, somite and notochord, after a brief digestion in Pancreatin (Gibco). Cleaned
neural tubes were transferred to 35-mm tissue culture dishes (Falcon) and maintained in Ham's F12 medium (Gibco), supplemented with 10% fetal calf serum, 3% 10-day chick embryo extract and 100 units/ml penicillin/streptomycin (Gibco). Cultures enriched for neuroblasts and glioblasts (early outgrowths) but deficient in melanoblasts were obtained by removing the neural tube 13 hours after explantation (Reedy et al., 1998). Melanoblast clusters were isolated from the dorsal neural tube 30 hours after explantation, as previously described (Loring et al., 1981).

For immunocytochemistry, neural crest cells were plated on glass coverslips coated with fibronectin or Fc fusion ligands (Davy and Robbins, 2000). Cells were then fixed in 4% PFA for 10 minutes at room temperature and permeabilised in 0.1% Triton X-100, 2% BSA in PBS. For staining of the actin cytoskeleton, cells were incubated for 30 minutes in rhodamine-conjugated phalloidin (Sigma) as recommended by the supplier. Focal adhesion proteins were detected with the VIN-11-5 anti-vinculin antibody (1:100; Sigma). Cy2-conjugated secondary antibody (1:100; Jackson ImmunoResearch) was used to detect the primary antibody.

**RT-PCR analysis**

cDNAs were isolated from cultured neural crest cells by using a Cells-to-cDNA kit (Ambion). Lysis buffer was added to neural crest cultures and incubated for 5 minutes. DNase I was added to cell lysates and incubated for 30 minutes at 37°C followed by incubation for 5 minutes at 75°C to inactivate the DNase I activity. First-strand cDNA synthesis was primed with random decamers or oligo(dT) using MMLV-reverse transcriptase. The following primers (Operon) were used for PCR: EphB1, sense primer 5'-AGGGTGGAAGAAGTGAGTG-3' and antisense primer 5'-TCATCAACCTGACCACAAAG-3'; for EphB2, sense primer 5'-AGGGGGATTGTGTGAGGT-3', and antisense primer 5'-CCCCCTGACGATTACCTA-3'; for EphB3, sense primer 5'-AGGAGTGTGGTGGAGGTG-3' and antisense primer 5'-CTCGGCGACAGTGTGTAC-3'; for GAPDH, sense primer 5'-GATGGGTGTCAACCATGAGAA-3' and antisense primer 5'-ATCAAGGTTGGAAAGTGGCTG-3'. Amplification of cDNAs was performed using 25 cycles with denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute. Final amounts of reagents in the reaction were 1 µg/ml each primer, 10 mM dNTPs and 1.5 mM MgCl₂ in a final volume of 50 µl.

**Whole-mount in situ hybridisation**

Antisense and sense EphB1, EphB2, EphB3, EphB5 and EphA4 probes were produced by linearization of plasmid (kind gifts from Dr Elena Pasquale, The Burnham Institute, La Jolla, CA) with appropriate restriction enzymes, followed by in vitro transcription with T7 RNA polymerase. In situ hybridisation was performed as previously described (Nieto et al., 1996), with the following modifications. White leghorn chicken embryos (California Golden Eggs, Sacramento) stages 14-23 (Hamburger and Hamilton, 1951) were collected in cold PBS and extraembryonic tissues were removed. Embryos were fixed in 4% paraformaldehyde in PBS and dehydrated in a graded series of PBT-methanol. Embryos were bleached with 6% hydrogen peroxide, rehydrated and treated with 5 µg/ml proteinase K for 15-30 minutes, depending on the embryonic stages. Hybridisation was carried out at 70°C in hybridisation buffer for 36 hours. An alkaline phosphatase-mediated color reaction was carried out using BM Purple (Boehringer Mannheim). Control hybridisation experiments using sense probes were performed for all the receptor genes and did not produce any specific signals (data not shown). Stained embryos were embedded in OCT. Frozen sections were stained with HNK-1 antibody to detect neural crest cells. Cy2-conjugated goat anti mouse IgG+IgM (Jackson Immunoresearch) was used as a secondary antibody.

**Trunk explants**

The trunks of stage-12 chick embryos were excised at the level of the most posterior 6 somites, as previously described (Krull et al., 1995; Krull and Kulesa, 1998). When older embryos were employed, segments from the thoracic level, equivalent in length to the stage-12 pieces, were excised and cultured. Explants were preincubated in a solution of ephrin-B1-Fc (10 µg/ml) or control human Fc fusion proteins and grown for 24 to 36 hours on Millicell polycarbonate membranes (Millipore Corp., Bedford, MA) at 37°C in a 5% CO₂ tissue culture incubator. Explants were then fixed in 4% PFA and immunostained with the monoclonal HNK-1 antibody (supernatant prepared from the cell line obtained from ATCC) to determine the distribution of neural crest cells, and a secondary antibody conjugated to horseradish peroxidase (1:1000; Promega).

**Transfilter chemotaxis assay**

Chemotaxis chambers (Chemicon), in which upper and lower wells were separated by an 8-µm pore-size polycarbonate membrane precoated on the bottom side with fibronectin, were used in the transfilter migration assays. Prior to their addition to the bottom well, purified Fc or ephrin-B1-Fc fusion proteins (10 µg/ml) were preclustered by incubation with 100 µg/ml of polyclonal goat anti-human IgG-Fc (Jackson) for 1 hour at room temperature in complete culture medium. The upper wells were filled with medium containing neural crest cells at a concentration of 2×10⁶ cells/ml. Plates were incubated for 6 hours at 37°C in a 5% CO₂ incubator. After migration, cells were then scraped from the upper side of the filter membrane in order to visualize only those cells that had migrated through the filter. Migratory cells were stained (Quantitative Cell Migration Assay kit; Chemicon), solubilised and quantified by optical density (OD) measurements at 550 nm.

**Cell attachment assay**

Microtitre plates were coated with fibronectin (10 µg/ml) alone, ephrin-B1-Fc or human-Fc fusion proteins (10 µg/ml) alone or in combination with fibronectin as described by Davy and Robbins (Davy and Robbins, 2000). Adhesion assays were performed essentially as described by Hertzler and McClay (Hertzler and McClay, 1999). Briefly, coated plates were treated with BSA (40 µg/ml) to block any nonspecific background binding. Each well received 2.5×10⁴ to 1×10⁵ cells suspended in F12 culture medium containing 10% fetal calf serum. Plates were sealed and immediately spun at 500 rpm for 3 minutes to attach 100% of the cells to the substratum. After 5 minutes at 37°C, the plates were inverted and spun again to remove non-adhering cells. Bound cells were stained and quantified by OD measurements at 550 nm. Three independent experiments with all variables in triplicate were performed. Results of three representative experiments are shown.

**RESULTS**

**Localisation of transmembrane ephrins along the dorsolateral pathway during early and late stages of neural crest cell migration**

At the onset of migration at the trunk level, neural crest cells take one of two possible pathways. First, they are directed along a ventromedial route through the somite. Twenty-four hours later, neural crest cells emigrating from the neural tube invade the dorsolateral migratory pathway between the dermomyotome and overlying ectoderm (Erickson et al., 1992). Previous descriptions of ephrin distribution during the early stages of neural crest cell migration (stages 12-15) showed that ephrin-B1 and ephrin-B2 mRNA are distributed in the posterior half of the somitic sclerotome, which the neural crest cells avoid (Krull et al., 1997; Wang and Anderson, 1997). This experimental analysis revealed that ephrin-B
causes repulsion of neural crest cell movement, leading to the migration of the cells only through the anterior half of each somite. To assess the possibility that ephrin-B ligands control the timing of migration into the dorsolateral path, we determined the distribution of transmembrane ephrins by labelling whole chick embryos of stages 14-24 with Eph-Fc fusion proteins. At stage 14, when neural crest cells are migrating only in the ventral pathway, ephrins are expressed by the dermomyotome (Fig. 1C), in addition to the posterior half of each sclerotome (Fig. 1A,B), as described previously (Krull et al., 1997; Wang and Anderson, 1997). The distribution of ephrins in the dorsolateral space suggests that they may prevent the invasion of the early migrating crest into this pathway. At later stages, when melanoblasts begin migrating into the dorsolateral space (stages 19-20), ephrins continue to be expressed by the dermomyotome (Fig. 1F). Thus, unlike the ventrally migrating crest, melanoblasts do not avoid the dorsolateral space where ephrin-B ligands are expressed.

Detection of Eph receptors for transmembrane ephrins in explanted trunk neural crest cells

EphB3 expressed by early migratory neural crest cells binds to ephrin-B1 ligand to mediate a repulsive interaction that restricts the migration of the cells exclusively to the ventral pathway (Krull et al., 1997). The fact that ephrin-B proteins are still expressed in the dorsolateral pathway during melanoblast migration suggested that melanoblasts do not express EphB receptors and are therefore insensitive to the inhibitory signal. We used gene-specific RT-PCR to determine which EphB receptors are expressed by different subpopulations of neural crest cells (early outgrowths and melanoblasts). It has been reported previously that EphB3 mRNA is present in early migratory cells (Krull et al., 1997). Our results indicate that EphB1, EphB2 and EphB3 mRNAs are expressed by early and late migratory neural crest cells (Fig. 2). The EphB2 and Ephb3 transcripts are abundant in both early outgrowths and melanoblasts. The PCR primers do not reveal any isoforms as none of the amplified products overlap with any of the splice variant domains already identified for these receptors (Pasquale, 1991; Sajjadi and Pasquale, 1993; Connor and Pasquale, 1995).

Expression of Eph receptors during early and late stages of neural crest cell migration

We analysed Eph receptor expression during trunk neural crest cell migration by whole-mount in situ hybridisation. At stage 14, only EphB3 is expressed in the ventral pathway. EphB3 is present in the anterior half of the somites (Fig. 3), as has been previously demonstrated (Krull et al., 1997). Transverse sections through the trunk region show strong expression of EphB3 in the sclerotome (Fig. 3A,B). EphB3 expression is associated with a stream of neural crest cells (identified by HNK-1 staining) migrating into the sclerotome (Fig. 3C). EphB3 is expressed weakly throughout the neural tube, as was previously reported (Baker et al., 2001). EphB1, B2 and B5 are expressed exclusively in the brain. No staining of the dorsal neural tube and somites is observed (Fig. 3). Results from RT-PCR indicate that early migratory cells also express EphB1 and B2 mRNAs but these receptors were not detected along the ventral pathway by in situ hybridisation (data not shown). It is possible that low levels of receptor are expressed by crest cells in situ that are below levels of detection. Strong expression of EphB1 is found in rhombomeres 3 and 5 (Fig. 3). Expression of EphB1 in the hindbrain has been documented in the mouse and chicken (Becker et al., 1994; Irving et al., 1996; Kury et al., 2000). EphA4, the only EphA class receptor that can also bind class B ephrins (Gale et al., 1996a; Gale et al., 1996b), is expressed in the hindbrain (r3 and r5), unsegmented paraxial mesoderm and the ventral edge of the dermomyotome (Fig. 3) (Hirano et al., 1998; Schmidt et al., 2000; Swartz et al., 2001). Transverse sections at the trunk level show strong expression of EphA4 in the lateral plate mesoderm (Fig. 3D), but no expression is observed in the neural tube or somites. EphA4 expression is not observed in
regions corresponding to pathways taken by neural crest cells (Fig. 3E).

At later stages (stage 23), Eph receptors are widely expressed in the developing embryo. In whole mounts, mRNA transcripts are located primarily in the brain (Fig. 3). EphB1, B2, B5 and A4 are strongly expressed in the dorsal neural tube, whereas EphB3 is expressed weakly. In the trunk, EphB receptors show moderate expression in the somites. EphA4 is also expressed as a stripe on the lateral edge of the somites (Fig. 3). Transverse sections confirm EphB mRNA expression in the dorsal neural tube and along the dorsolateral pathway during melanoblast migration (Fig. 3F-Y). In transverse sections, EphA4 is expressed in the dorsal neural tube and the dorsal edge of the dermomyotome (Fig. 3V). Although multiple Eph receptors are expressed in the dorsal neural tube from which melanoblasts detach, these same receptors are...
expressed in the dermis through which the melanoblasts migrate. Therefore, in the in situ studies we could not unequivocally determine which Eph receptors are expressed by melanoblasts.

To confirm the presence of Eph receptor proteins for ephrin-B ligands in early outgrowths and melanoblasts, we labelled neural crest cultures with soluble ephrin-B1-Fc-tagged fusion proteins. Specific cell surface-labelling of melanoblasts by ligand-Fc, but not control Fc proteins, was detected (Fig. 4C,D). In agreement with a previous report (Krull et al., 1997), early outgrowths were also found to bind ephrin-B1-Fc, which indicates the expression of EphB receptors (Fig. 4A,B).

Embryonic explant experiments reveal a dual role for transmembrane ephrins in neural crest migration

To understand the nature of the interaction between the Eph receptors expressed by melanoblasts and the transmembrane ephrins found in the dorsolateral pathway, we disrupted the Eph-ephrin interactions by adding soluble ephrin-B1-Fc to chick trunk explants. The soluble ligand occupies the receptors in vivo without activating them and prevents the receptors on neural crest cells from binding the endogenous form of the ligand (Krull et al., 1995; Krull et al., 1997; Koblar et al., 2000). Chick embryos were collected at stage 12, before neural crest cells start migrating through the somites, and at stage 18, immediately prior to melanoblast migration into the dorsolateral path. Whole trunk explants were excised and grown on Millicell inserts in the presence of ephrin-B1-Fc or Fc alone in culture medium.

Treatment of stage-12 explants with soluble ephrin-B1-Fc disrupts the segmental migration of neural crest cells, so that cells are found in both the anterior and posterior somite. Transverse sections confirmed the presence of cells in the anterior and posterior sclerotome (Krull et al., 1997). Neural crest cells were also found in the dorsolateral pathway, whereas in the controls, cells were only migrating ventromedially (Fig. 5A, B; Table 1). These results suggest that the early migratory neural crest cells are prevented from taking the dorsolateral pathway by the inhibitory action of the transmembrane ephrins present in this region. Conversely, when soluble ephrins are added at the onset of melanoblast migration in stage-18 explants, the migration of the cells into the dorsolateral path is inhibited (Fig. 5C; Table 2). In control explants, neural crest cells are observed in the dorsolateral pathway (Fig. 5D; Table

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**Table 1. Migration of early migratory neural crest cells in trunk explants treated with Ephrin-B1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of explants</th>
<th>Migration into the dorsolateral path*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc (control)</td>
<td>7</td>
<td>1.14</td>
</tr>
<tr>
<td>Ephrin-B1-Fc</td>
<td>9</td>
<td>24.6</td>
</tr>
</tbody>
</table>

*Average numbers of cells in the dorsolateral pathway in whole explants treated with ephrin-B1-Fc or Fc alone. Cells were counted from transverse sections of whole trunk explants stained with HNK-1 antibody.

**Table 2. Migration of melanoblasts in trunk explants treated with Ephrin-B1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of explants</th>
<th>Migration into the dorsolateral path*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc (control)</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>Ephrin-B1-Fc</td>
<td>6</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*Average numbers of cells in the dorsolateral pathway in whole explants treated with ephrin-B1-Fc or Fc alone. Cells were counted from transverse sections of whole trunk explants stained with HNK-1 antibody.
Ephrin ligands and neural crest cell migration

2) in a pattern identical to what would be observed in an intact embryo. These latter results suggest that transmembrane ephrins promote the migration of melanoblasts into the dorsolateral pathway.

Transmembrane ephrins promote melanoblast migration in vitro

The possible role of transmembrane ephrins as migration-promoting factors for melanoblasts was examined further using a transfilter chemotaxis assay. In this assay, preclustered ephrin-B1-Fc was added to the bottom well of a chemotaxis chamber and neural crest cells were added to the top well. In some control experiments preclustered ephrin-B1-Fc was also added to the upper well. Cells that migrated to the bottom side of the porous filter were stained, solubilized and quantified by OD measurements. Ephrin-B1-Fc induces a significant decrease of early migratory neural crest cell migration (white bars). In contrast, clustered ephrin-B1-Fc causes a significant increase in the transfilter migration of melanoblast cells (grey bars). This is a representative experiment and each data point is the mean ± s.e.m. of triplicate wells (P<0.05).

Surface-displayed ephrin-B1-Fc promotes melanoblast attachment to fibronectin

The above experiments showed that an interaction between the EphB receptors expressed by melanoblasts and the ephrin-B ligands does not result in a repulsive response. Instead, soluble ephrin-B ligands are chemoattractants for EphB-expressing melanoblast cells in vitro. Another possibility is that the Eph-ephrin interaction may promote melanoblast migration by increasing cell adhesion. To test this hypothesis we investigated whether the engagement of transmembrane ephrins by Eph-B receptors increases adhesion of melanoblasts to the substratum. Cells were subjected to adhesion assays on plates to which ephrin-B1-Fc or a mixture of fibronectin and ephrin-B-Fc had been preadsorbed. Ephrin-B1-Fc promoted melanoblast attachment to fibronectin (Fig. 7A,C). In contrast, early migratory neural crest cells increased their adhesion to fibronectin in the presence of ephrin-B1-Fc (Fig. 7A,B). These data are consistent with the results of the transwell chemotaxis assay, and suggest that transmembrane ephrins present in the dorsolateral pathway act as positive cues guiding the migration of melanoblasts.

Ephrin-B ligand induces changes in neural crest cell morphology

Ephrins have been shown to induce rearrangements of the actin cytoskeleton, which results in growth cone collapse.

Fig. 6. Effects of soluble ephrin-B1-Fc on neural crest cell migration. Pre-clustered Fc or ephrin-B1-Fc (10 μg/ml) was added to the bottom well of a chemotaxis chamber and neural crest cells were added to the top well. In some control experiments preclustered ephrin-B1-Fc was also added to the upper well. Cells that migrated to the bottom side of the porous filter were stained, solubilized and quantified by OD measurements. Ephrin-B1-Fc induces a significant decrease of early migratory neural crest cell migration (white bars). In contrast, clustered ephrin-B1-Fc causes a significant increase in the transfilter migration of melanoblast cells (grey bars). This is a representative experiment and each data point is the mean ± s.e.m. of triplicate wells (P<0.05).

Fig. 7. Effects of ephrin-B1-Fc on neural crest cell adhesion to fibronectin. (A) Cells were plated on surfaces coated with fibronectin plus Fc (10 μg/ml) or fibronectin plus ephrin-B1-Fc (10 μg/ml), as described in Materials and Methods. Ephrin-B1-Fc significantly increases adhesion of melanoblasts to fibronectin (A,C), but decreases the attachment of early outgrowths to fibronectin (A,B). Asterisks represent no significant difference between values (P<0.05). Early outgrowths (B). Melanoblasts (C).
Ephrin-B proteins also cause the collapse of early migratory neural crest filopodia (Krull et al., 1997). To examine the consequences of EphB-induced signalling on neural crest cell morphology, cells were plated on substrata consisting of fibronectin or ephrin-B1, and then stained with phalloidin to visualize the actin cytoskeleton. To determine the presence of focal adhesions, cells were stained with an anti-vinculin antibody. Early migrating neural crest cells plated on fibronectin had a flattened morphology, exhibited well-developed actin stress fibers, and formed abundant focal contacts (Fig. 8G-L). Early migratory neural crest cells plated on ephrin-B1 developed a rounded morphology accompanied by disruption of the actin cytoskeleton. In addition, no defined localisation of vinculin was apparent in these cells, suggesting a loss of focal adhesions (Fig. 8D-F). In contrast, melanoblasts plated on ephrin-B1 were more irregular in shape and were characterized by the formation of numerous microspikes (Fig. 8A-C). These cells also formed fewer focal contacts, as evidenced by a diffuse staining of vinculin, although in some cells vinculin localized to the tips of actin microspikes (Fig. 8B,C). The change in morphology was quantified by determining the percentage of total melanoblast cells with more than three branched processes and the presence of microspikes. Of the cells plated on fibronectin, 4% bore microspikes, whereas plating onto Ephrin-B-Fc resulted in 49% of the cells with microspikes.

**DISCUSSION**

Previous studies revealed that early migrating neural crest cells, which will differentiate into neurons and glial cells, are inhibited from migrating into the dorsolateral path. Conversely, later migrating neural crest cells are already specified as melanoblasts, and these are the only neural crest cells capable of invading the dorsolateral path. In this study, we show that ephrin-B ligands produced by the dermomyotome prevent early migrating neural crest cells from taking this path, whereas later in development ephrin-B ligands promote the migration of melanoblasts into the dorsolateral path. Ephrin-B proteins enhance melanoblast adhesion to fibronectin and stimulate the production of actin-filled microspikes, suggesting that ephrins promote melanoblast migration by an adhesive mechanism.

**Ephrin-B ligands prevent early migratory neural crest cells from invading the dorsolateral pathway**

The ephrin-B family of transmembrane ligands has been implicated in the segmental guidance of chick trunk neural
crest cells through the somites. These ligands are expressed in regions that early migratory neural crest cells avoid, such as the posterior half of the sclerotome (Krull et al., 1997; Wang and Anderson, 1997). We show that ephrin-B ligands are also present in the dorsolateral pathway when neural crest cells are migrating ventrally, and both in vitro and in vivo assays suggest that they prevent early migratory neural crest cells from invading the dorsolateral path. First, transfilter assays confirm that transmembrane ephrins can act as cell repellants for early migratory neural crest cells, and reduce adhesion to the substrate in an adhesion assay. Conversely, addition of soluble ephrin-B to chick trunk explants results in the inappropriate migration of early neural crest cells into the dorsolateral pathway. Together these results show that ephrin-B produced by the dermomyotome acts as a repulsive cue for early migratory crest, and blocks their migration into the dorsolateral pathway.

Ephrins are unlikely to act by themselves as other molecules that have been demonstrated to inhibit neural crest cell migration in vitro are distributed in the dorsolateral pathway. Specifically, PNA-binding proteins and chondroitin sulphate proteoglycan expression patterns in the chick trunk suggest a barrier function (Oakley et al., 1994; Pettway et al., 1996). F-spondin is an extracellular matrix molecule that is also produced by barrier tissues. Consistent with an inhibitory role for this protein, blocking F-spondin activity using function-blocking antibodies also results in the inappropriate migration of early neural crest cells into the dorsolateral pathway (Debby-Brafman et al., 1999). Semaphorins are molecules whose inhibitory activity in vitro has also been suggested to restrict migration of neural crest cells to the anterior somite (Eickholt et al., 1999). They are promising candidates for blocking the dorsolateral pathway as well, since they are expressed along this path during the early stages of neural crest cell migration (our unpublished results). It is likely that ephrin-B ligands operate in concert with these molecules to prevent early migratory cells from invading the dorsolateral pathway.

**Ephrin-B ligands promote the migration of melanoblasts**

In this report we show that ephrin-B expression is not downregulated when neural crest cells advance through the dorsolateral path (see also Koblar et al., 2000). Furthermore, previous studies show that when clusters of melanoblasts are placed into a young chick embryo at the time when neural crest cells are only migrating ventrally, the grafted cells invade the dorsolateral pathway precociously (Erickson and Goins, 1995), which we know is filled with ephrins. In the chick, ephrin-B1 remains expressed in the posterior half of the somite and along the dermomyotome at late stages of neural crest cell migration (Koblar et al., 2000). In addition, melanoblasts express several EphB receptors, including EphB3, which are also expressed by early migratory cells and are known to mediate a repulsive response that restricts early migratory neural crest cells to the anterior somite. These observations are not consistent with melanoblast migration being inhibited by ephrins, and instead suggest the ephrin-B ligands expressed in the dorsolateral path positively regulate melanoblast migration. Three independent assays were used to investigate this possibility.

Embryonic explants have been very useful in studies of cell migration and axon guidance (Krull and Kulesa, 1998). We disrupted the Eph/ephrin signalling by adding soluble ligand to trunk explants. Blocking receptor activation has a significant effect on the migration of melanoblast cells, as these cells are no longer found migrating on the dorsolateral pathway. This is direct evidence that ephrin-B promotes neural crest cell migration into the dorsolateral pathway.

To further substantiate this conclusion, we used a chemotaxis assay in which addition of soluble ephrin-B ligand inhibits the migration of early migratory cells as expected, but promotes the transfilter migration of melanoblasts. Ephrin-A1 has been shown to be a chemoattractant for endothelial cells (Pandey et al., 1995). Given that ephrins are cell-surface-associated molecules, it is not certain that there is a soluble form of ephrin in the embryo to generate a chemotactic gradient. However, a recent study by Hattori and colleagues (Hattori et al., 2000) demonstrated that ADAM-10 metalloprotease cleaves ephrin-A2 to generate a soluble form of this ligand. The in vivo proteolytic processing of these ephrins by ADAMs would result in the release of ligand fragments that may form a chemotactic gradient. Consistent with this, data from our lab shows that ADAM10 is highly expressed along the dorsolateral pathway during melanoblast migration (R. J. Hall and C. A. Erikson, personal communication). Studies are underway to determine the role of this metalloprotease in the regulation of neural crest cell migration.

Our data demonstrate that ephrin-B, as a soluble protein, is a chemotactic signal for melanoblasts, but does not rule out the possibility that neural crest cells migrating along the dorsolateral pathway are responding to an adhesive substrate of membrane-bound ephrin-B. Consistent with this, we show that engagement of EphB receptors by substratum-adsorbed ephrin-B ligands promotes melanoblast attachment to fibronectin. Signal transduction pathways downstream of the EphB receptors may lead to an increase in cell adhesion by modulating integrin affinity and/or interactions between integrins and intracellular proteins that are required for adhesion. Previous studies show that ephrin-A ligands can modulate integrin function leading to an increase adhesion of fibroblasts to fibronectin (Davy and Robbins, 2000). Also, ephrin-B ligands have been reported to promote endothelial cell attachment to extracellular matrix components through $\alpha_5\beta_3$ and $\alpha_5\beta_1$ integrins (Huynh-Do et al., 1999). Neural crest cells express several fibronectin receptors, including $\alpha_5\beta_3$ and $\alpha_5\beta_1$ (Testaz et al., 1999). In our study, these integrins could be involved in the increased adhesion via EphB engagement. Other molecules, such as F-spondin, PNA-binding glycoproteins and chondroitin sulphate glycoconjugates, are also expressed in the dermomyotome and display inhibitory effects in vitro (Davies et al., 1990; Debby-Brafman et al., 1991; Oakley et al., 1994). Guidance of melanoblasts along the dorsolateral pathway might therefore result from a composite of the effects of repellent and attractant signals. Subtle differences in the concentration of these molecules would be instrumental in creating specific substratum conditions favouring the migration of melanoblasts into the dorsolateral pathway.

There are several examples in other systems where ephrins have been implicated in cell adhesion events. In the retinotectal system, EphB2 and ephrin-B1 are expressed in dorsoventral gradients in a manner inconsistent with a role in repulsion; axons from the ventral retina express EphB2 and project to the...
dorsal tectum, which expresses high levels of ephrin-B1 protein (Holash and Pasquale, 1995; Braisted et al., 1997). Furthermore, EphB2/EphB3 double-mutant mice display cleft palate (Orioli et al., 1996) and neural folds fail to adhere in the dorsal midline in ephrin-A5 null mice (Holmberg et al., 2000), suggesting involvement of these molecules in cell-cell adhesion. Finally, activation of certain Eph receptors can promote cell-cell adhesion, and adhesion to a substrate in myeloid cells (Bohme et al., 1996) and retinal cells (Holash et al., 1997), respectively. Similarly, activation of EphB receptors in endothelial cells with clustered soluble ephrin-B upregulates cell adhesion, which triggers assembly of endothelial cells in culture to form a capillary network (Stein et al., 1998; Adams et al., 1999).

The binding of early migratory cells to ephrin-B results in cell rounding, substantial reduction of stress fibers and a nearly complete loss of vinculin localisation, suggesting a loss of focal adhesions. In contrast, binding of melanoblasts to ephrin-B results in the reorganization of the actin cytoskeleton, notably the generation of numerous F-actin-containing microspikes, which terminate in small focal contacts. Microspikes are structures usually found at the leading edge of migratory fibroblasts, epithelial cells and neuronal growth cones, where they are actively involved in cell migration (Adams, 1997; Fischer et al., 1997; Yamashiro et al., 1998). The small focal adhesions present in microspikes formed in melanoblasts upon contact with ephrin-B could be important in promoting adhesion to extracellular matrix proteins such as fibronectin and providing the traction force necessary to direct cells along the dorsolateral pathway. This is in agreement with the observation that nascent (small) focal contacts in extending lamellipodia generate strong forces responsible for driving cell migration (Benigno et al., 2001).

Ephrins in neural crest cell guidance

To our knowledge, this is the first report of transmembrane ephrins promoting neural crest cell migration. Furthermore, the results presented here show that ephrin-B ligands act as bifunctional guidance cues. They first prevent early migratory neural cells from invading the dorsolateral path, and then later stimulate the migration of melanoblasts into this pathway. A related situation occurs in cortical neurons that project through different layers of the cerebral cortex. Here, ephrin-A5 repels neurons from layers 2/3 and 5 of the cortex, but induces sprouting of axons from deeper layers (Castellani et al., 1998).

The mechanism by which Eph receptors and ephrins trigger different cellular responses in the neural crest cells is not known. Presumably one important downstream signalling target is the cytoskeleton, since the ephrin-B ligand induces the formation of actin microspikes in melanoblasts and not in the early migratory crest. The formation of microspikes is regulated by the activation of Rac/Rho/Cdc42 family of small GTPases (Hall, 1998; Nobes and Hall, 1998), and therefore it seems likely that the activation of the Eph receptor acts downstream on these small GTPases in melanoblasts. Indeed, Ephexin, a guanine nucleotide exchange factor (GEF), and several SH2-domain-containing proteins such as Src, Nck, and RasGAP, which bind activated Eph receptors, are components of signal transduction pathways that control the polymerization of the actin cytoskeleton via the Rac/Rho/Cdc42 family of GTPases (Bruckner and Klein, 1998; Holland et al., 1998; Shamah et al., 2001). Why the two signalling pathways diverge functionality in melanoblasts and the early migratory neural crest is unknown. One possibility is that they may be regulated by interactions with different Eph receptors and or/coreceptors, resulting in the assembly of receptor complexes with different signalling properties. Consistent with this, the EphB3/ephrin-B1 receptor-ligand complex appears to be responsible for the repulsive response that restricts neural crest cell entry into the posterior half-sclerotome. At later stages of development, ephrin-B1 produced by the dermomyotome (Koblar et al., 2000), may form a complex with EphB3 and/or other EphB receptors. Even though by in situ hybridisation Eph receptor expression is seen in the dorsal neural tube and in regions corresponding to the pathway taken by melanoblasts, we could not confirm that HNK-1-positive cells express multiple Eph receptors in situ. Interestingly, the formation of hetero-receptor complexes between members of the EphB family upon ephrin-B1 stimulation has recently been described (Freywald et al., 2002).

Variation in receptor structure may also underlie differences in function. In favour of this, a truncated isoform of EphA7 can block the repulsive interaction mediated by full-length EphA7, shifting the cellular response from repulsion to adhesion (Holmberg et al., 2000). Different isoforms encoded by alternatively spliced mRNAs have also been identified for several EphB receptors. For example, a variant form of EphB3 with a juxtamembrane insertion of 15 amino acids has been isolated (Sajjadi and Pasquale, 1993). Different isoforms of EphB2 are transcribed in the chicken: a truncated form lacking the kinase domain and a form that has an insertion of 16 amino acids in the juxtamembrane region (Sajjadi and Pasquale, 1993; Connor and Pasquale, 1995). It is not known whether neural crest cells express any of these variants. We are presently investigating what modulates the nature of the distinct cellular responses observed between these subpopulations of neural crest cells.

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