The Journal of Neuroscience, September 1, 1997, 17(17):6717–6728

Differential Expression of Distinct Members of Rho Family GTP-Binding Proteins during Neuronal Development: Identification of *Rac1B*, a New Neural-Specific Member of the Family

Maria Luisa Malosio, Daniela Gilardelli, Simona Paris, Chiara Albertinazzi, and Ivan de Curtis

Cell Adhesion Unit, Department of Biological and Technological Research (DIBIT), San Raffaele Scientific Institute, 20132 Milano, Italy

Previous studies on small GTP-binding proteins of the Rho family have revealed their involvement in the organization of cell actin cytoskeleton. The function of these GTPases during vertebrate development is not known. With the aim of understanding the possible role of these proteins during neuronal development, we have cloned and sequenced five members expressed in developing chick neural retinal cells. We have identified four chicken genes, c*RhoA*, c*RhoB*, c*RhoC*, and *cRac1A*, homologous to known human genes, and a novel *Rac* gene, *cRac1B*. Analysis of the distribution of four of the identified transcripts in chicken embryos shows for the first time high levels of expression of Rho family genes in the vertebrate developing nervous system, with distinct patterns of distribution for the different transcripts. In particular, *cRhoA* and *cRac1A* gene expression appeared ubiquitous in the whole embryo, and the *cRhoB* transcript was more prominent in populations of neurons actively extending neurites, whereas the newly identified *cRac1B* gene was homogeneously expressed only in the developing nervous system. Temporal analysis of the expression of the five genes suggests a correlation with the morphogenetic events occurring within the developing retina and the retinotectal pathway. Expression of an epitope-tagged cRac1B in retinal neurons showed a diffuse distribution of the protein in the cell body and along neurites.

Taken as a whole, our results suggest important roles for ubiquitous and neural-specific members of the Rho family in the acquisition of the mature neuronal phenotype.

Key words: Rho GTPases; neuronal development; chick embryo; neural retinal cells; retinotectal pathway; dorsal root ganglia

The actin cytoskeleton plays a fundamental role in several aspects of cell life, including adhesion, migration, and cytokinesis. Several actin binding proteins take part in the organization of the actin cytoskeleton and contribute to its dynamic properties. Recent studies have shown that components of the Rho family, which belong to the Ras superfamily of small GTPases, are involved in the reorganization of the actin cytoskeleton and of the associated sites of cell adhesion to the extracellular matrix (Hall, 1994). In particular, microinjection experiments have demonstrated that RhoA is essential for the assembly of focal adhesions and the associated actin stress fibers (Ridley and Hall, 1992) and that Rac1 is required for growth factor-induced membrane ruffling (Ridley et al., 1992), whereas Cdc42 triggers the formation of filopodia (Nobes and Hall, 1995). Neurite outgrowth can be considered as a particular form of cell motility in which actin dynamics during growth cone navigation evolves into stabilization of the cytoskeleton and neurite elongation (Tanaka and Sabry, 1995). The behavior of growth cones can therefore be compared with that of the leading edge of spreading or migrating fibroblasts, in which the dynamic adhesive interactions with the substrate are accompanied by a continuous reorganization of the actin cytoskeleton.

In line with this interpretation, recent evidence has accumulated that suggests a role for the Rho family GTPases in neuritogenesis (Mackay et al., 1995; Luo et al., 1996). In fact, activation of Rho proteins by lysophosphatidic acid, thrombin, or sphingosine-1-phosphate leads to growth cone collapse and retraction of neurites in N1E-115 neuroblastoma cells (Jalink et al., 1994; Postma et al., 1996), and these effects can be prevented by pretreatment of the cells with the *Clostridium* botulinum C3 exoenzyme, which specifically ADP-ribosylates Rho proteins. Furthermore, two *Drosophila* homologs of the Rho family GTPases, Drac1 and Dcdc42, are highly expressed in the *Drosophila* developing nervous system, and mutants of these proteins cause distinct defects in neuronal development (Luo et al., 1994). These data raise the possibility that components of the Rho family of small GTPases may play a role in neuronal development in vertebrates as well. So far, however, the nature and distribution of the different GTPases of the Rho family expressed during vertebrate development remain undefined. With the aim of studying the role of Rho GTPases in the development of the neuronal phenotype, we have now identified by molecular cloning various components expressed in chicken developing neurons. The identified cDNAs have been used for *in situ* hybridization analysis to look at the expression of the corresponding transcripts in the entire chicken embryo.

Received Jan. 19, 1997; revised June 6, 1997; accepted June 11, 1997.

This work was supported by Telethon–Italy (Grant No. 791). M.L.M. was supported by a postdoctoral fellowship from the University of Milano. We are grateful to Dr. Elena Rugarli (TIGEM, Milano, Italy) and Dr. Elena Zanaria (University of Pavia) for providing chick embryo sections for *in situ* hybridization; Dr. C. Nottenburg (Fred Hutchinson Cancer Research Center, Seattle, WA) for the E10 chick cDNA library; Dr. B. Ranscht (La Jolla Cancer Research Foundation, La Jolla, CA) for the E13 chick brain cDNA library; and Dr. M. A. Impagnatiello for the pcDNA-I-Amp-HA plasmid. We also thank Dr. R. M. Alvarado-Mallart and Dr. E. Gallego (Institut National de la Santé et de la Recherche Médicale U-106, Paris, France) for their help in the interpretation of the results from the *in situ* hybridization studies, and Dr. Edoardo Boncinelli and Dr. Jacopo Meldolesi for critical reading of this manuscript.

Correspondence should be addressed to Ivan de Curtis, Cell Adhesion Unit, Department of Biological and Technological Research (DIBIT), San Raffaele Scientific Institute, via Olgettina 58, 20132 Milano, Italy.

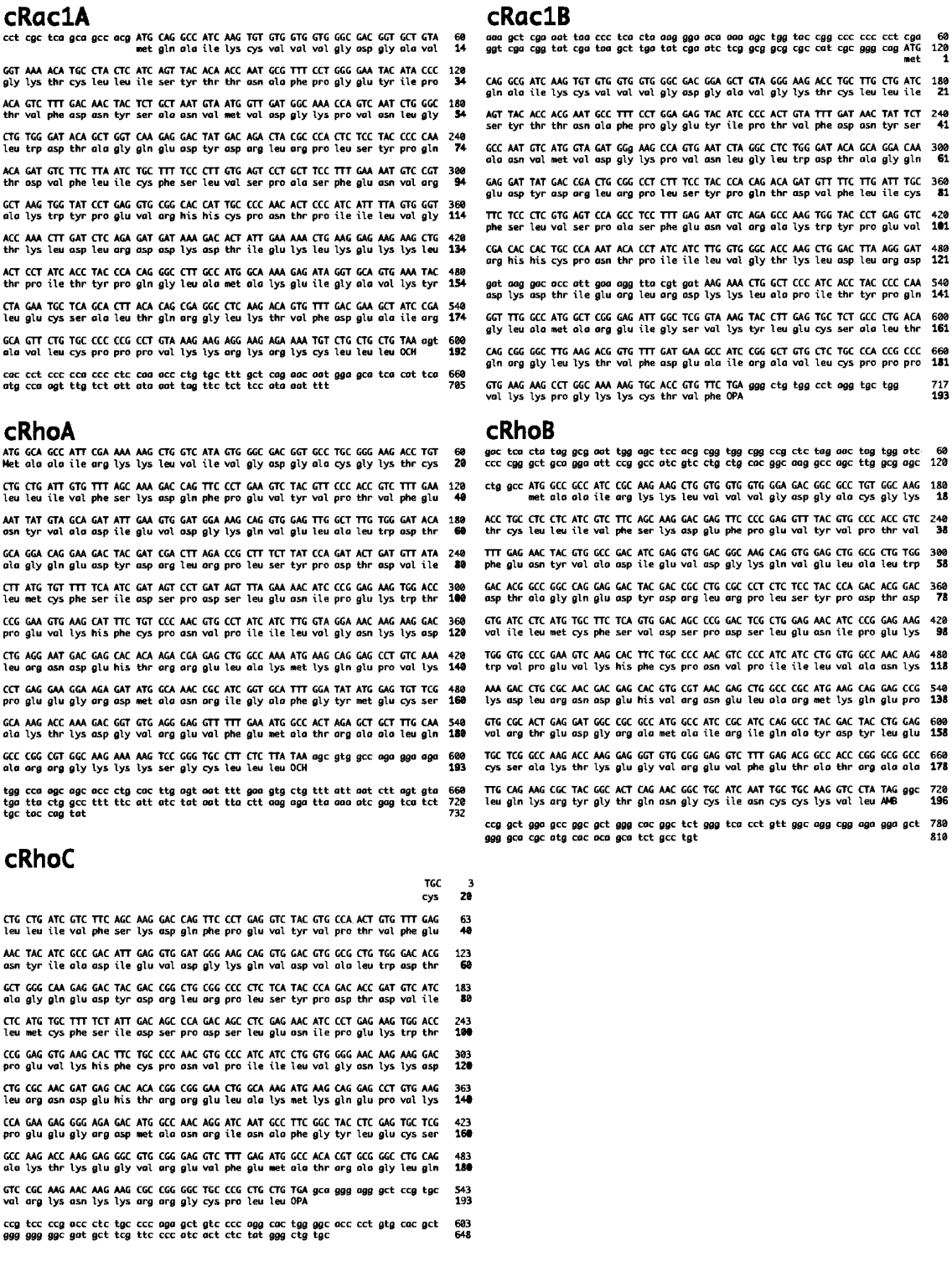

Figure 1. Nucleotide sequences of the chick Rho family GTPases cDNAs expressed in embryonic neural retina, and deduced primary sequences of the encoded polypeptides. The sequence data are available from GenBank under accession numbers U79757 (*cRhoA*), U79758 (*cRhoB*), U79759 (*cRhoC*), U79755 (*cRac1A*), and U79756 (*cRac1B*).

The data presented in this paper show that developing retinal neurons express the mRNAs of at least five different components of the Rho family of GTPases, including a new Rac protein, which are differentially expressed during the development of the neural retina. Furthermore, the analysis of the distribution of four of the identified transcripts shows that they are strongly and specifically expressed in various areas of the developing CNS and peripheral nervous system.

MATERIALS AND METHODS

Reagents. Fertilized chicken eggs were purchased from Allevamento Giovenzano (Vellezzo Bellini, Italy). *Taq* polymerase was from Promega

A

```
cRhoA     MAAIRKKLVI VGDGACGKTC LLIVFSKDQF PEVYVPTVFE NYVADIEVDG KQVELALWDT AGQEDYDRLR
cRhoB     MAAIRKKLVV VGDGACGKTC LLIVFSKDEF PEVYVPTVFE NYVADIEVDG KQVELALWDT AGQEDYDRLR
cRhoC                       C LLIVFSKDQF PEVYVPTVFE NYIADIEVDG KQVDVALWDT AGQEDYDRLR
cRac1A    MQAI--KCVV VGDGAVGKTC LLISYTTNAF PGEYIPTVFD NYSANVMVDG KPVNLGLWDT AGQEDYDRLR
cRac1B    MQAI--KCVV VGDGAVGKTC LLISYTTNAF PGEYIPTVFD NYSANVMVDG KPVNLGLWDT AGQEDYDRLR

cRhoA     PLSYPDTDVI LMCFSIDSPD SLENIPEKWT PEVKHFCPNV PIILVGNKKD LRNDEHTRRE LAKMKQEPVK
cRhoB     PLSYPDTDVI LMCFSVDSPD SLENIPEKWV PEVKHFCPNV PIILVANKKD LRNDEHVRNE LARMKQEPVR
cRhoC     PLSYPDTDVI LMCFSIDSPD SLENIPEKWT PEVKHFCPNV PIILVGNKKD LRNDEHTRRE LAKMKQEPVK
cRac1A    PLSYPQTDVF LICFSLVSPA SFENVRAKWY PEVRHHCPNT PIILVGTKLD LRDDKDTIEK LKEKKLTPIT
cRac1B    PLSYPQTDVF LICFSLVSPA SFENVRAKWY PEVRHHCPNT PIILVGTKLD LRDDKDTIER LRDKKLAPIT

cRhoA     PEEGRDMANR IGAFGYMECS AKTKDGVREV FEMATRAALQ ARRGKKKSG- --CLIL
cRhoB     TEDGRAMAIR IQAYDYLECS AKTKEGVREV FETATRAALQ KRYGTQNGCI NCCKVL
cRhoC     PEEGRDMANR INAFGYLECS AKTKEGVREV FEMATRAGLQ VRKNKKRRG- --CPLL
cRac1A    YPQGLAMAKE IGAVKYLECS ALTQRGLKTV FDEAIRAVLC PPPVKKRKRK --CLIL
cRac1B    YPQGLAMARE IGSVKYLECS ALTQRGLKTV FDEAIRAVLC PPPVKKPGKK --CIVF
```

B

	cRhoA	cRhoB	cRhoC	cRac1A	cRac1B	hRhoA	hRhoB	hRhoC	hRac1	hRac2
cRhoA	100									
cRhoB	72.1	100								
cRhoC	76.2	77.8	100							
cRac1A	56.6	56.8	59.2	100						
cRac1B	57	57	58.9	78.5	100					
hRhoA	83.5	75.7	77.8	56.6	56.6	100				
hRhoB	72.9	90.3	78.5	56.6	56.5	69.9	100			
hRhoC	79	79.9	85.3	62	62.7	78.4	79.6	100		
hRac1	63	57	58.3	88.5	80.7	62.2	64.3	62	100	
hRac2	62.9	67	63.7	77.4	78.5	58.9	67	66.2	78.2	100

C

	cRhoA	cRhoB	cRhoC	cRac1A	cRac1B	hRhoA	hRhoB	hRhoC	hRac1	hRac2
cRhoA	100									
cRhoB	85.5	100								
cRhoC	92.5	83.3	100							
cRac1A	59.2	56.7	56.3	100						
cRac1B	57	56.7	54.6	93.8	100					
hRhoA	100	86	92	59.2	57.6	100				
hRhoB	84.5	97.5	82.2	56.7	56.7	85	100			
hRhoC	91.7	85.5	95.4	58.6	56.6	91.7	85	100		
hRac1	59.7	56.7	56.3	100	93.7	59.2	56.7	58.7	100	
hRac2	56.3	55.2	53.5	92.2	89	55.8	55.2	55.5	92.2	100

Figure 2. Amino acid and nucleotide sequence comparisons. *A*, Amino acid sequence alignment of cRhoA, cRhoB, cRhoC, cRac1A, and cRac1B deduced polypeptides. Identical amino acids are shown in **bold type**. *B*, Percentages of identity between the coding regions of chick cDNAs *cRhoA*, *cRhoB*, *cRhoC*, *cRac1A*, and *cRac1B* and those of human cDNAs *RhoA*, *RhoB*, *RhoC*, *Rac1*, and *Rac2*. *C*, Percentages of amino acid identities between chick proteins cRhoA, cRhoB, cRhoC, cRac1A, and cRac1B and human proteins RhoA, RhoB, RhoC, Rac1, and Rac2.

(Madison, WI), Klenow fragment of DNA polymerase was from Pharmacia (Uppsala, Sweden), and restriction enzymes were from Boehringer Mannheim (Mannheim, Germany). [α-^{35}S]dATP and [α-^{32}P]dCTP were from Amersham (Buckinghamshire, UK). Other chemicals were purchased from Sigma–Aldrich (Milan, Italy). Laminin was purified from Engelbreth–Holm Swarm sarcoma as published (Timpl et al., 1979).

Isolation of PCR clones. Total RNA was extracted from E6 retinas by the RNAzolB method (Chomczynski and Sacchi, 1987). Five micrograms of total RNA were converted into complementary DNAs by oligo-dT-priming [oligo-dT_{12-18}; Life Technologies-BRL, Milano, Italy] in the presence of Moloney murine leukemia virus reverse transcriptase (Life Technologies-BRL). Aliquots of the resulting single-stranded cDNA (7.5 ng) were mixed with pairs of degenerate synthetic oligonucleotides and subjected to thermal cycling. Two different sets of degenerate oligonucleotides were used for the amplifications: a set containing the sequences 5′-TTYWSMAARGAYCAGTTCCC (RhoA-1) and 5′-TCACBGGY TCCTGYTTCAT (RhoA-2) coding for amino acid positions 25–31 and 134–140, respectively, of the human RhoA protein, and a more degenerate set of oligonucleotides containing the sequences 5′-AAR ACNTGYYTNCTSAT (RhoF-1) and 5′-GCHGARCAYTCVADRTA (RhoF-2) coding for amino acid positions 18–23 and 156–161, respectively, of different Rho proteins. PCRs contained 50 μl of 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM desoxyribonucleotide triphosphate (dNTP), 100 pmol of each oligonucleotide, 2.5 U of *Taq* polymerase (Promega), and 1.5 μl of cDNA. The first five cycles were performed under low stringency annealing conditions (94°C, 1 min; 43°C, 1 min; 72°C, 1 min); the following 40 cycles were performed at higher stringency (94°C, 1 min; 50°C, 1 min; 72°C, 1.5 min). The cRhoC clone was isolated by using the "touchdown" PCR technique with RhoF1 and RhoF2 primers (Don et al., 1991). Amplified DNA fragments were subcloned into a pBluescript KS$^-$ T-vector (Marchuk et al., 1991). Plasmid clones containing inserts of appropriate size were subjected to direct sequencing by the dideoxy method (Sanger et al., 1977) and analyzed by the GCG Wisconsin Sequence Analysis Package.

Isolation of cDNA clones from λgt10 libraries. Four full-length clones and a partial clone coding for the different chicken Rho and Rac proteins were isolated either from an embryonic day (E) 10 chicken or an E13 chicken brain cDNA library in λgt10 [obtained from Dr. C. Nottenburg (Fred Hutchinson Cancer Research Center, Seattle, WA) and Dr. B. Ranscht (Cancer Research Institute, La Jolla, CA), respectively]. The libraries were plated on *Escherichia coli* strain LE392, and replica filters were screened in duplicate at high stringency according to a modified procedure of Church and Gilbert (1984). Prehybridization was performed for 3 hr at 65°C in hybridization buffer (125 mM Na_2HPO_4, 1 mM EDTA, 250 mM NaCl, 7% SDS, 10% PEG-8000, 1% BSA, 100 μg/ml denatured salmon sperm DNA). Hybridizations were performed at 65°C

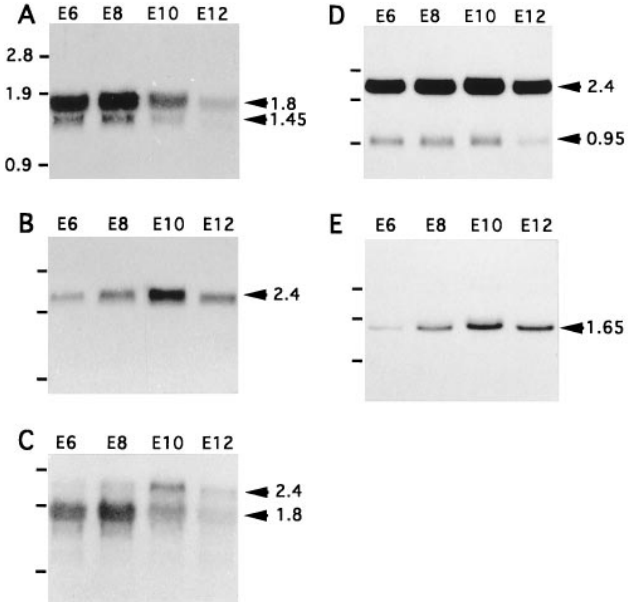

Figure 3. Northern blot analysis of GTPase mRNA levels during neural retina development. Total RNA extracted from E6, E8, E10, and E12 neural retinas was electrophoresed on a 1% agarose gel and transferred to filters, as described in Materials and Methods. Filters were incubated with ^{32}P-labeled probes specific for *cRhoA* (*A*), *cRhoB* (*B*), *cRhoC* (*C*), *cRac1A* (*D*), or *cRac1B* (*E*). RNA markers (in kilobases) are indicated to the *left* of each blot; the size of the transcripts (in kilobases) is indicated to the *right*.

in the same buffer in the presence of 0.5–1 $\times$ 10^6 cpm/ml of ^{32}P-labeled probe. Washings were in 0.2× SSC at 65°C. The cDNAs corresponding to the different chicken PCR products were labeled by random priming (Feinberg and Vogelstein, 1983) at a specific activity between 7 $\times$ 10^8 and 1.2 $\times$ 10^9 cpm/μg. cDNAs inserts from positive purified phages were extracted by *Eco*RI digestion and subcloned into pBluescript KS$^-$. Both strands were sequenced with the T7 sequencing kit (Pharmacia, Uppsala, Sweden) using different specific oligonucleotide primers.

Northern blot analysis. Total RNA was prepared from E6, E8, E10, and E12 chick neural retinas by the RNAzolB method (Chomczynski and Sacchi, 1987). Northern blot analysis of total RNA (20 μg/lane) was performed as described previously (Lehrach et al., 1977; Malosio et al., 1991). Hybridizations and washes were performed under the same high stringency conditions used for the screening of the libraries. Hybridizations took place in hybridization buffer supplemented with ^{32}P-labeled probes (0.5–1.0 $\times$ 10^6 cpm/ml) for 12–16 hr at 65°C. After high stringency washes (0.2 $\times$ SSC at 65°C), x-ray films were exposed for 3–7 d to the hybridized filters. RNA blots were reprobed with an 18 S ribosomal RNA probe. Quantitations of hybridized bands were performed by computer densitometry (Molecular Dynamics, Sunnyvale, CA). The values for the different GTPase transcripts were normalized to the corresponding values obtained for the 18 S ribosomal RNA.

In situ *hybridization.* The 370- to 400-bp-long cDNAs obtained by PCR, coding for the different Rho proteins, were used for the preparation of sense and antisense RNA probes to be used for *in situ* hybridizations. The specificity of hybridization of these probes had been assessed previously by Northern blotting. After linearization of the pBluescript plasmids with the appropriate restriction enzyme, T3 and T7 polymerases were used to generate high specificity ^{35}S-labeled riboprobes by incorporation of [α^{35}S]rUTP (Amersham, Arlington Heights, IL) into the transcribed RNA (RNA transcription kit, Stratagene, La Jolla, CA). The DNA template was removed by digestion with DNase I and the labeled RNA probe was purified through a Bio-spin column (Boehringer Mannheim). The purified probe was supplemented with 20 mM DTT, and an aliquot was counted in scintillation fluid.

For *in situ* hybridization, paraffin sections of chick embryos were dewaxed, deproteinated, and post-fixed in 4% paraformaldehyde (Rugarli et al., 1993). The ^{35}S-labeled riboprobes were diluted at 4.2–6.3 $\times$ 10^7 cpm/ml (~60 ng/ml) in hybridization buffer containing 50% (v/v) formamide, 0.3 M NaCl, 10 mM Tris-HCl, pH 7.6, 10 mM NaH_2PO_4, pH 6.8, 5 mM EDTA, pH 8.0, 0.2% (w/v) Ficoll 400, 0.2% (w/v) polyvinylpyrrolidone, 10% (w/v) dextran sulfate, 50 mM DTT, 0.5 mg/ml poly ribo A, and 50 μg/ml yeast tRNA, and added to the sections. Sections were incubated overnight at 55°C in a humidified chamber. Stringency washes at 64°C included several washes in 2× SSC, 50% formamide, 20 mM β-mercaptoethanol, in 4× SSC, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 30 min incubation with RNase A (10 μg/ml). Slides were air-dried and exposed to x-ray films for 3–6 d. Subsequently, slides were dipped

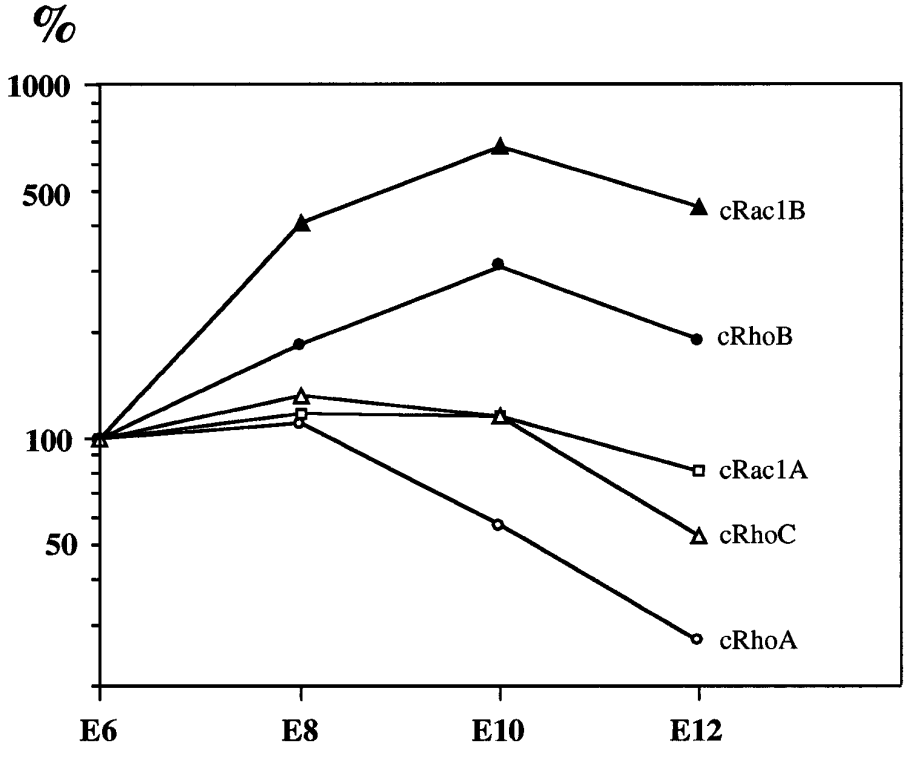

Figure 4. Quantitation of the levels of expression of the *cRhoA*, *cRhoB*, *cRhoC*, *cRac1A*, and *cRac1B* transcripts during retina development. Quantitation was obtained by densitometry on autoradiograms from two experiments such as those shown in Figure 3. For *cRhoA*, *cRhoC*, and *cRac1A* transcripts, the values for both hybridizing bands were added (Fig. 3, *A, C*, and *D*, respectively). The values for the different GTPase transcripts were normalized to the values obtained from the corresponding 18 S ribosomal RNA hybridizations and plotted on a semilogarithmic scale. The E6 expression levels of the different transcripts were considered as 100%. Each value represents the mean obtained from two blots. Quantitation indicated that changes during neural retina development of the individual transcripts for *cRhoA* (Fig. 3*A*) and *cRac1A* (Fig. 3*D*) were similar (quantitation not shown); on the other hand, the changes during development of the two transcripts of 2.4 and 1.8 kb recognized by the *cRhoC* probe (Fig. 3*C*) were different, showing highest levels of expression at E10 and E8, respectively (quantitation not shown).

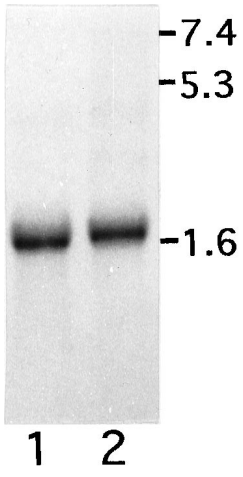

Figure 5. Expression of *cRac1B* mRNA in neurons and glia from developing neural retina. Fifteen micrograms of total RNA extracted from cultures enriched in retinal glial cells (*lane 1*) or in retinal neurons (*lane 2*) prepared from E7 retinas were electrophoresed on a 1% agarose gel and transferred to filters, as described in Materials and Methods. Filters were incubated with a ^{32}P-labeled probe specific for *cRac1B*. RNA markers (in kilobases) are indicated to the *right*.

into Kodak NTB-2 emulsion and exposed at 4°C for 15–21 d. Sections hybridized to sense probes for the different genes were processed in parallel and used as controls for nonspecific hybridization. After development, the slides were counterstained with Hoechst 33258 and analyzed by dark-field illumination and by UV fluorescence.

Cell culture. Cultures enriched in retinal neurons and in retinal glia (Biscardi et al., 1993) were prepared from E7 chick retinas. Neural retinas were dissected and trypsinized, and cultures of retinal neurons were obtained under serum-free conditions as described (de Curtis et al., 1991). After 18 hr in culture, neuronal cells were used to prepare total RNA as described above. For glial cells, cells from trypsinized retinas were cultured in DMEM with 5% fetal calf serum. Confluent monolayers were transferred to new culture dishes to dilute neurons; remaining neurons were washed off the glial monolayers. Neuron-free monolayers were then used for total RNA preparation as described above.

Expression of cRac1B in retinal cells. The full-length cDNA for *cRac1B* was subcloned into pcDNA-I-Amp vector (Invitrogen, Carlsbad, CA) containing a sequence including the YDVPDYA amino acids of the influenza hemagglutinin (HA), and the pcDNA-I-HA-Rac1B plasmid obtained was used for transfections of primary retinal cells.

For transfections, we used a protocol modified from Boussif et al. (1995). Approximately 300,000 retinal cells obtained from E6 chick neural retinas were plated in each 1.5-cm-diameter well containing a glass coverslip coated with 200 μg/ml poly-D-lysine and 40 μg/ml laminin. Cells were cultured overnight at 37°C, 5% CO_2 as described (de Curtis et al., 1991), to induce neurite extension. Cells were then incubated with 200 μl/well of 150 mM NaCl containing 150 nmol of polyethylenimine (PEI 50 kDa; Sigma) and 5 μg of pcDNA-I-HA-Rac1B plasmid in 0.5 ml of transfection medium [50% retinal growth medium (RGM), 50% DMEM, and 5% fetal calf serum]. After 3 hr of culture, the medium was replaced with serum-free RGM, and the cells were cultured for an additional 24 hr. Cells were then fixed with paraformaldehyde or with cold (−20°C) methanol and processed for indirect immunofluorescence as described by Cattelino et al. (1995). Cells were incubated for 1 hr at room temperature with the following primary antibodies: a monoclonal antibody against the HA-tag, a polyclonal antibody against the 200 kDa neurofilament protein (Sigma), and a polyclonal antibody against the extracellular portion of the integrin α6 subunit (de Curtis and Reichardt, 1993). Cells were subsequently incubated for 30 min with TRITC-conjugated sheep anti-mouse IgG together with FITC-conjugated sheep anti-rabbit IgG (Boehringer Mannheim) and observed using a Zeiss-Axiophot microscope.

RESULTS

Cloning of five Rho family members expressed in chicken embryonic neural retina

Our first aim has been the identification of members of the Rho family of GTPases expressed in developing neurons. For this purpose we have used RT-PCR using two sets of degenerate oligonucleotides to amplify fragments of transcripts of Rho family genes from RNA prepared from developing chick retinas, which have then been used to isolate cDNA clones from λgt10 cDNA libraries.

Fragments of Rho family cDNAs were amplified by PCR from cDNAs prepared from E6 chick neural retina mRNAs. The PCR reactions were performed in the presence of either one of two sets of degenerated oligonucleotides: the oligonucleotides RhoA-1 and RhoA-2 corresponding to the FSKD(Q/E)FP and MKQEPV(K/R) peptides, specific for the human RhoA, B, and C proteins, and the oligonucleotides RhoF-1 and RhoF-2 corresponding to the KTCLLI and Y(L/M/V)ECSA peptides, specific for all known human Rho family members. Restriction analysis and sequencing of ~100 cDNA fragments obtained by PCR identified five different DNA sequences encoding proteins with a high degree of homology to known human Rho family members. The five different PCR fragments were used to screen two λgt10 cDNA libraries, one from E10 chick embryo and one from E13 chick brain. In this way, several λ phage clones were found that contain coding regions corresponding to the five identified chicken Rho family genes. Sequence analysis of the isolated clones (Fig. 1), and the comparison with the sequences of known human Rho proteins (Fig. 2*B,C*), allowed us to identify open reading frames coding for the predicted full-length polypeptides of four of the five genes. Several unsuccessful attempts were made to isolate from two available chick cDNA libraries a full-length clone for a fifth cDNA, for which no 5′ terminal sequence could be found (Fig. 2*A, cRhoC*).

Comparison at the nucleotide and polypeptide levels of the five chicken genes with the human Rho and Rac sequences (Fig. 2*B,C*) revealed that three chicken Rho and two chicken Rac homologs had been isolated. We propose to name the five chicken genes *cRhoA*, *cRhoB*, *cRhoC*, *cRac1A*, and *cRac1B*. At the amino acid level, cRhoA is 100% identical to human RhoA, whereas cRhoB and cRhoC show 97.5% and 95.4% identity, respectively, to their human counterparts. For *cRhoC*, comparison with the respective human gene indicated that the sequence corresponding to the 19 amino terminal amino acid residues is missing. Interestingly, both *cRac1A* and *cRac1B* show the highest degree of identity with the human *Rac1* sequence (88.5% and 80.7%, respectively). Comparison of the polypeptide sequences derived from the chick clones with the human sequences (Fig. 2*C*) confirmed that the cRac1A polypeptide is 100% identical to the human Rac1 protein, whereas the cRac1B polypeptide is 93.7% identical to human Rac1, and only 89% identical to human Rac2. In particular, the C-terminal portion of the cRac1B polypeptide sequence showed a much higher degree of identity for human Rac1 than for human Rac2 (not shown). We propose that *cRac1B* represents a new *Rac* gene.

Developmental regulation of expression of GTPases in the chicken retina

To characterize in more detail the expression of the five identified Rho family transcripts during retinal development, we have analyzed their expression by Northern blot analysis (Fig. 3). Filters with total RNA prepared from neural retinas isolated from dif-

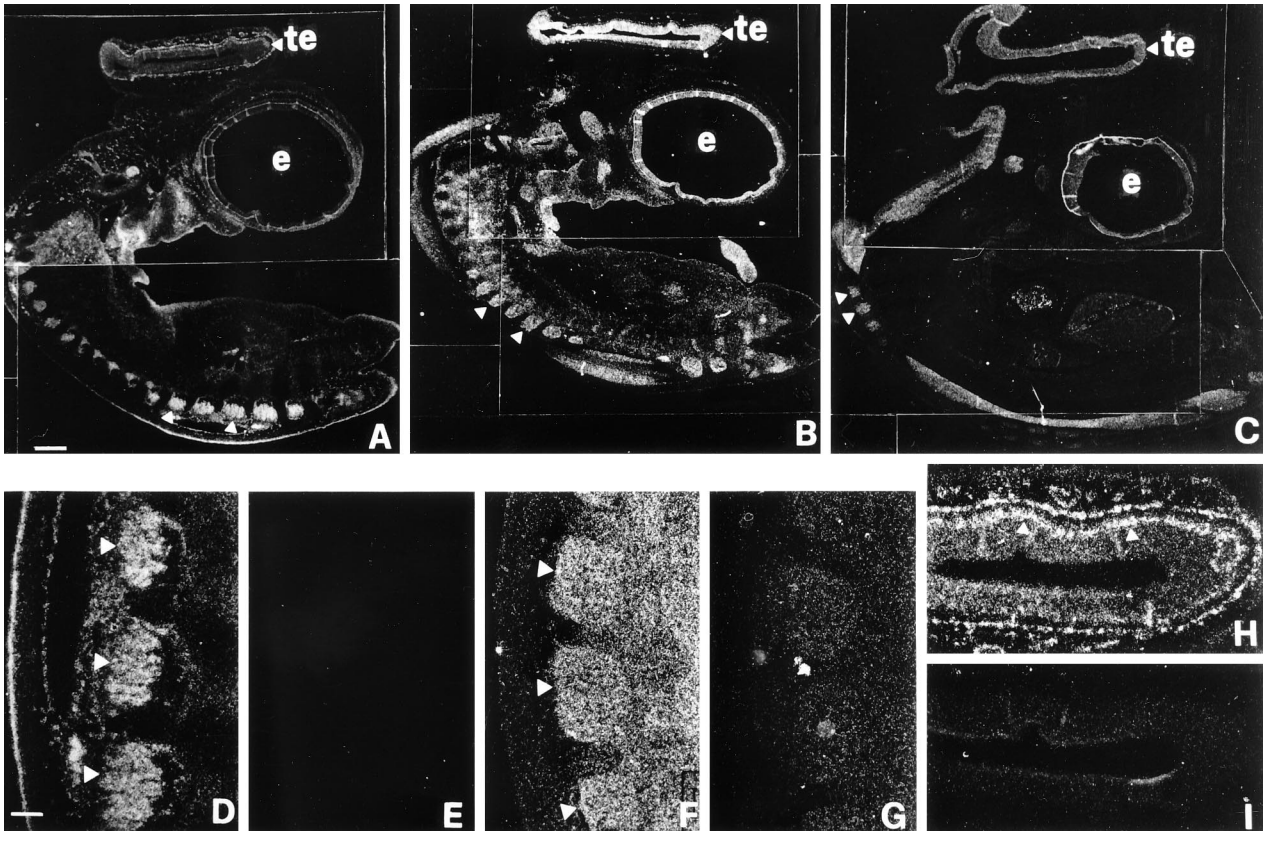

Figure 6. *In situ* hybridization for different GTP-binding proteins of the Rho family in E6.5 chick embryos. Parasagittal sections were incubated with antisense probes for *cRhoB* (*A*), *cRac1A* (*B*), and *cRac1B* (*C*). Differences can be detected in the overall distribution of the mRNA for these three proteins. At this stage, the three different mRNAs were strongly expressed in the developing nervous system. DRGs show high levels of expression of the three mRNAs (*arrowheads*). The expression of *cRhoB* (*D*) and *cRac1A* (*F*) mRNAs in the DRGs is shown at higher magnification. *E* and *G* include similar fields from sections incubated with sense probes for *cRhoB* and *cRac1A*, respectively. In the tectum (*te*), *cRac1A* (*B*) and *cRac1B* (*C*) mRNAs show a homogeneous distribution. In *H*, a higher magnification of the area of the tectum shown in *A* reveals that *cRhoB* mRNA is strongly expressed in an external layer (*arrowheads*) corresponding to presumptive postmitotic neuroepithelial cells. *I* shows the area of the tectum from a control section incubated with a sense probe for *cRhoB*. *e*, Eye. Scale bars: *A–C*, 100 μm; *D–I*, 25 μm.

ferent developmental stages (E6, E8, E10, and E12) were probed with random-primed ^{32}P-labeled cDNAs. Distinct RNA hybridization patterns were obtained with each probe. A single band corresponding to 2.4 and 1.65 kb transcripts was detected for *cRhoB* and *cRac1B*, respectively (Fig. 3*B,E*), whereas two different bands were detected for *cRhoA*, *cRhoC*, and *cRac1A* (Fig. 3, *A, C*, and *D*, respectively). Two different RNA blots were probed for each transcript and quantitated by densitometric scanning (Fig. 4). The data presented in Figure 4 were obtained after normalizing the value for each transcript with the corresponding value obtained after hybridization for the 18 S RNA (not shown). For *cRhoA*, *cRhoC*, and *cRac1A*, quantitations at each developmental stage represent the sum of the two transcripts (Fig. 3*A,C,D*). The results show that the expression of the five transcripts is regulated differently during maturation of the retina. In particular, *cRhoB* and *cRac1B* transcripts were upregulated during retinal development, whereas the other transcripts were downregulated (Fig. 4).

For the detection and quantitation of the *cRhoC* transcripts, the blots had to be exposed for autoradiography 10 times longer compared with those for the other transcripts (Fig. 3*C*), suggesting that this gene is not as abundantly expressed as the other Rho family genes in the developing neural retina; on the other hand, we found that *cRhoC* was highly expressed in non-neuronal chick cells (data not shown).

Expression of *cRac1B* mRNA in neurons and glia from developing neural retina

To check whether the newly identified, neural-specific cRac1B GTP-binding protein was present also in non-neuronal cells of the CNS, we prepared cultures enriched either in neurons or in glial cells from E7 neural retinas, as described in Materials and Methods. Northern blot analysis from gels loaded with the same amount of total RNA isolated from the two different cell preparations showed that similar amounts of the 1.65 kb *cRac1B* transcript were present in glial cells and neurons at this stage of development (Fig. 5, *lanes 1* and *2*, respectively).

Differential distribution of Rho proteins in developing chicken embryos

The differential expression of the identified Rho family transcripts during retinal development encouraged us to further characterize their expression in the developing chick nervous system by *in situ* hybridization. Because *cRhoC* was poorly expressed in the neural retina compared with the other four genes, we limited the distribution studies to the four abundantly expressed *cRhoA, cRhoB, cRac1A,* and *cRac1B* genes. Sections obtained from E6.5 and E8.5 chicken embryos were analyzed. The overall pattern of expression at E6.5 showed clear differences among the transcripts (Fig. 6). Those for *cRhoA* (not shown) and *cRac1A* (Fig. 6*B*) were quite homogeneously distributed throughout the em-

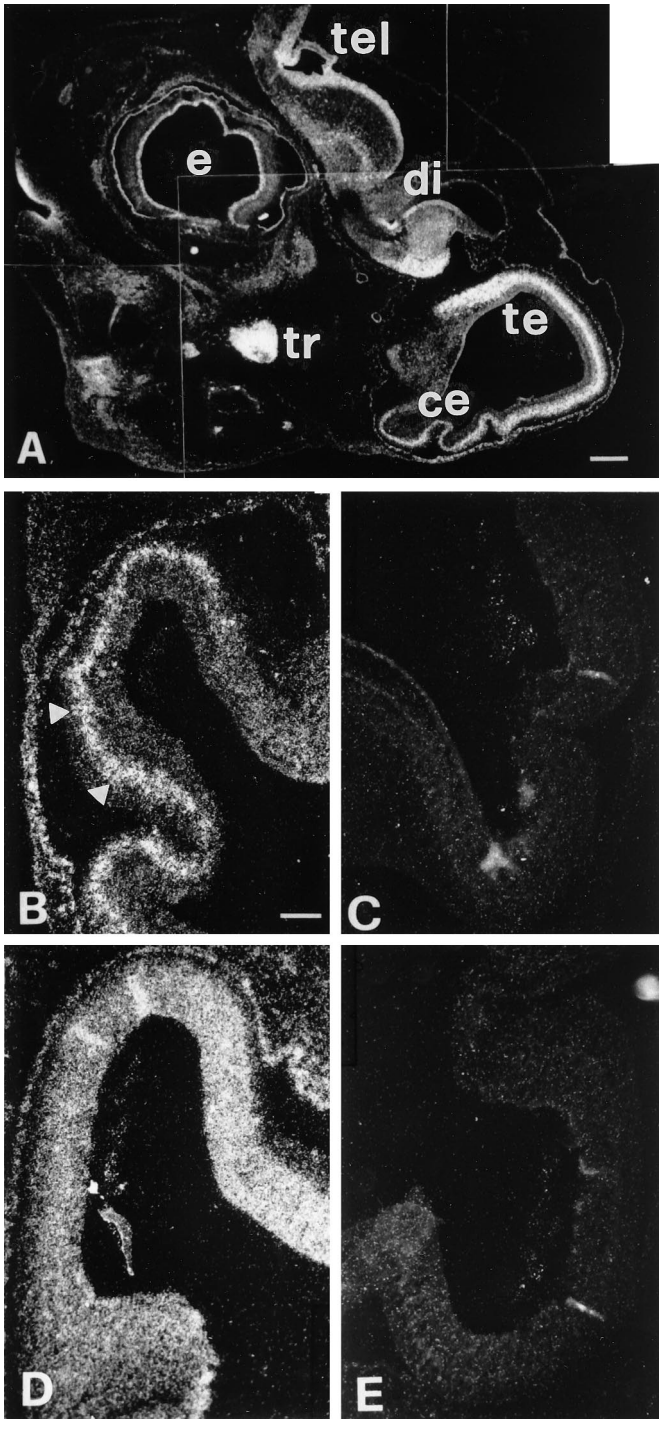

Figure 7. *In situ* hybridization for different GTP-binding proteins of the Rho family in E8.5 chick embryos. *A*, *In situ* hybridization of *cRhoB* mRNA in parasagittal sections from E8.5 chick embryo head. Several structures of the developing nervous system show strong expression of *cRhoB*, including the retina in the eye (*e*), the telencephalon (*tel*), the diencephalon (*di*), the tectum (*te*), the cerebellum (*ce*), and the trigeminal ganglion (*tr*). *B*, Higher magnification of the developing cerebellum shows stronger expression of *cRhoB* mRNA in the presumptive developing Purkinje cell layer (*arrowheads*). *D*, The developing cerebellum from a section similar to the one shown in *B* shows a homogeneous expression of *cRhoA* mRNA. In *C* and *E*, sections incubated with sense probes for *cRhoB* and *cRhoA*, respectively, are shown as controls. Scale bars: *A*, 100 μm; *B–E*, 25 μm.

bryo, whereas the distribution of *cRhoB* (Fig. 6*A*) and *cRac1B* (Fig. 6*C*) transcripts was more restricted. In particular, *cRac1B* transcript seemed concentrated in the developing nervous system, including the retina, the tectum, the spinal cord, the dorsal root ganglia (DRGs), and the trigeminal ganglion (Fig. 6*C*). The same structures were labeled also by *cRhoB*, *cRac1A* (Fig. 6, *A* and *B*, respectively), and *cRhoA* (not shown) antisense probes. At higher magnification, DRGs labeling for *cRhoB* (Fig. 6*D*) appeared concentrated on the more dorsal half of the structures, quite different from the distributions of *cRac1A* (Fig. 6*F*), *cRac1B*, and *cRhoA* (not shown), which were homogeneous throughout the ganglia. Differences were also observed in the labeling of the developing tectum. In fact, although the overall distribution of the *cRac1A* and *cRac1B* transcripts in this structure appeared homogeneous (Fig. 6, *B* and *C*, respectively), at higher magnification *cRhoB* staining was stronger in the external layer, presumably corresponding to postmitotic neurons derived from the neuroepithelium (Fig. 6*H*).

Similar to what we observed at E6.5, in E8.5 embryos the patterns of distribution of the transcripts for *cRhoA* and *cRac1A* were more homogeneous than those for *cRhoB* and *cRac1B* (not shown). In contrast, the *cRac1B* transcript was highly concentrated in the developing nervous system (not shown), whereas the distribution of *cRhoB*, although somewhat more widespread in comparison with *cRac1B*, showed several interesting features within different structures of the developing nervous system. Figure 7*A* shows a low-power magnification of a parasagittal section of an E8.5 chicken head, incubated with an antisense probe for *cRhoB*. Several structures of the developing nervous system, including a very bright area below the eye corresponding to the trigeminal ganglion, express high levels of the transcript. Higher magnification of the developing cerebellum at E8.5 showed that *cRhoB* was highly concentrated in the presumptive Purkinje cell layer (*arrowheads*, Fig. 7*B*), whereas *cRhoA* was distributed homogeneously throughout the entire region (Fig. 7*D*). The distribution of both *cRac1A* and *cRac1B* transcripts was similar to that of *cRhoA*, although the signal was not as strong (not shown). Higher magnification of the eye region showed a clear concentration of the *cRhoB* transcript in the retinal ganglion cell (RGC) layer of E8.5 retinas (*arrows*, Fig. 8*C*). In contrast, *cRhoA* was distributed homogeneously within the neural retina (Fig. 8*A*). The distribution of *cRac1B* was similar to that of *cRhoB*, although weaker (not shown), whereas the expression pattern of *cRac1A* was similar to that of *cRhoA* (not shown). At E6.5, the distribution of the different transcripts in the neural retina was similar to that observed in E8.5 embryos, although the concentration of the *cRhoB* transcript in the RGC layer was not as distinct, probably because of the presence of a less defined ganglion cell layer at this stage (not shown).

The distribution of the transcripts in the spinal cord was analyzed in transversal/oblique sections from E6.5 and E8.5 embryos. Interestingly, three different patterns were revealed by *in situ* analysis. At E6.5, the expression of *cRac1B* was quite homogeneous throughout the section of the spinal chord (Fig. 9*D*). In contrast, *cRhoA* and *cRac1A* (Fig. 9, *A* and *C*, respectively) were concentrated around the ventricular zone, where proliferation is occurring, and in the ventral area of the spinal chord, where motor neurons are located. Finally, a third pattern was observed for *cRhoB* (Fig. 9*B*), which was highly expressed in the ventral portion of the spinal cord, including the floor plate and the area with motor neurons. At this stage, DRGs visible on the side of the spinal cord were positive for all four tested GTP-binding protein

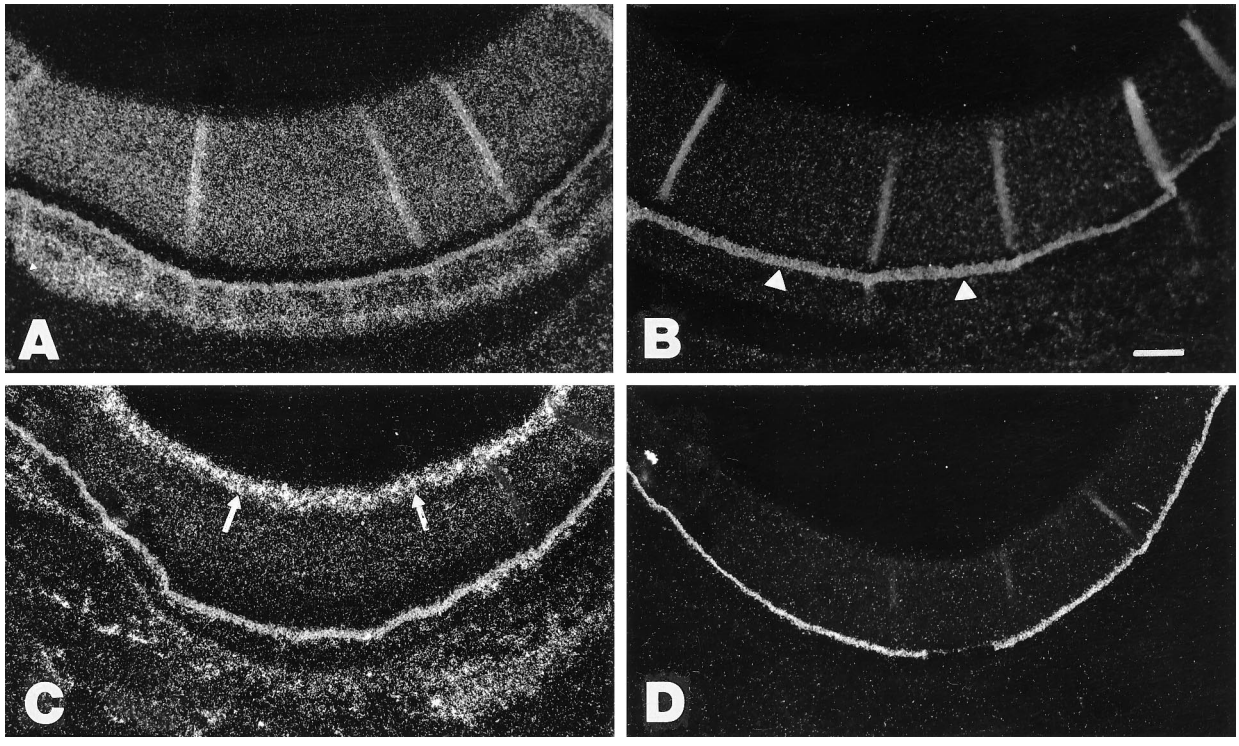

Figure 8. Expression of Rho GTPases in the developing chick retina. Expression of *cRhoA* (*A, B*) and *cRhoB* (*C, D*) mRNAs in the developing chick retina. Antisense (*A, C*) and sense (*B, D*) probes obtained from the respective cDNAs were incubated with sections from E8.5 chick embryos. Diffuse staining of the neural retina is observed for *cRhoA* (*A*), whereas stronger labeling is observed in the RGC layer (*arrows*) for *cRhoB* (*C*). The nonspecific signal given by the retinal pigmented epithelium is indicated by *arrowheads* in *B*. Scale bar, 25 μm.

mRNAs. At E8.5 the pattern of distribution of *cRhoA* and *cRac1A* was similar to that observed at E6.5, although the differences in the intensity of the signal among distinct areas were not as clear (not shown); a stronger, still homogeneous signal was found for *cRac1B* (Fig. 9*F*), whereas the distribution of the *cRhoB* transcript seemed more restricted than in E6.5 spinal cord and was localized to the motor neuron region and the floor plate (Fig. 9*E*).

Distribution of the cRac1B polypeptide in retinal neurons

The distribution of the cRac1B protein in retinal neurons was studied by expressing an epitope-tagged form of the protein. Cultured primary retinal neurons were transiently transfected with the pcDNA-I-HA-Rac1B vector containing the sequence encoding for an HA-tagged cRac1B protein. We obtained the best transfection efficiencies by using PEI 50 kDa on E6 retinal cells that had been cultured for ~12 hr before treatment. Immunofluorescence with an anti-HA antibody showed that cRac1B was homogeneously distributed in retinal cells (Fig. 10). In particular, cRac1B was also visible along neurites and in all their protrusions. Double immunofluorescence staining was used to identify transfected neurons expressing a 200 kDa neurofilament polypeptide (Fig. 10*A,B*). Neurofilament-negative cells expressing the HA-Rac1B construct were also present in culture (not shown). The distribution of the epitope-tagged cRac1B in neurons showed a pattern similar to that of the integrin α6 subunit, which is expressed on the surface of these cells (Fig. 10*C,D*), suggesting a possible association of the Rac1B polypeptide with the plasma membrane.

DISCUSSION

Five major conclusions can be drawn from the data presented in this paper. First, developing neural retinal cells express mRNAs coding for at least five components of the Rho family of GTPases: three coding for Rho proteins and two coding for Rac proteins. Second, the comparison of the cDNAs with the human homologs indicates that one of the Rac proteins represents a novel *Rac* gene. Third, the levels of expression of the five transcripts are differentially regulated during the development of the retina. Fourth, four of the identified transcripts show distinct patterns of distribution in developing chick embryos, with particularly high levels of expression of all the transcripts, and prominent localization of the newly identified *cRac1B* gene in the developing nervous system. Finally, the expression of an epitope-tagged form of cRac1B in primary retinal neurons reveals a homogeneous distribution of the polypeptide in the cell body and along neurites. These results demonstrate for the first time that Rho family GTPases are highly expressed in the developing CNS and peripheral nervous system, suggesting that these GTPases play an important role during the development of the vertebrate nervous system.

Several extracellular cues, including extracellular matrix glycoproteins, can induce dramatic morphological changes in the developing neurons, which result in the formation of neurites (Sanes, 1989; de Curtis, 1991; Reichardt and Tomaselli, 1991). We have shown previously that the dramatic effects of laminin on neurite extension from retinal neurons in culture are mediated by the α6β1 integrin laminin receptor (de Curtis and Reichardt, 1993). The molecular mechanisms underlying these processes remain poorly understood. Recent studies in non-neuronal cells

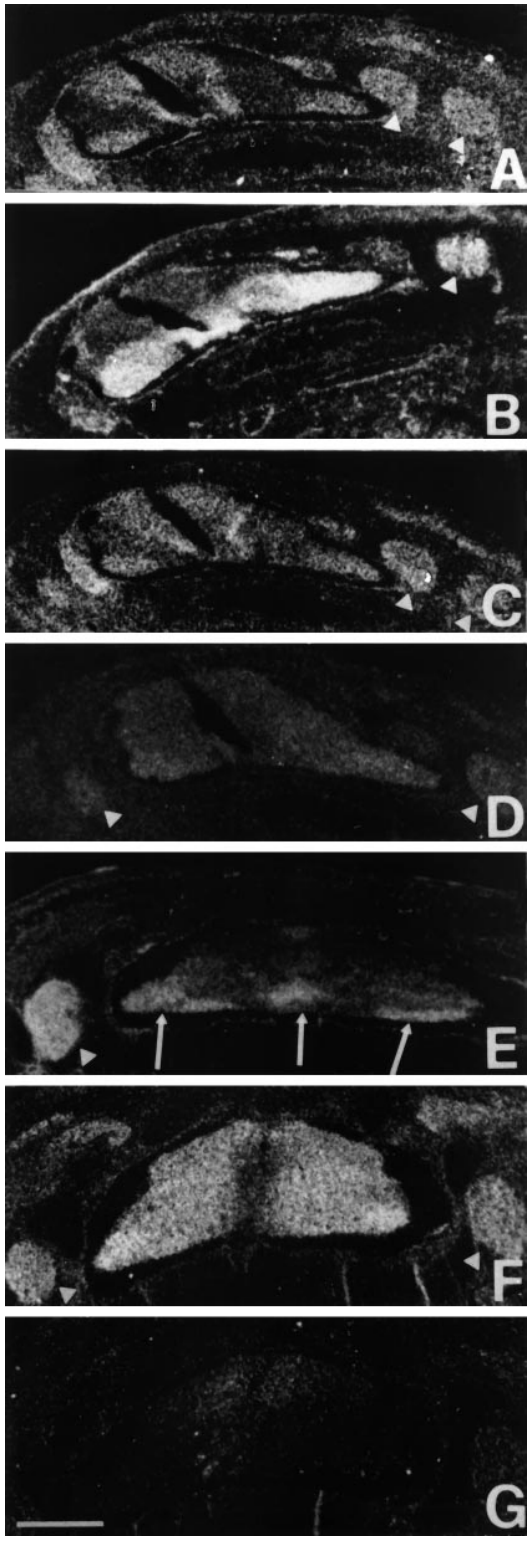

Figure 9. Expression of Rho GTPases in the developing spinal cord. Sections including the spinal cord of E6.5 (*A–D*) and E8.5 (*E–G*) chick embryos were incubated with antisense probes for *cRhoA* (*A*), *cRhoB* (*B, E*), *cRac1A* (*C*), and *cRac1B* (*D, F*), and with a sense probe for *cRac1B* (*G*) as a control. Different patterns of expression can be observed for the different mRNAs. DRGs (*arrowheads*) can be observed on the sides of the spinal cord. In *A–D* the more oblique sections included two DRGs on one side of the spinal cord. In *E*, *arrows* indicate the localization of *cRhoB* transcript in E8.5 spinal cord, mainly restricted to the motor neuron regions and to the floor plate (*central arrow*). Scale bar, 100 μm.

have shown that Rho family GTPases regulate the formation of actin-based structures such as filopodia, lamellipodia, and stress fibers (Nobes and Hall, 1995). Although stress fibers are not found in growth cones, filopodia and lamellipodia are actin-dependent processes also involved in growth cone navigation. Moreover, recent data postulate the involvement of Rho family GTPases in the regulation of actin-mediated growth cone migration (Jalink et al., 1994; Postma et al., 1996). In addition to their role in cytoskeletal reorganization, Rho family GTPases have been involved in the regulation of the activity of transcription factors and in membrane traffic (Ridley, 1996). Furthermore, a number of possible effectors for these GTPases have been identified recently (for review, see Ridley, 1996).

With the aim of studying the role of these GTPases during the development of the neuronal phenotype, we have looked for cDNA clones of Rho family GTPases expressed in primary neurons. We have used E6 retinas as the source of mRNA for this study, because this is the stage at which cultured retinal neurons respond to laminin by extending neurites. Three of the identified small GTP-binding proteins expressed by neural retinal cells correspond to the chick homologs of the already known human *RhoA*, *RhoB*, and *RhoC* genes (Madaule and Axel, 1985). Two other cDNAs were related to *Rac* genes and predicted two different Rac proteins, one showing complete identity (cRac1A) and the other showing a high degree of identity (cRac1B) to human Rac1. We think that the cRac1B protein does not correspond to the chick homolog of human Rac2, because cRac1B shows a higher degree of identity to the human Rac1 than to human Rac2 at both the nucleotide and protein level. Furthermore, although the *Rac1* gene is known to be expressed in various tissues and cell lines, the expression of *Rac2* is restricted to cells of the hemopoietic lineage (Didsbury et al., 1989; Shirsat et al., 1990; Moll et al., 1991).

The temporal expression of the transcripts coding for the different identified chicken GTPases during the development of the neural retina has been investigated. Our data show that the five transcripts are differentially regulated between E6 and E12; in fact, although the expression of *cRhoA* decreased after E8 and that of *cRac1A* and *cRhoC* decreased after E10, the expression of *cRac1B* and *cRhoB* showed an increase between E6 and E10 and a decrease afterward. Between E6 and E12, neural retinal cells migrate and organize into the different layers that are recognizable in the mature retina. Furthermore, the RGCs, which form at E6 the only clearly identifiable neuronal layer of the retina, do actively extend their axons toward their target, the optic tectum. The first axons of the RGC layer reach the optic tectum at E6, and by E12 all of them have reached the target. Extracellular matrix components are expressed along virtually the entire embryonic retinotectal pathway (McLoon, 1984; Adler et al., 1985; Cohen et al., 1987; Halfter and Fua, 1987; McLoon et al., 1988; Bartsch et al., 1995). In the optic stalk, laminin expression is transient and correlates with the ability of RGCs to use laminin as a substrate (Cohen et al., 1987, 1989). Similarly, expression of tenascin in the tectum at the time of innervation by RGC axons has been correlated with the capacity of these neurons to extend neurites on tenascin in culture (Bartsch et al., 1995). Interestingly, *in situ* hybridization revealed accumulation of *cRhoB* transcript in the RGC layer that was particularly evident at E8.5, and also of *cRac1B*, although the accumulation was less dramatic, whereas *cRhoA* and *cRac1A* were homogeneously distributed in the whole neural retina. Moreover, the

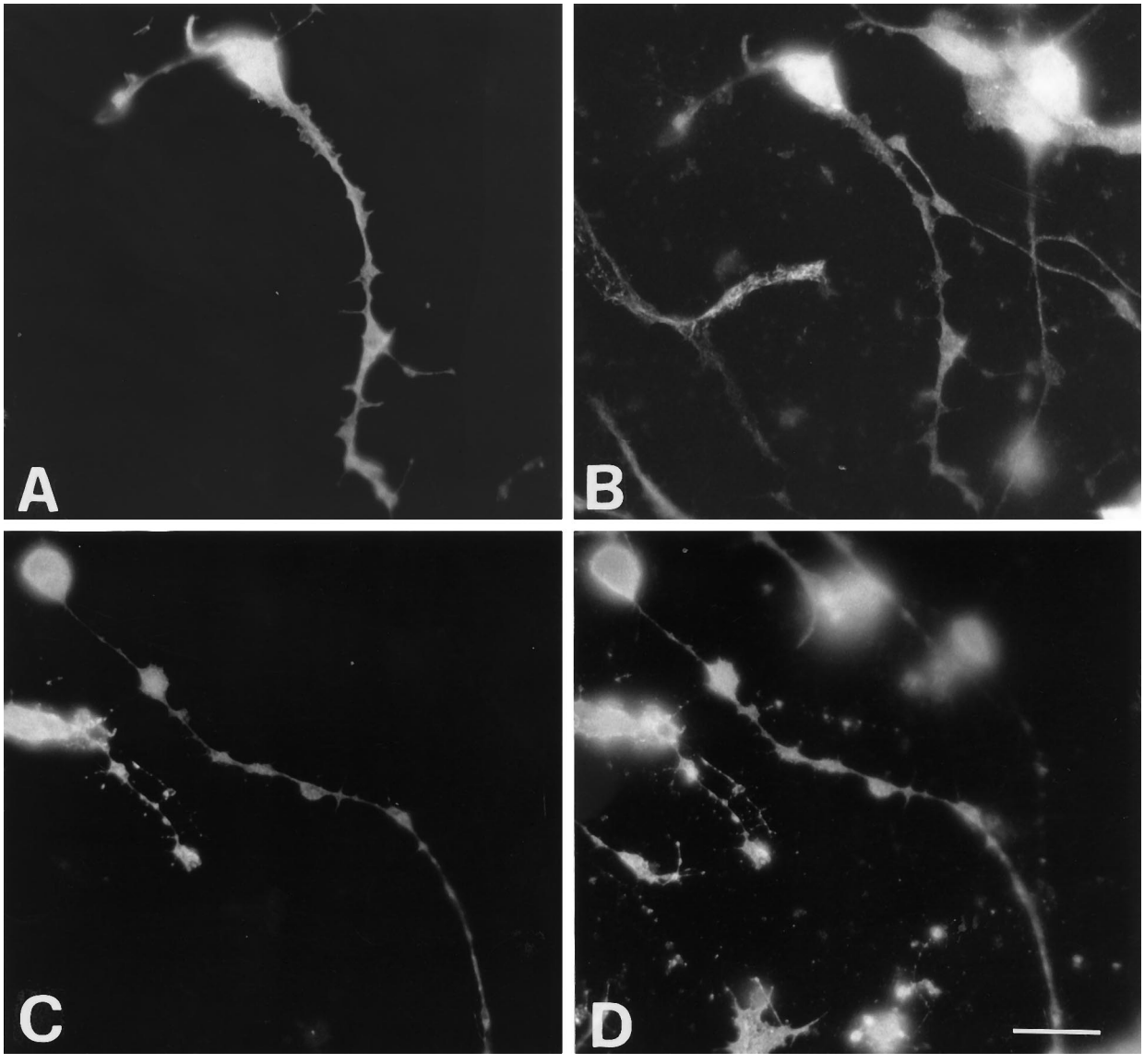

Figure 10. Distribution of cRac1B in cultured retinal neurons. Retinal neurons grown on laminin were transfected with the pcDNA-I-HA-Rac1B plasmid as described in Materials and Methods, and the cells were analyzed by immunofluorescence 24 hr after transfection. In *A* and *B*, cells were fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100. In *C* and *D*, cells were fixed and permeabilized with cold (−20°C) methanol. Primary antibodies were monoclonal antibody against HA (*A, C*), polyclonal antibody against 200 kDa neurofilament protein (*B*), and polyclonal antibody against the integrin α6 subunit (*D*). Same fields are represented in *A* and *B* and in *C* and *D*). Scale bar, 10 μm.

expression of all studied Rho family GTPases was decreased by E12, when retinal layers have formed and all RGC axons have reached the optic tectum. Because Rho and Rac proteins have been implicated in the organization of the actin cytoskeleton, one hypothesis is that the observed expression of these proteins in RGCs may be required for the process of neuritogenesis, which occurs at this time of development.

Another aim of this study was the analysis of the distribution of the identified GTPases in the developing chick embryo. A striking result from this study is the observation that *cRhoA*, *cRhoB*, *cRac1A*, and *cRac1B* are strongly expressed in the developing nervous system. In fact, *in situ* hybridization on sections from E6.5 and E8.5 embryos showed strong labeling of both CNS and peripheral nervous system. At both stages, DRGs showed high levels of expression of the transcripts, and a more detailed analysis showed differences in the pattern of expression that were particularly evident for *cRhoB* and *cRac1A*. The observed strong *cRhoB* expression in trigeminal ganglia may be correlated with the innervation of the target by the axons of the trigeminal sensory neurons that is actively occurring at this stage (Windle and Austin, 1936; Moody et al., 1989). Clear differences were detected in the localization of the different GTPase transcripts within the spinal cord. The specific localization of *cRhoB* transcripts in layers of the developing CNS, in contrast to the homogeneous distribution of the transcripts of other GTPases within the same structures, also suggests specific and different functions of distinct members of the Rho family during neuronal development. Such a conclusion is corroborated by recent studies in invertebrates that have shown *Caenorhabditis elegans* RhoA to be expressed at highest levels during embryogenesis and particularly enriched in the pharyngeal nerve ring and at the tip of the head

containing chemosensory and mechanosensory neurons (Chen and Lim, 1994). Furthermore, the *Drosophila DRac1* and *DCdc42*, are also highly expressed in the nervous system, where they are involved in axonal outgrowth (Luo et al., 1994). Interestingly, we found that all four genes analyzed in this paper are expressed in the chicken developing cerebellum and that *cRhoB* is concentrated in the presumptive Purkinje cell layer. This might correlate with the recent observation that perturbation of Rac1 activity in mice Purkinje cells leads to modifications of the axonal and dendritic structures of these cells (Luo et al., 1996).

Expression of an epitope-tagged cRac1B has allowed the analysis of the distribution of this new neural-specific Rac in primary neurons. The cellular localization of cRac1B is similar to that of the integrin $\alpha 6$ subunit, a known plasma membrane component, suggesting a possible association of cRac1B with the plasma membrane of neurons, although further work is required to prove association of this protein with the plasma membrane. Like the other members of the family, cRac1B has a C-terminal motif that can be isoprenylated and could account for its possible association to the plasma membrane. In particular, cRac1B is uniformly expressed along actin-rich neurites and their protrusions. This localization could correspond to a prerequisite for the rapid reorganization of the actin cytoskeleton during filopodia extension, a process required for neurite extension or neurite branching, and future work will be aimed at exploring this issue.

In conclusion, the results presented in this paper have shown for the first time that various members of the Rho family of small GTP-binding proteins are differentially and specifically expressed in the CNS and peripheral nervous system of chicken embryos in concomitance with complex events of neuronal differentiation. In view of the widely accepted role of these proteins in multiple aspects of cell physiology, these observations strongly support an important role for Rho family GTPases in the acquisition of the mature neuronal phenotype.

REFERENCES

Adler R, Jerdan J, Hewitt AT (1985) Responses of cultured neural retinal cells to substratum-bound laminin and other extracellular matrix molecules. Dev Biol 112:100–114.

Bartsch S, Husmann K, Schachner M, Bartsch U (1995) The extracellular matrix molecule tenascin: expression in the developing retinotectal system and substrate properties for retinal ganglion cell neurites in vitro. Eur J Neurosci 7:907–916.

Biscardi JS, Cooper NGF, Maness PF (1993) Phosphotyrosine-modified proteins are localized in Müller cells of the chick neural retina. Exp Eye Res 56:281–289.

Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA 92:7297–7301.

Cattelino A, Longhi R, de Curtis I (1995) Differential distribution of two cytoplasmic variants of the $\alpha 6\beta 1$ integrin laminin receptor in the ventral plasma membrane of embryonic fibroblasts. J Cell Sci 108:3067–3078.

Chen W, Lim L (1994) The *Caenorhabditis elegans* small GTP-binding protein RhoA is enriched in the nerve ring and sensory neurons during larval development. J Biol Chem 269:32394–32404.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995.

Cohen J, Burne JF, McKinlay C, Winter J (1987) The role of laminin and the laminin/fibronectin receptor complex in the outgrowth of retinal ganglion cell axons. Dev Biol 122:407–418.

Cohen J, Nurcombe V, Jeffrey P, Edgar D (1989) Developmental loss of functional laminin receptors on retinal ganglion cells is regulated by their target tissue, the optic tectum. Development 107:381–387.

de Curtis I (1991) Neuronal interactions with extracellular matrix. Curr Opin Cell Biol 3:824–831.

de Curtis I, Reichardt LF (1993) Function and spatial distribution in developing chick retina of the laminin receptor $\alpha 6\beta 1$ and its isoforms. Development 118:377–388.

de Curtis I, Quaranta V, Tamura RN, Reichardt LF (1991) Laminin receptors in the retina: sequence analysis of the chick integrin $\alpha 6$ subunit. J Cell Biol 113:405–416.

Didsbury J, Weber RF, Bokoch GM, Evans T, Snyderman R (1989) Rac, a novel ras-related family of proteins that are botulinum toxin substrates. J Biol Chem 264:16378–16382.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008.

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specificity activity. Anal Biochem 132:6–13.

Halfter W, Fua CS (1987) Immunohistochemical localization of laminin, neural cell adhesion molecule, collagen type IV and T-61 antigen in the embryonic retina of the japanese quail by in vivo injection of antibodies. Cell Tissue Res 249:487–496.

Hall A (1994) Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu Rev Cell Biol 10:31–54.

Jalink K, van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenar WH (1994) Inhibition of lysophosphatidate- and trombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. J Cell Biol 126:801–810.

Lehrach H, Diamond D, Wozney JM, Boedtker H (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions: a critical reexamination. Biochemistry 16:4743–4751.

Luo L, Liao YJ, Jan LY, Jan YN (1994) Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev 8:1787–1802.

Luo L, Hensch TK, Ackerman L, Barbel S, Jan LY, Jan YN (1996) Differential effects of Rac GTPase on Purkinje cell axons and dendritic trunks and spines. Nature 379:837–840.

Mackay FJG, Nobes CD, Hall A (1995) The Rho's progress: a potential role during neuritogenesis for the Rho family of GTPases. Trends Neurosci 18:496–501.

Madaule P, Axel R (1985) A novel ras-related gene family. Cell 41:31–40.

Malosio ML, Grenningloh G, Kuhse J, Schmieden V, Schmitt B, Prior P, Betz H (1991) Alternative splicing generates two variants of the α 1 subunit of the inhibitory glycine receptor. J Biol Chem 266:2048–2053.

Marchuk D, Drumm M, Saulino A, Collins FS (1991) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res 19:1154.

McLoon SC (1984) Development of the retinotectal projection in chicks. In: Organizing principles of neural development (Sharma SC, ed), pp 325–342. New York: Plenum.

McLoon SC, McLoon LK, Palm SL, Furcht LT (1988) Transient expression of laminin in the optic nerve of the developing rat. J Neurosci 8:1981–1990.

Moll J, Sansig G, Fattori E, van der Putten H (1991) The murine rac1 gene: cDNA cloning, tissue distribution and regulated expression of rac1 mRNA by disassembly of actin microfilaments. Oncogene 6:863–866.

Moody SA, Quigg MS, Frankfurter A (1989) Development of the peripheral trigeminal system in the chick revealed by an isotype-specific anti-beta-tubulin monoclonal antibody. J Comp Neurol 279:567–580.

Nobes CD, Hall A (1995) Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81:53–62.

Postma FR, Jalink K, Hengeveld T, Moolenar WH (1996) Sphingosine-1-phosphate rapidly induces Rho-dependent neurite retraction: action through a specific cell surface receptor. EMBO J 15:2388–2395.

Reichardt LF, Tomaselli KJ (1991) Extracellular matrix molecules and their receptors: functions in neural development. Annu Rev Neurosci 14:531–570.

Ridley AJ (1996) Rho: theme and variations. Curr Biol 6:1256–1264.
Ridley AJ, Hall A (1992) The small GTP-binding protein Rho regulates the assembly of focal adhesion and actin stress fibers in response to growth factors. Cell 70:389–399.
Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70:401–410.
Rugarli EI, Lutz B, Kuratani SC, Wawersik S, Borsani G, Ballabio A, Eichele G (1993) Expression pattern of the Kallmann syndrome gene in the olfactory system suggests a role in neuronal targeting. Nat Genet 4:19–26.
Sanes JR (1989) Extracellular matrix molecules that influence neural development. Annu Rev Neurosci 12:491–516.
Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.
Shirsat NV, Pignolo RJ, Kreider BL, Rovera G (1990) A member of the ras gene superfamily is specifically expressed in T, B and myeloid hemopoietic cells. Oncogene 5:769–772.
Tanaka E, Sabry J (1995) Making the connection: cytoskeletal rearrangements during growth cone guidance. Cell 83:171–176.
Timpl R, Rhode H, Gehron-Robey P, Rennard S, Foidart J-M, Martin G (1979) Laminin, a glycoprotein from basement membrane. J Biol Chem 254:9933–9937.
Windle WF, Austin AM (1936) Neurofibrillar development in the central nervous system of chick embryos up to 5 days' incubation. J Comp Neurol 63:431–463.