

# A role for *rhoB* in the delamination of neural crest cells from the dorsal neural tube

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## SUMMARY

The differentiation of neural crest cells from progenitors located in the dorsal neural tube appears to involve three sequential steps: the specification of premigratory neural crest cell fate, the delamination of these cells from the neural epithelium and the migration of neural crest cells in the periphery. BMP signaling has been implicated in the specification of neural crest cell fate but the mechanisms that control the emergence of neural crest cells from the neural tube remain poorly understood. To identify molecules that might function at early steps of neural crest differentiation, we performed a PCR-based screen for genes induced by BMPs in chick neural plate cells. We describe the cloning and characterization of one gene obtained from this screen, *rhoB*, a member of the rho

family GTP-binding proteins. *rhoB* is expressed in the dorsal neural tube and its expression persists transiently in migrating neural crest cells. BMPs induce the neural expression of *rhoB* but not the more widely expressed rho family member, *rhoA*. Inhibition of rho activity by C3 exotoxin prevents the delamination of neural crest cells from neural tube explants but has little effect on the initial specification of premigratory neural crest cell fate or on the later migration of neural crest cells. These results suggest that *rhoB* has a role in the delamination of neural crest cells from the dorsal neural tube.

Key words: rho, Neural crest, BMP, Chick

## INTRODUCTION

The neural crest constitutes a transient population of migratory progenitor cells that differentiate into a wide variety of peripheral neurons and non-neuronal cells (Le Douarin, 1982). Many of the cellular and molecular mechanisms that control the diversification and differentiation of neural crest cells during their peripheral migration have been defined (Anderson, 1997). However, the earlier steps that contribute to the specification of premigratory neural crest cells within the neural tube and to the delamination of these cells from the dorsal neural epithelium remain less well understood.

Fate mapping studies have shown that neural crest cells originate from the border of the neural plate and epidermal ectoderm (Rosenquist, 1981). This observation raised the possibility that signals derived from the epidermal ectoderm might be involved in the specification of neural crest cell fate. Support for this idea came from transplantation experiments in which the apposition of epidermal ectoderm and neural plate tissue induced the generation of neural crest cells in amphibian and avian embryos (Moury and Jacobson, 1989; Selleck and Bronner-Fraser, 1995). In vitro studies have indicated further that signals from the epidermal ectoderm are sufficient to induce neural crest differentiation in chick neural plate tissue (Dickinson et al., 1995; Liem et al., 1995). Members of the bone morphogenetic protein (BMP) family are strong

candidates as mediators of this epidermal signal. In chick embryos, *BMP4* and *BMP7* are expressed in the epidermal ectoderm at the time of neural crest induction and both BMP proteins can mimic the neural crest inducing activity of the epidermal ectoderm in vitro (Liem et al., 1995). Furthermore, proteins that inhibit *BMP4* and *BMP7* activity block the ability of the epidermal ectoderm to induce neural crest cells (Liem et al., 1997), providing evidence for the requirement of BMP signaling in the initiation of neural crest cell differentiation.

The pathway by which cells in the neural plate respond to BMP signaling with the acquisition of a premigratory neural crest cell fate, however, is not well understood. Genes encoding several transcription factors, notably *Msx* and *Zic* genes, are expressed in the dorsal neural tube and some of these have been implicated in the specification of neural crest cell fate (Brewster et al., 1998; Foerst-Potts and Sadler, 1997; Nagai et al., 1997; Nakata et al., 1997; Shimeld et al., 1996; Wang et al., 1996). Following their specification, neural crest cells delaminate from the dorsal region of the neural epithelium and acquire mesenchymal properties as they begin their migration into the periphery (Duband et al., 1995). In chick embryos, the zinc finger transcription factor *slug* is expressed in the dorsal folds of the neural plate, in the dorsal neural tube and later in migrating neural crest cells (Liem et al., 1995; Nieto et al., 1994). Perturbation of *slug* function in vivo by antisense oligonucleotides has been reported to inhibit the emergence of

neural crest cells from the dorsal neural tube of chick embryos (Nieto et al., 1994). The delamination of neural crest cells also appears to involve a reorganization of the cytoskeleton and changes in the expression of cell surface adhesion molecules and receptors (Duband et al., 1995). In particular, the expression of two members of the cadherin family, *N-cadherin* and *cadherin6B*, is detected at sites of premigratory neural crest cell generation and these genes are downregulated at the onset of neural crest cell migration (Nakagawa and Takeichi, 1995; Revel and Brown, 1976). The loss of these two *cadherins* is accompanied by the onset of high level expression of *cadherin7* (Nakagawa and Takeichi, 1995). Although there is evidence for the involvement of these transcription factors and cell surface proteins in the differentiation of neural crest cells, the extent to which their pattern of expression is regulated by BMP signaling remains unclear.

In this study, we have attempted to identify additional molecules that might participate in the early steps of neural crest differentiation using a PCR-based screen for genes induced by BMPs in neural plate cells. We describe here the cloning and functional characterization of one gene obtained from this screen, *rhoB*, a member of the *rho* gene family. *rho* proteins are small GTP-binding proteins of the ras superfamily and the three major members of this subfamily, *rho*, *rac* and *cdc42*, appear to control many aspects of cellular functions including adhesion, morphology, motility and cell-cycle progression (Hall, 1998; Van Aelst and D'Souza-Schorey, 1997). The *rho* proteins have also been implicated in the transduction of extracellular signals that control gene transcription (Hill et al., 1995; Perona et al., 1997). Our results show that *rhoB* is expressed selectively by cells in the dorsal neural tube, that its expression persists transiently in migrating neural crest cells and that the expression of *rhoB* in neural tissue is induced by BMPs. Blockade of *rho* activity prevents the delamination of neural crest cells from the dorsal neural epithelium in vitro. These results suggest that *rhoB* has a role in the delamination of neural crest cells from the dorsal neural tube.

## MATERIALS AND METHODS

### Explant cultures

Neural plate explants from Hamburger-Hamilton (HH) stage 10 chick embryos (Hamburger and Hamilton, 1951) were isolated essentially as described (Yamada et al., 1993). After dissection, explants were cultured in collagen matrix (Vitrogen) or fibronectin (Boehringer Mannheim)-treated 10 mm tissue culture wells, with F12 medium containing penicillin/streptomycin, glutamine (Speciality Media), 2 mM glucose and Mito<sup>+</sup> Serum Extender (Collaborative Biomedical Products). Neural tube tissue from stage 10 chick embryos was dissected, treated with dispase and then cultured on fibronectin (Boehringer Mannheim)-treated chamber slides or 10 mm tissue culture wells.

To quantify the emigration of neural crest cells from explants cultured in collagen gels, 16 pairs of explants (with and without C3) were analyzed under a dissecting microscope. To determine the number of slug<sup>+</sup> and DAPI<sup>+</sup> cells, each explant was sectioned serially at 10  $\mu$ m, stained and the number of cells in each section was then counted. A total of 3 pairs of explants (with and without C3) were analyzed. The concentration of C3 used in these neural plate explant assays was ~50  $\mu$ g/ml. For the analysis of neural crest delamination, C3 (150–200  $\mu$ g/ml) was added to the medium at the beginning of the

culture period. For the analysis of neural crest migration, C3 (~100  $\mu$ g/ml) was added to the culture media 6 hours after plating. To measure the rate of cell migration, the distances between the center of each explant and cells that had migrated farthest away from the explant were recorded at each time point (~40 cells/explant). A total of 3 pairs of explants (with and without C3) were measured.

### Differential display screen

A differential display screen was performed essentially as described (Liang et al., 1993; Liang and Pardee, 1992) using  $\alpha$ -[<sup>32</sup>P]dATP (Amersham) and a combination of nineteen 5' primers and three 3' primers. The sequences of 5' primers used are:

- |                       |                       |
|-----------------------|-----------------------|
| 1. 5'-CTGATCCATG-3',  | 11. 5'-TACAACGAGG-3', |
| 2. 5'-CTTGATTGCC-3',  | 12. 5'-GACCGCTTGT-3', |
| 3. 5'-CTGCTCTCAA-3',  | 13. 5'-GGAGGGTGTT-3', |
| 4. 5'-CTAGACTAGC-3',  | 14. 5'-GAACGGACTC-3', |
| 5. 5'-GATCGATGGA-3',  | 15. 5'-AGCGCCATTG-3', |
| 6. 5'-GTCATGCACA-3',  | 16. 5'-CTTCACCCGA-3', |
| 7. 5' AGCTTCGATC-3',  | 17. 5'-GGGATATCGG-3', |
| 8. 5' ACTGGATTCG-3',  | 18. 5'-TTCCCGGGTT-3', |
| 9. 5'-TGGATCCAAG-3',  | 19. 5'-AATGGCGCAG-3', |
| 10. 5'-TCACGTAAGC-3', |                       |

The sequence of 3' primers used are: T<sub>12</sub>MA, T<sub>12</sub>MG, T<sub>12</sub>MC, (M=A or G or C).

This screen generated 121 PCR fragments of 200–700 bp in length that were detected at higher levels in cultured [d] or [i]+ Ds11 explants when compared to [i] explants cultured alone. These 121 fragments were used as probes to screen Southern blots of PCR-amplified [i], [d] and [i]+ Ds11 cDNAs using the original primer combinations by which these fragments were derived. After this second round of screening, the number of positive bands was reduced to 30. These DNA fragments were then used as probes to isolate cDNA clones from a stage 17 chick neural tube cDNA library (Basler et al., 1993). cDNAs corresponding to 25 of the original 30 PCR-derived fragments were isolated and used to generate probes for determining the expression patterns of the corresponding genes by in situ hybridization.

### In situ hybridization

HH stage 10 and 20 chick embryos were processed for whole-mount in situ hybridization performed as described (They et al., 1995). HH stage 24 chick embryos were fixed, embedded and sectioned at 10  $\mu$ m first before processing for in situ hybridization.

### Generation of anti-rhoB antibody

To generate an antibody against chick rhoB, the 3' coding region of *rhoB* cDNA (amino acids 114–196) was subcloned into pQE42 expression vector (Qiagen). The resulting fusion protein consists of dehydrofolate reductase (DHFR) and the C-terminal of rhoB. Purified protein was injected (100  $\mu$ g/mouse) every 3 weeks into Balb/C mice. After six injections, spleen cells were fused with NS1 myeloma cells for generation of monoclonal antibodies. One clone directed against rhoB, mAb 4H7, was obtained.

### Immunohistochemistry

Chick embryos were dissected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 2 hours, washed with PBS and equilibrated with 30% sucrose in 0.1 M PB, then mounted in OCT embedding medium. Neural plate explants in collagen gels were fixed for 20 minutes at 4°C, washed and embedded as described above. All tissues were sectioned on a cryostat at 10  $\mu$ m. Neural tube explants grown on chamber slides were fixed for 20 minutes and washed with PBS. For staining with mAb 4H7 (anti-rhoB), sections were treated with 6 M guanidine HCl in 0.1 M PB for 10 minutes at 20°C, washed with PBS, blocked with 10% goat serum and 0.1% Triton X-100 in PBS.

mAb 4H7 was used at 1:40 dilution, mAb HNK-1 was used at 1:10 dilution, mAb 1E6 (anti-slug) was used at 1:100 dilution in 1% heat-inactivated goat serum and 0.1% Triton X-100 in PBS. Rhodamine-conjugated phalloidin (Molecular Probes) was used at 1:100 dilution. FITC- and Cy3-conjugated goat anti-mouse secondary antibodies were used as directed (Jackson Immuno Research Laboratory).

### Western blot analysis

Plasmids containing human *rhoA* (L63), *rac1* (V12) and *cdc42* cDNAs in pGEX-2T vectors were provided by Dr Alan Hall. These pGEX fusion plasmids were transformed into BL21 bacterial cells while the pQE fusion plasmid (*rhoB*) was transformed into M15 bacterial cells. Bacterial lysate containing the chick *rhoB* (L114-L196)/DHFR, human *rhoA*/GST, *rac1*/GST and *cdc42*/GST fusion proteins were separated on 12% SDS-PAGE gels. Proteins were transferred to nitrocellulose filters using Tris/glycine/SDS transfer buffer. After blocking, mAb 4H7 was used at 1:6000 dilution and incubated with membranes at 4°C overnight. Alkaline phosphatase-conjugated anti-mouse IgG (Boehringer Mannheim) was used at a 1:400 dilution. The chromogenic reaction was performed with x-phosphate/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) (Boehringer Mannheim).

### RT-PCR assay

Four explants per collagen gel were collected in 1 ml Trizol reagent (Gibco BRL) and RNA was purified according to the manufacturer's protocol. RNA from each sample was divided equally for RT reactions and for controls lacking RT. 1-4 µl of the 30 µl total volume of the RT reaction was used for each PCR reaction. Amplification was performed at 17-18 cycles. PCR products were transferred to Hybond-N<sup>+</sup> membranes (Amersham) and hybridized with corresponding probes. Quantitation of each product was performed with a phosphorimager 'Storm 860' (Molecular Dynamics). The sequence for each primer pair is:

*rhoB* 5': 5'-ATGGCCGCCATCCGCAAGAAGCTG-3'.  
*rhoB* 3': 5'-CTATAGGACCTGACGCAATTGATGC-3'.  
*rhoA* 5': 5'-GCAGCTATGGCAGCCATTTCG-3'.  
*rhoA* 3': 5'-GACTTTTTCTTGCCACGCCG-3'.  
*cadherin6B* 5': 5'-GGCTCTTATTGCCATTCTTCTC-3'.  
*cadherin6B* 3': 5'-TTAAGAGTCTTTGTCACGTGCCAT-3'.  
*cadherin7* 5': 5'-GAGCCCTGATAGCAATATTGGCT-3'.  
*cadherin7* 3': 5'-CTATGAGTATAAGCAGTCTGGACC-3'.  
*slug* 5': 5'-ACCCATTACTGTGTGGACTACAA-3'.  
*slug* 3': 5'-TTGGATCTGTCTGCGAAAGCCCTG-3'.  
*s17* 5': 5'-AGAAGGCGGCGGGTGATCATCG-3'.  
*s17* 3': 5'-GTTTATTGTAAAAGCAACATAACG-3'.

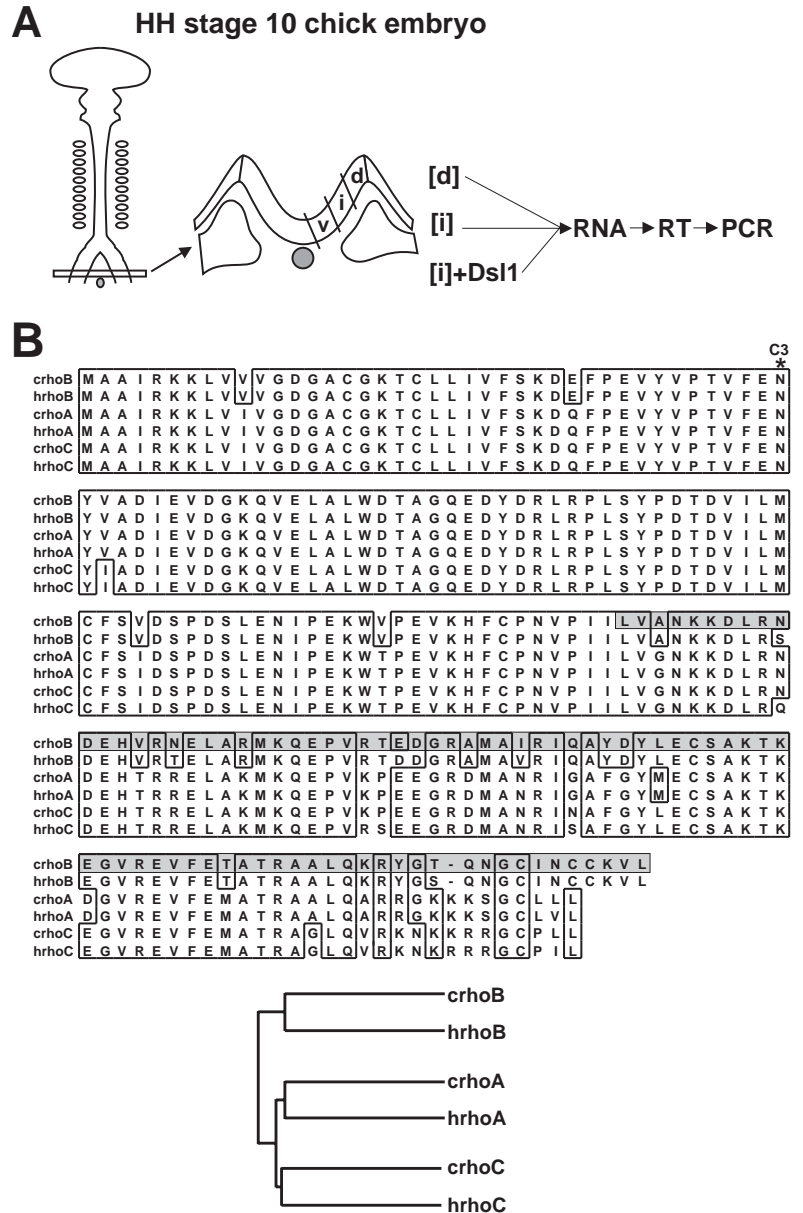
### Purification of C3 exotoxin

A plasmid encoding C3 toxin was provided by Dr Larry Feig. C3 protein was purified as described (Dillon and Feig, 1995). Centriprep 10 (Amicon) columns were used to change the buffer to F12 medium and to concentrate the protein. The amount of protein was quantified with a Bio-Rad protein assay using BSA as the standard.

### Assay of rho inhibition by C3-mediated ADP-ribosylation

To analyze the C3-mediated inhibition of rho activity [d] explants and [i] explants cultured with BMP (Dsl1) were treated with ~50 µg/ml C3. Samples were collected in 0.2%

Triton X-100, 50 mM Tris pH 7.4, 5 mM MgCl<sub>2</sub>, 50 mM NaCl, and 1 mM PMSF at 4.5 hours and 9 hours after plating. For pNT explants, C3 was added at 150-200 µg/ml, samples were collected at 3 hours and 6 hours after plating. For aNT explants, ~100 µg/ml C3 was added 6 hours after plating. Neural tube explants were removed with a tungsten needle at 3 hours and 6 hours after the addition of C3, and migrating neural crest cells were collected. ADP-ribosylation assays were performed as described (Ridley and Hall, 1992). Quantitation



**Fig. 1.** Isolation and sequences of chick *rho* genes. (A) Experimental design. Neural plate tissue at the caudal end of HH stage 10 chick embryo was dissected into [d] and [i] regions. RNA purified from [d] and [i] explants cultured alone and [i] explants cultured with Dsl1 was used as templates for reverse transcription and subsequent PCR reactions to identify genes expressed highly in [d] and [i]+Dsl1 explants. (B) Amino acid sequence alignment and PILEUP of chick and human rhoB, rhoA, and rhoC proteins. '\*' indicates the Asn-41 which is the site of ADP-ribosylation by C3. Shaded area indicates the C-terminal portion used to generate the anti-rhoB monoclonal antibody 4H7. GenBank accession numbers: rhoA(AF098513), rhoB(AF098515), rhoC(AF098514).

was performed with a phosphoimager 'Storm 860' (Molecular Dynamics).

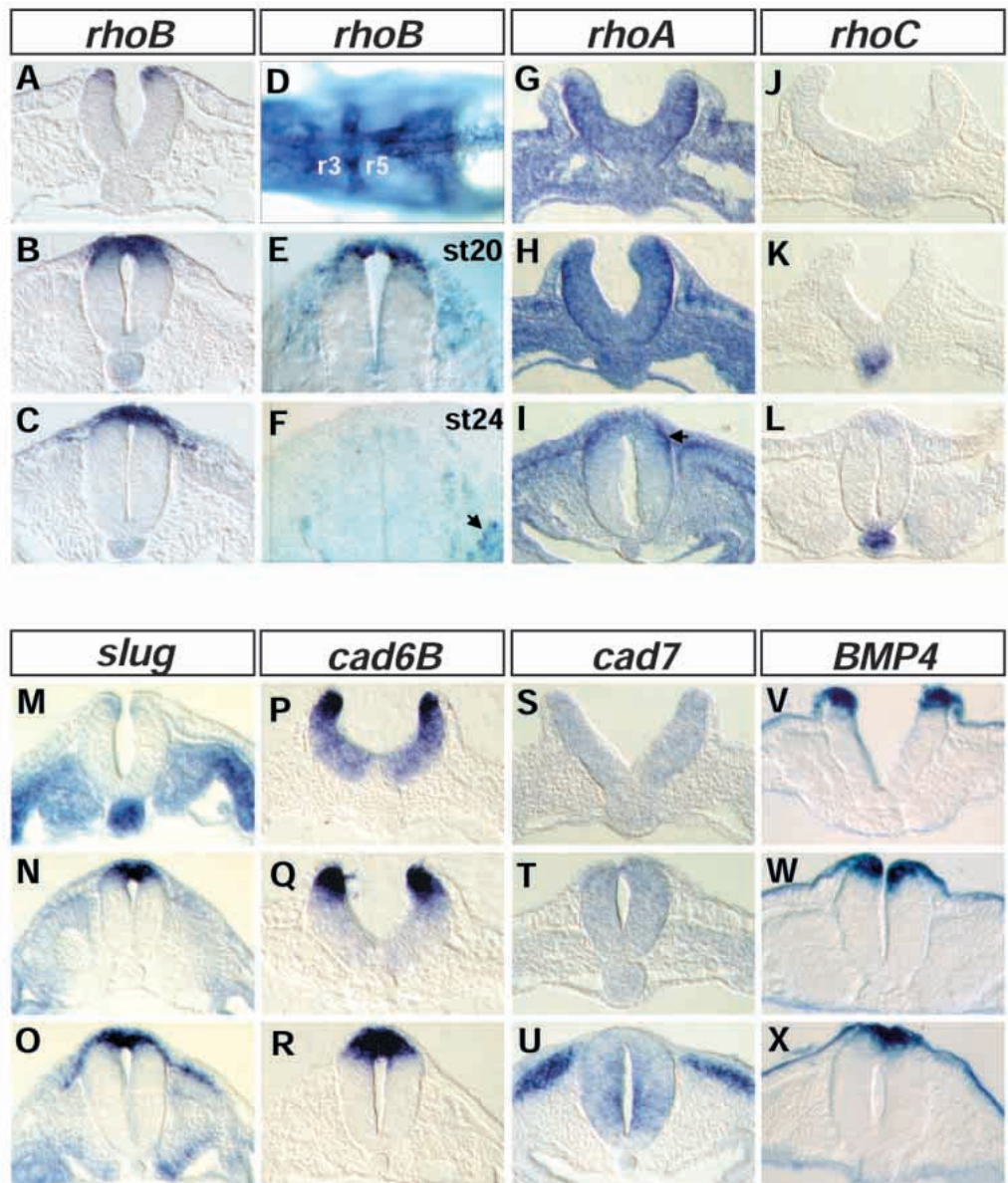
## RESULTS

### Isolation of BMP-induced neural genes

We used a PCR-based differential display assay (Liang et al., 1993; Liang and Pardee, 1992) to identify genes that define early stages of neural crest cell differentiation. This assay was designed on the basis of previous studies showing that cells in neural plate explants can be induced to differentiate into neural

crest cells upon exposure to BMPs (Basler et al., 1993; Liem et al., 1995). Caudal neural plate tissue from stage 10 chick embryos was dissected into dorsal [d] and intermediate [i] regions (Liem et al., 1995; Yamada et al., 1993) and cultured in serum-free medium on a fibronectin substratum (Fig. 1A). Under control conditions, [i] explants did not give rise to migratory neural crest cells but when cultured in the presence of Dsl1 (a member of the BMP family) or other BMPs, many migratory neural crest cells were generated (Basler et al., 1993). In contrast, [d] explants grown alone express *BMP* genes (Liem et al., 1995) and thus generate migratory neural crest cells in the absence of exogenous BMP protein (Basler et al., 1993). RNA was isolated

**Fig. 2.** Patterns of expression of *rhoB*, *rhoA*, *rhoC*, *slug*, *cadherin6B*, *cadherin7* and *BMP4* during neural tube development. All panels, except D-F, show transverse sections of the neural plate and neural tube of HH stage 10 chick embryos. (A-C) *rhoB* expression is restricted caudally to the dorsal tips of the neural fold (A), and rostrally to the dorsal neural tube (B) and migrating neural crest cells (C). (D) Whole-mount in situ hybridization analysis of *rhoB* in the hindbrain of HH stage 10 chick embryos. *rhoB* is expressed in migrating neural crest cells at rhombomeres 2, 4 and 6 levels. (E) Transverse section of a HH stage 20 chick embryo spinal cord showing *rhoB* expression in the dorsal region and in migrating neural crest cells. (F) Transverse section of a HH stage 24 chick embryo spinal cord showing that *rhoB* is no longer expressed dorsally. Arrow indicates the expression of *rhoB* in a subset of cells in the DRG. (G-I) *rhoA* is ubiquitously expressed in the neural plate (G) and at neural fold (H) stages, however the level of expression decreases in ventral neural tube after neural tube closure (I). Arrow in I indicates the higher level expression of *rhoA* by cells in the lateral part of dorsal neural tube than by cells at the dorsal midline. (J-L) *rhoC* is expressed in notochord (K,L), but only at a very low level in neural tissue (J-L). (M-O) Neural expression of *slug* was detected at the tips of the neural folds (M), the dorsal neural tube (N) and in migrating neural crest cells (O). (P-R) *Cadherin6B* is expressed throughout the neural plate except the floor plate region, albeit with a higher level of expression dorsally (P). The level of expression ventrally decreases at more rostral level of the embryo (Q). After neural tube closure, expression is restricted to the dorsal neural tube (R), but is absent from migrating neural crest cells. (S-U) *Cadherin7* is expressed at a very low level in the neural plate and neural tube (S, T), but at a high level in migrating neural crest cells (U). (V-X) *BMP4* is expressed at the dorsal tips of the neural folds (V,W), and dorsal neural tube (X), but not in migrating neural crest cells. The blue staining along the surface of ectoderm is an edge artifact.



separately from [i] explants cultured alone or with DsII and from [d] explants cultured alone (Fig. 1A). cDNAs derived from these RNA samples were then subjected to differential display PCR analysis (see Materials and Methods). We focus here on one clone obtained from this screen, *A19-1*, a gene that is expressed in a highly selective pattern by cells in the dorsal neural tube and by neural crest cells.

Sequence analysis revealed that clone *A19-1* encodes a member of the rho family of small GTP-binding proteins (Madaule and Axel, 1985) and is most closely related to mammalian *rhoB* (Fig. 1B). Vertebrates contain two other closely related *rho* genes, *rhoA* and *rhoC* (Chardin, 1988). To establish the identity of clone *A19-1*, we isolated two additional chick *rho* genes. Comparison of the sequence of these three chick genes indicated that clone *A19-1* encodes chick *rhoB* and that the two additional genes encode chick *rhoA* and *rhoC* (Fig. 1B). The sequences of these chick *rho* genes have recently been reported in an independent study (Malosio et al., 1997).

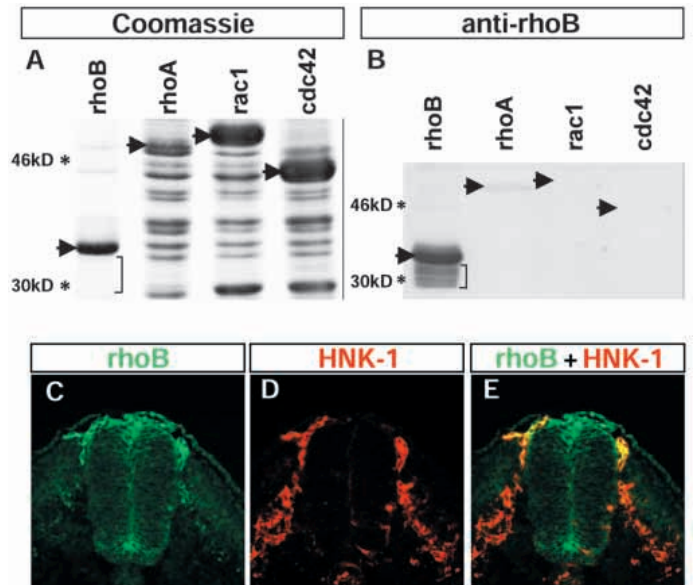
### ***rhoB* expression defines early stages of neural crest differentiation**

We first examined the pattern of *rhoB* expression during early neural development with reference to other markers of neural crest differentiation. In stage 10 chick embryos, the expression of *rhoB* was detected in cells at the dorsal tips of the neural folds (Fig. 2A). After neural tube closure, a high level of *rhoB* expression was detected in cells at the dorsal midline of the neural tube (Fig. 2B), in a domain that overlapped with that of the genes encoding the zinc finger transcription factor *slug* (Fig. 2M,N) and *BMP4* (Fig. 2V,W). The expression of *cadherin6B*, in contrast to that of *slug* and *rhoB*, was detected initially throughout the neural plate with the exception of the ventral midline and became restricted to the dorsal neural folds just before neural tube closure (Fig. 2P,Q). At a more rostral level of stage 10 embryos, where the emigration of neural crest cells had commenced, *rhoB* expression was detected in migratory neural crest cells located close to the neural tube (Fig. 2C,D). The expression of *rhoB* by cells at the dorsal midline of the spinal cord persisted at stage 20 (Fig. 2E) but was absent by stage 24 (Fig. 2F), a time at which the vast majority of neural crest cells have migrated from the neural tube. Thus, the expression of *rhoB* appears to define an early stage of neural crest cell differentiation.

In contrast to the restriction in *rhoB* expression to regions of neural crest cell generation, the expression of *rhoA* was detected throughout the neural plate and neural folds as well as in surrounding mesodermal tissues (Fig. 2G,H). After neural tube closure, the level of *rhoA* expression in the ventral neural tube was markedly decreased (Fig. 2I). The highest level of expression of *rhoA* dorsally was detected in lateral regions of the neural tube and not, as with *rhoB*, at the dorsal midline (Fig. 2I arrow; data not shown). *rhoC* was expressed at a high level in the notochord but only at very low levels in the neural tube (Fig. 2J-L). Thus, of the three *rho* genes examined, the expression pattern of *rhoB* is most closely associated with the position of neural crest differentiation.

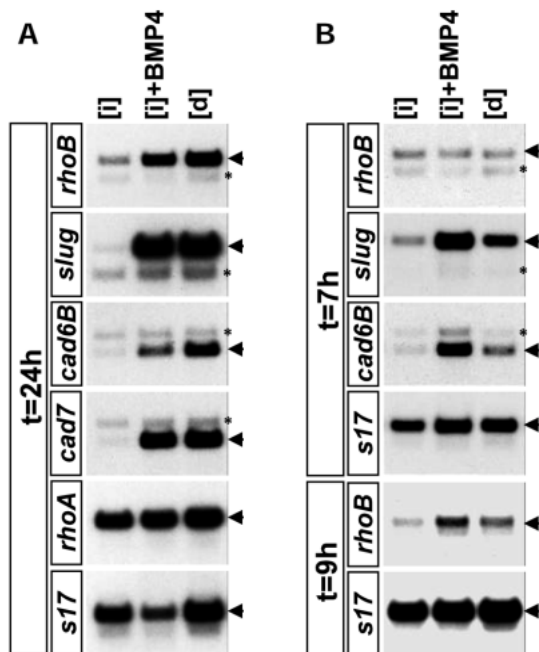
### ***rhoB* is expressed transiently in migrating neural crest cells**

To define the pattern of *rhoB* protein expression, we generated



**Fig. 3.** Immunocytochemical detection of *rhoB* expression. (A-B) Analysis of the specificity of anti-*rhoB* mAb 4H7. (A) Coomassie-stained bacterial lysate of *rhoB*, *rhoA*, *rac1* and *cdc42* fusion proteins resolved on a SDS-PAGE gel. \* marks the position of 46 kDa and 30 kDa molecular weight markers. Arrows indicate the position of each fusion protein. 'J' marks the position of *rhoB* degradation products. (B) Immunocytochemical detection of *rhoB*. Arrows indicate the position of each fusion protein. mAb 4H7 does not cross react with *rac1* or *cdc42*. Slight cross-reactivity is detected with *rhoA*. However, a comparison of the amount of *rhoA* and degraded *rhoB* protein present with the difference in their staining intensity indicates that the cross-reactivity of mAb 4H7 with *rhoA* is negligible. (C-E) Pattern of expression of *rhoB* in HH stage 14 chick embryos. (C) mAb 4H7 detects premigratory neural crest cells at the dorsal midline of the neural tube and migrating neural crest cells. A lower level of *rhoB* was also detected in the floor plate, consistent with the pattern of *rhoB* RNA expression detected by in situ hybridization on embryos at this stage (data not shown). (D) mAb HNK-1 detects migrating neural crest cells. (E) Combined confocal image of C and D, showing that early migrating neural crest cells co-express HNK-1 and *rhoB* and that the level of expression of *rhoB* diminishes as neural crest cells migrate away from the neural tube.

a monoclonal antibody (4H7) directed against the carboxy-terminus of chick *rhoB*. The selectivity of this reagent for detection of *rhoB* was established by western blot analysis (see Fig. 3A,B). The expression pattern of *rhoB* in the spinal cord of stage 14-17 chick embryos was compared with that of the neural crest marker HNK-1 (Tucker et al., 1984). *rhoB* was detected both in cells at the dorsal midline of the neural tube and in migrating neural crest cells (Fig. 3C). The expression of HNK-1 by neural crest cells, however, was evident only after their emigration from the dorsal neural tube (Fig. 3D). The co-expression of *rhoB* and HNK-1 was detected only in neural crest cells that had recently emigrated from the neural tube (Fig. 3E). The expression of *rhoB* was lost in neural crest cells that had migrated further from the neural tube, whereas the expression of HNK-1 persisted (Fig. 3E). Thus, *rhoB* expression defines the premigratory and early migratory phases of neural crest differentiation.



**Fig. 4.** RT-PCR analysis of BMP4-induced gene expression in neural plate explants. (A) Analysis of [d] and [i] explants cultured for 24 hours alone or with BMP4. BMP4 induces the expression of *rhoB*, *slug*, *cadherin6B*, and *cadherin7* expression in [i] explants. Expression of *rhoA* is not induced significantly by BMP4. The chick *s17* ribosomal protein gene is used as an internal control. Arrows indicated the position of each transcripts analyzed. '\*' indicates the position of control products from competitive templates. (B) Analysis of [d] and [i] explants cultured alone or with BMP4 for 7 hours or 9 hours. Induction of *slug* and *cadherin6B*, but not *rhoB* is apparent after 7 hours in culture. The induction of *rhoB* can be detected 9 hours after culturing with BMP4. Similar results were obtained in 3 separate experiments.

### *rhoB* expression is induced by BMPs

The differentiation of neural crest cells appears to be initiated by BMP-mediated signals from the epidermal ectoderm (Liem et al., 1995). We therefore examined the effect of BMPs on the expression of *rhoB* and other early markers of neural crest differentiation. The expression of *rhoB* was detected at low levels in [i] explants grown alone for 24 hours but the level of expression was increased markedly (~7 fold) in [i] explants exposed to BMP4 (Fig. 4A), as expected from the design of the original screen. In contrast, the level of expression of *rhoA* was not significantly elevated by BMP4 exposure (Fig. 4A). The selective induction of *rhoB* by BMPs is consistent with the preferential association of *rhoB* expression in vivo with sites of neural crest differentiation. A marked induction in the level of expression of *slug*, *cadherin6B* and *cadherin7* was also detected in [i] explants exposed to BMP4 (Fig. 4A). High levels of expression of *rhoB*, *slug*, *cadherin6B* and *cadherin7* were also detected in [d] explants grown in the absence of added BMPs (Fig. 4A).

We next examined the temporal sequence of gene expression during the initial specification of premigratory neural crest cells, focusing on *rhoB* and *slug*, the two most selective markers of this step of neural crest cell differentiation. Marker gene expression was analyzed 3 hours, 7 hours and 9 hours after

exposure of [i] explants to BMPs. After 3 hours, there was no induction of either *slug* or *rhoB* expression (data not shown). After 7 hours, induction of *slug* but not *rhoB* expression was evident (Fig. 4B). The induction of *rhoB* was detectable after 9 hours exposure to BMP4 (Fig. 4B). These results provide evidence that *slug* expression is induced prior to that of *rhoB* during the specification of premigratory neural crest cells.

### Inhibition of rho activity perturbs neural crest development

To examine the involvement of rhoB in the early differentiation of neural crest cells, we attempted to block rho activity through the use of the *Clostridium botulinum* exotoxin C3. The C3 protein blocks rho activity through ADP-ribosylation of the Asn-41 residue conserved in all rho proteins (Aktories and Hall, 1989; Sekine et al., 1989). Importantly, C3 inactivates rho proteins without inhibiting rac or cdc42 activity (Ridley and Hall, 1992). Moreover, the inhibition of rho activity in intact cells can be achieved in vitro by exposure of intact cells to medium containing C3 (Jalink et al., 1994; Yamamoto et al., 1993).

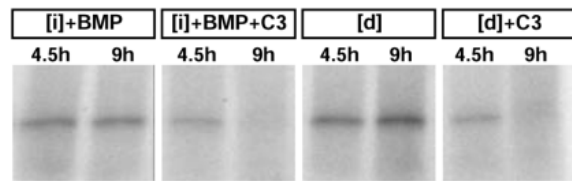
We used an ADP-ribosylation assay (Ridley and Hall, 1992) to monitor the time course of inhibition of rho function in neural plate explants after addition of C3 to the culture medium. In [i] explants cultured in the presence of BMP (Dsl1) and in [d] explants, ~50% of rho proteins were ADP-ribosylated by C3 (~50 µg/ml) at 4.5 hours and nearly complete inactivation was evident by 9 hours (Fig. 5A). These results indicate C3 treatment efficiently ADP-ribosylates endogenous rho proteins in neural plate tissue in vitro.

To begin to determine the effect of inhibition of rho activity on the early differentiation of neural crest cells, we assayed the emergence of neural crest cells from [i] explants in the presence of BMP4, with or without C3. In control explants, the migration of neural crest cells from [i] explants exposed to BMP4 was first detected at 18 hours and by 24 hours, many cells had migrated from the explants (Fig. 5C). In the presence of C3 (~50 µg/ml), the number of migratory neural crest cells induced by 24 hours BMP4 exposure was reduced by over 80% (Fig. 5D,E). This result suggests that rho activity is required for the generation of migratory neural crest cells in response to BMP signaling. However, it does not resolve whether rho activity is required for the specification of premigratory neural crest cells, for the subsequent delamination of these cells or for the later migration of neural crest cells. We examine the potential involvement of rho proteins in each of these three steps of neural crest differentiation in the following sections.

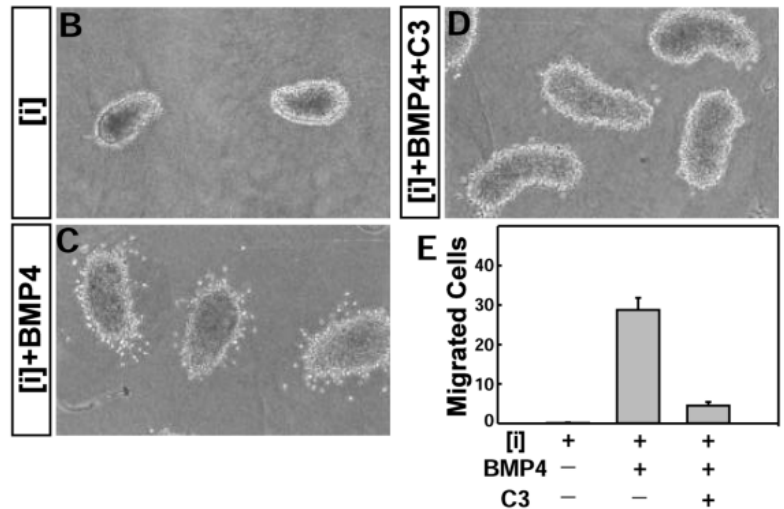
### Inhibition of rho activity does not prevent the specification of premigratory neural crest cells

The BMP-mediated induction of *rhoB* expression occurs later than that of *slug* (Fig. 4B), suggesting that rho activity may not be involved in the initial specification of premigratory neural crest cells. To address this issue more directly, we used RT-PCR analysis and immunohistochemistry to examine if the C3-mediated blockade of rho activity affects the induction of *slug* expression by BMPs in [i] explants. Since rho function has been implicated in cell cycle progression (Olson et al., 1995), we also monitored the expression level of the gene encoding the *s17* ribosomal protein and counted DAPI-stained nuclei to assess total cell number in these explants. In the presence of

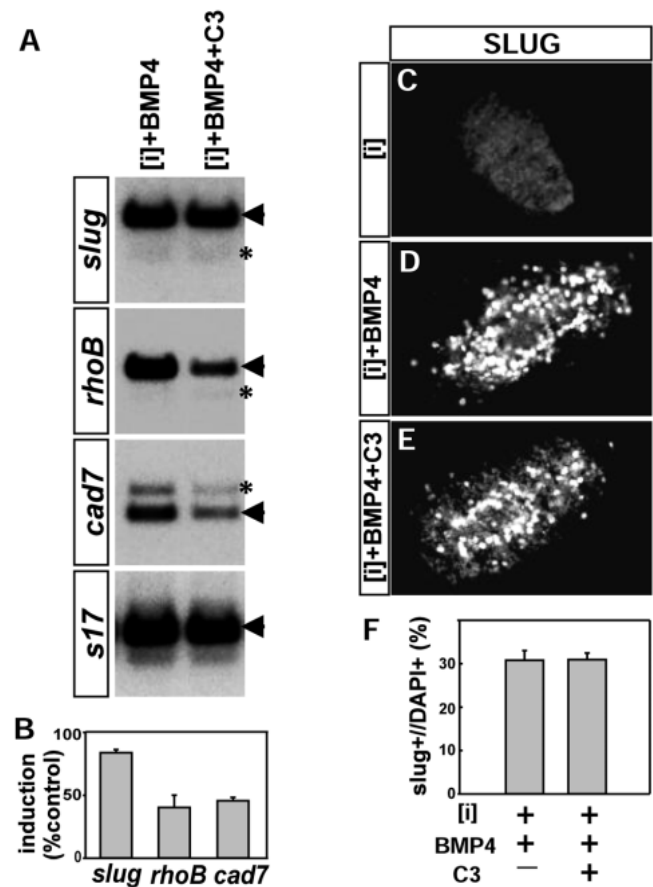
## A ADP-ribosylation assay



**Fig. 5.** Inactivation of rho proteins and inhibition of neural crest cell differentiation in neural explants by C3 exotoxin. (A) Analysis of ADP-ribosylation of rho proteins in neural plate explants by C3 toxin. In [d] explants grown alone or [i] explants cultured with Ds11 ([i]+BMP), the level of active rho proteins is constant at 4.5 hours and 9 hours. The addition of C3 virtually completely inactivates rho proteins in these explants within 9 hours. (B-E) Effect of C3 on neural crest migration in neural plate explants cultured for 24 hours in collagen matrix. (B) [i] explants cultured alone do not give rise to neural crest cells. (C) [i] explants cultured with BMP4 generate many neural crest cells that migrate from the explants. (D) [i] explants cultured with both BMP4 and C3 show a marked reduction in the number of migrating neural crest cells. (E) Quantitation of migrated cells/explant in the presence or absence of C3. Bars indicate standard error ( $n=16$ ).



**Fig. 6.** Influence of C3 on BMP-induced expression of *slug* and other dorsal markers. (A) RT-PCR assay of *rhoB*, *slug* and *cadherin7* expression in [i] region neural plate explants cultured with BMP4 in the presence or absence of C3 for 24 hours. BMP4 induces the expression of *slug*, *rhoB* and *cadherin7*. The addition of C3 slightly reduced the induction of *slug* by BMP4 but has a much greater effect on that of *rhoB* and *cadherin7*. Arrows indicate the position of each transcript analyzed. \* indicates the position of control products from competitive templates. (B) Quantitation of the expression level of *slug*, *rhoB* and *cadherin7* in [i] explants cultured with BMP4 and C3 as compared to that of [i] explants cultured with BMP4 alone. A 20% reduction in the level of expression of the ribosomal protein gene *s17* was detected in explants grown in the presence of C3. The percentage induction of *slug*, *rhoB* and *cadherin7* has been normalized with reference to the level of *s17* expression in the presence of C3. Bars indicate standard error ( $n=3$ ). (C-F) Immunohistochemical detection of slug expression in [i] explants. (C) [i] explants cultured alone do not generate slug<sup>+</sup> cells. (D) Many slug<sup>+</sup> cells are detected in [i] explants cultured with BMP4. (E) Slug<sup>+</sup> cells are present in [i] explants cultured with BMP4 and C3. (F) Quantitative analysis of slug<sup>+</sup> cells in [i] explants cultured with BMP4 in the presence or absence of C3. As with *s17*, we detected a decrease in the number of DAPI<sup>+</sup> nuclei in explants grown in the presence of C3. Normalizing the number of slug<sup>+</sup> cells in each explant to the number of DAPI<sup>+</sup> nuclei indicates that the percentage of slug<sup>+</sup> cells in each explant is not markedly reduced by the addition of C3. Bars indicate standard error ( $n=3$ ).



C3, there was a ~20% decrease in the level of *s17* expression (Fig. 6A, legend). Nevertheless, the level of expression of *slug* in [i] explants grown in the presence of both BMP4 and C3 was ~80% of that in [i] explants cultured in BMP4 alone (Fig. 6A,B). Similarly, the BMP-mediated induction of slug protein expression persisted in [i] explants grown in the presence of C3 (Fig. 6C-F). Since *slug* is an early marker of avian neural crest cell differentiation (Nieto et al., 1994), these experiments support the idea that rho proteins are not required for the initial specification of neural crest cell fate.

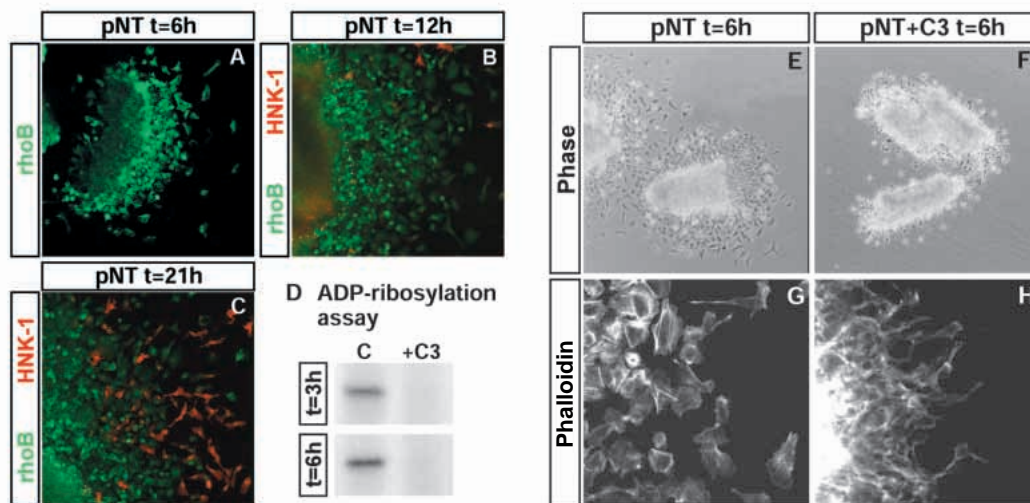
We also analyzed the effects of C3 on *cadherin7*, *rhoB* and *rhoA* expression in [i] explants grown for 24 hours in the presence of BMP4. In explants grown in the presence of C3 the expression of *cadherin7*, a gene expressed at high levels only by migrating neural crest cells, was reduced to ~40% of controls (Fig. 6A,B). This result provides evidence that the expression of markers of later steps in neural crest differentiation is sensitive to the blockade of rho activity. The level of expression of *rhoB* itself was reduced to ~30% of controls in the presence of C3 (Fig. 6A,B). The basis of the reduction in *rhoB* expression is not clear but this observation raises the possibility that C3 may reduce rhoB function in neural cells both by inhibiting rho protein activity and by reducing the level of *rhoB* transcript. In contrast, the level of expression of *rhoA* was not reduced by the presence of C3 (data not shown).

### Inhibition of rho activity blocks the delamination of neural crest cells

We next determined whether rho protein activity is required for

later steps in neural crest cell differentiation, focusing first on the delamination of neural crest cells from the dorsal neural epithelium. To test this, neural tube explants were isolated from stage 10-11 embryos at the level of the five most recently formed somites (pNT explants). At this axial level, the emigration of neural crest cells has not yet started (Delannet and Duband, 1992; Loring and Erickson, 1987; Tosney, 1978). The time course of rhoB and HNK-1 expression by emigrating neural crest cells was first examined in control pNT explants. Neural crest cell emigration was detected by 3 hours in culture (data not shown) and extensive migration was observed by 6 hours (Fig. 7A,E). At this time, rhoB was expressed at high levels by cells close to the border of the neural tube explant (Fig. 7A). In contrast, HNK-1 was not expressed by neural crest cells at this stage (data not shown). Over the period from 6-21 hours in vitro, the expression of rhoB was progressively downregulated in neural crest cells as they migrated away from the border of the neural tube explant. In a complementary manner, the expression of HNK-1 increased with time and the extent of neural crest cell migration (Fig. 7B,C, data not shown).

We next examined the influence of C3 on the delamination of neural crest cells from such pNT explants. In order to block rho activity before the onset of neural crest emigration from neural tube explants, a higher concentration of C3 (150-200 µg/ml) was used. At this concentration, the inhibition of rho proteins in pNT explants was essentially complete by 3 hours (Fig. 7D). At 6 hours, there was an 87% reduction in the number of neural crest cells that emerged from pNT explants



**Fig. 7.** C3 exotoxin inhibits the delamination of neural crest cells in vitro. (A-C) Dynamic expression of rhoB and HNK-1 in HH stage 10-11 posterior neural tube (pNT) explants. (A) Neural crest migration has commenced 6 hours after plating of pNT. rhoB expression in the dorsal region of the explant and in migrating neural crest cells is detected at this time. (B) The expression of rhoB by neural crest cells decreases as cells migrate away from the explant at 12 hours after plating. Some HNK-1<sup>+</sup> cells are detected at this time. (C) The expression of rhoB is still detectable in cells at the edge of the explant and in neural crest cells close to the explant but not in more distant neural crest cells at 21 hours after plating. Many HNK-1<sup>+</sup> cells can be seen at this time. White dotted lines in B-C mark the margin of the explants. (D) ADP-ribosylation analysis of rho proteins in pNT explants by C3 toxin. rho proteins are essentially completely ADP-ribosylated after 3 hours culture with ~200 µg/ml of C3 toxin. (E-H) Effect of C3 on the delamination of neural crest cells from posterior neural tube (pNT) explants. (E) Phase-contrast image of pNT explants cultured on a fibronectin substratum for 6 hours. Cells have migrated from the edge of the explants, forming a dense monolayer surrounding the explants. Scattered neural crest cells can be seen migrating away from the explants. (F) Phase-contrast image of pNT explants cultured on fibronectin substratum in the presence of C3 (~150 µg/ml) for 6 hours. The emigration of cells is severely reduced. Cells that emerged from the explants appear impaired in their ability to attach to the substratum and are rounded. (G) Rhodamine-phalloidin labeling of actin filaments in the pNT explants shows the presence of actin stress fibers in emerging neural crest cells. (H) Rhodamine-phalloidin labeling shows the virtual absence of actin stress fibers in cells located at the edge of pNT explants cultured with C3.



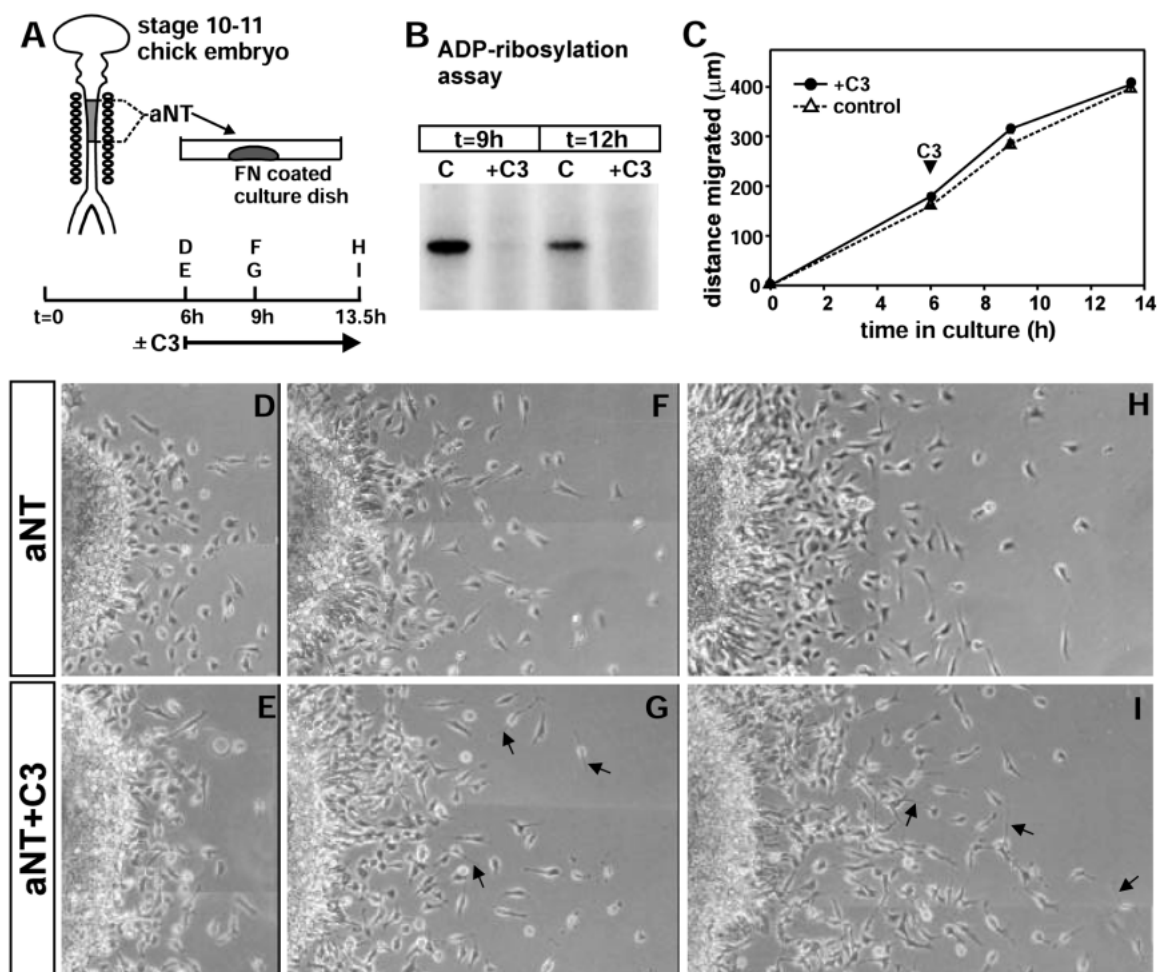
cultured in the presence of C3 (Fig. 7F). These findings provide evidence that the inhibition of rho activity results in a blockade of the delamination of neural crest cells from the dorsal neural tube.

rho proteins have been implicated in the assembly of the actin cytoskeleton (Ridley and Hall, 1992). We therefore used fluorescently labelled phalloidin to examine whether C3 modifies the actin cytoskeleton of neural crest cells located at the border of pNT explants. In control explants, emerging neural crest cells were flattened and exhibited a well-organized filamentous actin cytoskeleton, (Fig. 7G). In contrast, in the presence of C3 (~150  $\mu\text{g}/\text{ml}$ ), prospective neural crest cells at the margin of the neural tube explants did not exhibit prominent actin stress fibers and many cells had an elongated morphology (Fig. 7H). Thus, inhibition of rho activity appears to perturb the actin cytoskeleton of emerging neural crest cells

and produces a marked change in their morphology. These cytoskeletal and morphological changes may contribute to the failure of neural crest delamination when rho activity is inhibited.

### The later migration of neural crest cells persists when rho activity is inhibited

The rapid downregulation of rhoB expression after the emergence of neural crest cells from the neural tube in vivo (Fig. 3C,E) and in vitro (Fig. 7A-C) suggests that rhoB activity is not required for the later migration of neural crest cells. It remains possible, however, that other rho proteins contribute to the migration of neural crest cells. To assess this, we examined the influence of C3 treatment on neural crest cell migration in vitro. In these assays, we isolated regions of neural tube from a more rostral axial level at which



**Fig. 8.** C3 does not affect the migration of neural crest cells. (A) Experimental design. Anterior neural tube explants (aNT) from stage 10-11 chick embryos were plated on a fibronectin substratum. After 6 hours C3 (~100  $\mu\text{g}/\text{ml}$ ) was added to the experimental group. Phase-contrast images of these explants were recorded at 6 hours, 9 hours and 13.5 hours after plating. (B) ADP-ribosylation analysis of rho proteins in migrating neural crest cells exposed to C3 toxin. The inactivation of rho proteins by C3 was ~93% at 3 hours and was virtually completed at 6 hours. (C) Quantitative analysis of neural crest migration. The migration rate of cells distant from the explants is not significantly different with or without C3. ( $n=3$ ). (D-I) Photomicrographs of aNT explants cultured alone (D,F,H) or with C3 (E,G,I). (D,E) Neural crest cells have emerged from aNT explants cultured on fibronectin substratum by 6 hours. Addition of C3 occurs at this time. (F) In aNT explants cultured alone for 9 hours, cells have migrated further from the explant. (G) Migrating cells with elongated processes can be seen (arrows) 3 hours after the addition of C3 (9 hours in culture). (H) Continued cell migration in aNT explants cultured for 13.5 hours. (I) More cells exhibit an elongated morphology (arrows) 7.5 hours after the addition of C3 (13.5 hours in culture). However, the rate of migration of these cells is not significantly affected by the presence of C3.

neural crest emigration has commenced, to permit a more rapid initial emergence of neural crest cells. Segments of the neural tube were therefore isolated from the level of the 6th to the 10th most recently formed somites in stage 10-11 chick embryos (aNT explants) (Fig. 8A), plated on a fibronectin substratum and grown in vitro for 13.5 hours. C3 (~100 µg/ml) was added to experimental cultures with a delay of 6 hours after plating to permit the delamination and initial migration of neural crest cells. At this concentration, there was a >90% inactivation of rho proteins 3 hours after the addition of C3 ( $t=9$  hours from the onset of culture) and a complete inactivation by 6 hours (Fig. 8B).

Phase-contrast images of the same culture fields were obtained 6 hours, 9 hours and 13.5 hours after the onset of culture and the extent of migration of neural crest cells was compared in the presence and absence of C3 (Fig. 8D-I). We focused on those cells that had migrated from aNT explants at early stages and thus were located furthest from the explant. In these neural crest cells the expression of *rhoB* has been downregulated (Fig. 7A-C, data not shown). In control cultures, the rate of neural crest cell migration was relatively constant over the entire 13.5 hours culture period, with a mean migration rate of ~30 µm/hour (Fig. 8C). In cultures exposed to C3 from 6 hours, neural crest cell migration persisted over the subsequent 7.5 hours culture period. Moreover, the mean rate of migration in the presence of C3 was not significantly different from the rate detected prior to C3 addition, or from the rate of cell migration in control cultures (Fig. 8C). Thus, the later migration of neural crest cells appears not to be markedly affected by C3. These data, taken together with the downregulation of rhoB expression in neural crest cells soon after their migration in vitro and in vivo indicate that rhoB activity is not required for the later migration of these cells. They also argue against a critical role for other rho proteins in this migratory step, at least as assayed in vitro on a fibronectin substratum.

## DISCUSSION

To identify molecules involved in early steps of neural crest differentiation, we performed a screen for BMP-induced neural genes and identified *rhoB*, a gene that encodes a member of the rho family of GTP-binding proteins (Madaule and Axel, 1985). rho proteins have been implicated in the control of neuronal cell morphology, axonal growth and dendritic arborization (Jalink et al., 1994; Kozma et al., 1997; Nishiki et al., 1990; Threadgill et al., 1997; Tigyí et al., 1996). This study shows that *rhoB* is expressed in premigratory neural crest cells and transiently in migrating neural crest cells. Moreover, the expression of *rhoB* in neural plate cells is induced by BMPs. The inhibition of rho function in vitro blocks the delamination of neural crest cells from the dorsal neural epithelium but appears not to inhibit their initial specification or their later migration. These findings suggest that rhoB activity has a role in the early differentiation of neural crest cells in response to BMP signaling.

### rho proteins appear not to function in the specification of premigratory neural crest cells

rho proteins have been shown to function as intermediates in

several cytoplasmic signal transduction pathways that link extracellular mitogenic and inductive signals to nuclear transcriptional responses. rhoA has been implicated in the serum-response pathway that activates transcription of the *c-fos* gene (Hill et al., 1995). rho proteins have also been implicated in the activation of the transcription factor NF-κB (Perona et al., 1997) and in photoreceptor cell differentiation in *Drosophila* (Hariharan et al., 1995). These observations raised the question of whether the early expression of *rhoB* by premigratory neural crest cells might have a function in the initial specification of neural crest cell fate in response to BMP-mediated signaling. Our results show the blockade of rho activity does not inhibit the BMP-induced generation of slug<sup>+</sup> premigratory neural crest cells. Moreover, the induction of expression of *slug* precedes that of *rhoB* in response to BMP signaling. These data argue against a role for *rhoB* at this initial step of neural crest cell differentiation.

### A role for rhoB in the delamination of neural crest cells

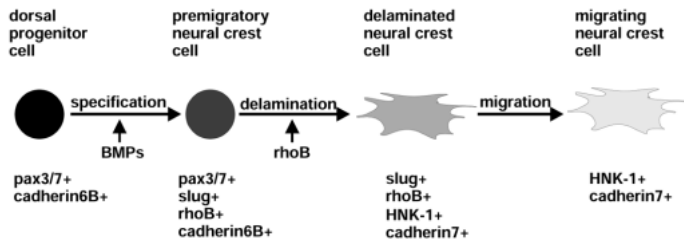
The delamination of neural crest cells from the dorsal neural epithelium represents a specialized instance of the more general phenomenon of epithelial-mesenchymal transition (Duband et al., 1995; Hay, 1995). During this transition, premigratory neural crest cells undergo changes in cell shape and cell-cell interactions and acquire the ability to interact with extracellular matrix (Duband et al., 1995).

Our studies provide evidence that rho activity is involved in the delamination of neural crest cells from the dorsal neural epithelium. The inactivation of rho function in prospective neural crest cells at the border of neural tube explants results in a disorganization of the actin cytoskeleton and impairs the ability of cells to migrate from the neural tube explant. rho proteins have been shown to regulate assembly of the actin cytoskeleton and focal adhesions: events that are important for cell morphology and adhesion (Nobes and Hall, 1995; Ridley and Hall, 1992). Thus, rho activity might contribute to the changes in cell shape and adhesion that occur during the delamination of neural crest cells by regulating actin polymerization and the formation of focal adhesions and stress fibers. Nevertheless, details of the pathway by which rho proteins control neural crest cell delamination remain unclear. In other cell types, rho proteins have been shown to interact with several downstream effector proteins. In particular, the serine/threonine kinases p160ROCK and ROCKII have been shown to be important for focal adhesions and stress fibers formation (Ishizaki et al., 1997; Leung et al., 1996). In addition, the p140mDia, a formin-related protein, has been shown to bind both rho proteins and profilin and may function in the regulation of actin polymerization by rho proteins (Watanabe et al., 1997).

The use of C3 toxin does not permit us to distinguish the contribution of individual rho proteins to the delamination of neural crest cells. However, the preferential association of *rhoB* expression with sites of neural crest differentiation in vivo supports the idea that rhoB is the primary target of C3 action in premigratory neural crest cells.

### Inhibition of rho activity does not significantly alter the migration of neural crest cells in vitro

The inhibition of rho function has been shown to inhibit the



**Fig. 9.** A model for the regulation of sequential stages of early neural crest differentiation. Progressive stages in the differentiation of a Pax3/7<sup>+</sup>, cadherin6B<sup>+</sup> dorsal neural progenitor cell into a HNK1<sup>+</sup>, cadherin7<sup>+</sup> migratory neural crest cell. BMPs appear to specify premigratory neural crest cell fate, to induce the expression of *slug* and *rhoB* and to maintain the expression of *cadherin6B* and *Pax3/7*. Slug and rhoB appear to function during the delamination of neural crest cells from the dorsal neural tube. The expression of *cadherin6B* is downregulated at this step. Both HNK-1 and *cadherin7* are expressed by migrating neural crest cells. As neural crest cell migration proceeds, the expression of *slug* and *rhoB* is extinguished.

motility of a wide variety of cell types (Hinsch et al., 1993; Stasia et al., 1991; Takaishi et al., 1993). However, inhibition of rho activity appears to have little effect on the migration of neural crest cells, at least on a fibronectin substratum in vitro. One possible explanation for the absence of a requirement for rho activity is that neural crest cells employ other C3-insensitive GTPases, rac or cdc42, for their later migration. Distinct cell types have been shown to rely on different members of this GTPase superfamily for similar migratory behaviors. For example, in mouse keratinocytes, HGF-induced cell motility appears to be mediated by rho, but not by rac or ras (Takaishi et al., 1994), whereas in MDCK cells HGF-induced cell motility is mediated by ras or rac but not by rho (Ridley et al., 1995). Similarly, rac and cdc42 but not rho have been shown to induce cell motility in mammary epithelial cells (Keely et al., 1997). Thus, rac- or cdc42-dependent activities may be sufficient to promote the later migration of neural crest cells, independent of rho proteins.

### BMP signaling and the induction of neural crest differentiation

Our studies on the expression of markers characteristic of sequential stages of neural crest cell development provide evidence that BMP signaling is sufficient to activate a molecular program of neural crest differentiation (Fig. 9). The induction of *slug* expression by BMPs in vitro precedes that of other markers examined, placing *slug* upstream of *rhoB* in a temporal hierarchy of neural crest cell gene expression in the chick embryo. The precise role of *slug* in the specification of neural crest cells, however, remains unclear. In chick embryos, the antisense oligonucleotide-mediated ablation of *slug* activity has been reported to perturb neural crest cell differentiation (Nieto et al., 1994). However in mouse embryos, *slug* expression is initiated only after neural crest cells have emerged from the neural tube and the targeted inactivation of the *slug* gene does not obviously affect early stages of neural crest cell differentiation (Jiang et al., 1998). It is possible that, in mouse, the closely related zinc finger gene *snail* serves a function in neural crest development similar to that proposed for the chick *slug* gene (Sefton et al., 1998; Smith et al., 1992).

The sequential expression of *cadherin6B* and *cadherin7* has been reported to control the delamination and later migration of neural crest cells (Nakagawa and Takeichi, 1995, 1998). Our results indicate that the pattern of expression of *cadherin6B* and *cadherin7* in the neural tube is controlled, at least in part, by BMP signaling. In contrast to the initial restriction in *slug* and *rhoB* expression to the dorsal neural folds, the expression of *cadherin6B* is initially detected over a wide domain of the neural plate, absent only from ventral midline cells. It is possible therefore that the restriction of *cadherin6B* expression to the site of generation of premigratory neural crest cells involves both the BMP-mediated upregulation of *cadherin6B* dorsally and the Shh-mediated repression of gene expression ventrally. Alternatively, neural plate cells may extinguish *cadherin6B* expression over time unless exposed to BMP signals. *Cadherin7* expression is excluded from the domain of premigratory neural crest cells, appearing only after cells have migrated away from the dorsal neural tube (Nakagawa and Takeichi, 1995). Thus, the induction of *cadherin7* expression in neural plate explants by BMPs is likely to be a secondary consequence of the generation of premigratory neural crest cells.

Finally, our studies showing that the level of expression of *rhoB* is markedly elevated by BMP signaling parallel studies of other cell types in which *rhoB*, but not *rhoA* or *rhoC*, is induced rapidly by growth factors (Jahner and Hunter, 1991). It is possible therefore that rhoB is used generally as an inducible source of rho proteins under conditions in which a high level of rho activity is demanded. In the context of neural crest differentiation, the high level of rhoB activity appears to reflect a function for rho proteins during the delamination of neural crest cells from the dorsal neural tube.

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