

# Overexpression of a Neural-specific Rho Family GTPase, cRac1B, Selectively Induces Enhanced Neuritogenesis and Neurite Branching in Primary Neurons

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**Abstract.** Rho family GTPases have been implicated in cytoskeletal reorganization during neuritogenesis. We have recently identified a new gene of this family, *cRac1B*, specifically expressed in the chicken developing nervous system. This GTPase was overexpressed in primary neurons to study the role of cRac1B in the development of the neuronal phenotype. Overexpression of cRac1B induced an increment in the number of neurites per neuron, and dramatically increased neurite branching, whereas overexpression of the highly related and ubiquitous cRac1A GTPase did not evidently affect neuronal morphology. Furthermore, expression of an inactive form of cRac1B strikingly inhibited neurite formation. The specificity of cRac1B action observed in neurons was not observed in fibroblasts, where both GTPases produced similar effects on cell

morphology and actin organization, indicating the existence of a cell type-dependent specificity of cRac1B function. Molecular dissection of cRac1B function by analysis of the effects of chimeric cRac1A/cRac1B proteins showed that the COOH-terminal portion of cRac1B is essential to induce increased neuritogenesis and neurite branching. Considering the distinctive regulation of cRac1B expression during neural development, our data strongly support an important role of cRac1B during neuritogenesis, and they uncover new mechanisms underlying the functional specificity of distinct Rho family GTPases.

**Key words:** small GTPases • neurites • cytoskeleton • actin • development

**N**EURITOGENESIS is a central event during neuronal development, and it 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). Therefore, the behavior of growth cones can be compared with that of the leading edge of spreading or migrating fibroblasts, where the dynamic adhesive interactions with the substrate are accompanied by a continuous reorganization of the actin cytoskeleton.

In cells, the organization of the actin cytoskeleton is regulated by several actin-binding proteins that contribute to its dynamic properties. Rho family GTPases belonging to the Ras superfamily of small GTPases have been shown to contribute to the organization of the actin cytoskeleton and of the associated sites of cell adhesion to the extracel-

lular matrix (Hall, 1994). Recently, evidence has accumulated for a role of Rho proteins in neuronal development and in the regulation of neuritogenesis (Luo et al., 1997). In N1E-115 neuroblastoma cells, the regulation of Rho by activating (e.g., lysophosphatidic acid) or inhibitory (*Clostridium botulinum* C3 exoenzyme) factors can affect growth cone behavior (Jalink et al., 1994; Postma et al., 1996). Furthermore, analysis in *Drosophila* and *Caenorhabditis elegans* have shown that Rho family GTPases regulate neuritogenesis and axonal guidance in these organisms (Luo et al., 1994; Zipkin et al., 1997), while perturbation of Rac1 activity in mice Purkinje cells leads to modifications of the axonal and dendritic structures of these cells (Luo et al., 1996). More recently, Rac1 has been implicated in the regulation of growth cone behavior (Lamoureux et al., 1997), in collapsin-1-induced growth cone collapse (Jin et al., 1997), and in the regulation of dendritic growth (Threadgill et al., 1997).

The hypothesis that Rho GTPases play an important role during vertebrate neuronal development has been reinforced by our recent finding that four members of the family are highly expressed in the chick embryonic ner-

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vous system (Malosio et al., 1997). Interestingly, one of these GTPases is the newly identified cRac1B, which is highly homologous to the chicken Rac1 (that we will refer to as cRac1A in this paper), and whose expression in the developing nervous system is specific.

With the aim of investigating the hypothesized role of the neural-specific cRac1B protein in the development of the neuronal phenotype, functionally active and inactive forms of the GTPases have now been expressed in neuronal cells. The data presented in this paper show that cRac1B has specific effects on neuritogenesis, since it increases the number of neurites per cell, and dramatically increases neurite branching in primary retinal neurons cultured on laminin. These effects are not observed upon cRac1A overexpression. Expression studies in non-neuronal cells show that the specificity of cRac1B-induced cytoskeletal rearrangements is lost in chicken embryo fibroblasts (CEFs),<sup>1</sup> where both GTPases induce dramatic changes in cell shape. Moreover, expression of cRac1A/cRac1B chimeras has allowed us to identify the COOH-terminal portion of the cRac1B polypeptide as the region sufficient to induce the specific effects observed in neurons. Our data indicate that cRac1B plays an important role in the maturation of the neuronal phenotype, and identify a region of the GTPase essential to confer functional specificity.

## Materials and Methods

### Reagents

Fertilized chicken eggs were purchased from Allevamento Giovenzano (Vellezzo Bellini, Italy). Taq polymerase was from Promega Corp. (Madison, WI), Klenow fragment of DNA polymerase was from Pharmacia Biotech Sevrage (Uppsala, Sweden), and restriction enzymes were from Boehringer Mannheim GmbH (Mannheim, Germany).  $\alpha$ -<sup>32</sup>S-labeled dATP, [ $\alpha$ -<sup>32</sup>P]dCTP, and <sup>125</sup>I-protein A were from Amersham Intl. (Buckinghamshire, United Kingdom). Other chemicals were purchased from Sigma-Aldrich (Milan, Italy). Laminin was purified from Engelbreth-Holm Swarm sarcoma as published (Timpl et al., 1979).

### Cell Culture

Neural retinal cells were prepared from embryonic day 6 (E6) chick retinas. Neural retinas were dissected and trypsinized, and cultures of retinal neurons were obtained under serum-free conditions on substrates coated with poly-D-lysine and laminin-1 as described (de Curtis et al., 1991; de Curtis and Malanchini, 1997). After 18 h in culture, neuronal cells were used for transfection experiments, as described below. CEFs were isolated from E10 embryos and cultured at 37°C, 5% CO<sub>2</sub> in DME containing 5% FCS, 1% chicken serum, 100 U/ml penicillin and streptomycin, 2 mM glutamine. CEFs up to the fifth passage were used for transfections.

### Northern Blot Analysis

Total RNA was prepared from E4, E6, E8, E10, E12, E15, E18, and adult chicken brains, or from different organs from E10 chick embryos, by a single-step RNA isolation method (Chomczynski and Sacchi, 1987). Northern blot analysis of total RNA (20  $\mu$ g/lane) was performed as previously described (Lehrach et al., 1977). Hybridization took place in hybridization buffer supplemented with <sup>32</sup>P-labeled probes (0.5–1  $\times$  10<sup>6</sup> cpm/ml) for 12–16 h at 65°C. After high stringency washes (twice with SSC at 65°C), x-ray films were exposed for 1–3 d to the hybridized filters.

1. *Abbreviations used in this paper:* CEF, chicken embryo fibroblast; E6, embryonic day 6; F-actin, filamentous actin; GST, glutathione-S-transferase; PEI, polyethylenimine; RGM, retinal growth medium.

## Antibodies

The polyclonal antibody specific for the cRac1B GTPase was obtained by injecting rabbits with a peptide corresponding to the COOH-terminal portion of this GTPase (peptide CPPPVKKPGKKCTVF) conjugated to key-hole limpet hemocyanin. The anti-Flag-M5 mAb recognizing the FLAG peptide sequence MDYKDDDDK was from Kodak (New Haven, CT); the anti- $\beta$ -galactosidase mAb was from Boehringer Mannheim GmbH.

## Production of Glutathione-S-Transferase (GST) Fusion Proteins for Rho Family GTPases

Full-length cDNAs coding for chicken cRac1A, cRac1B, cRhoA, and cRhoB polypeptides were obtained by PCR from the corresponding clones in pBluescript KS<sup>-</sup> vector (Malosio et al., 1997), and subcloned into the pGEX-4T-1 vector (Pharmacia Biotech, Uppsala, Sweden). The resulting pGEX-cRac1A, pGEX-cRac1B, pGEX-cRhoA, and pGEX-cRhoB plasmids were used to obtain GST fusion proteins by expression into *Escherichia coli* BL21 cells. After induction of the expression with isopropyl- $\beta$ -D-thiogalactopyranoside (Sigma-Aldrich) for 18 h at 23°C, cells were resuspended in PBS, and then lysed by sonication. Lysates were centrifuged for 10 min at 11,000 g, and GST fusion proteins were purified from the supernatants on glutathione-agarose beads (Sigma-Aldrich).

## Preparation of the Constructs for the Expression of the GTPases in Eukaryotic Cells

The cDNAs coding for chicken cRac1A, cRac1B, and cRhoB (obtained from pGEX-cRac1A, pGEX-cRac1B, and pGEX-cRhoB, respectively), and for  $\beta$ -galactosidase (obtained from the pInd-LacZ plasmid; Invitrogen, Carlsbad, CA) were subcloned into the pFLAG-CMV-2 expression vector (Kodak) containing the sequence coding for the FLAG peptide recognized by the anti-Flag-M5 mAb (Kodak). The resulting pFLAG-cRac1A, pFLAG-cRac1B, pFLAG-cRhoB, and pFLAG-LacZ plasmids code for fusion proteins containing the FLAG sequence at the NH<sub>2</sub> terminus. The plasmids pFLAG-N17-cRac1A, pFLAG-N17-cRac1B, and pFLAG-V12-cRac1B were obtained by mutation of T17 into N17 of cRac1A and cRac1B, and of G12 into V12 of cRac1B, using degenerate oligonucleotides in combination with the QuickChange™ site-directed mutagenesis kit (Stratagene GmbH, Heidelberg, Germany).

Constructs coding for the cRac1A/cRac1B chimeric polypeptides were obtained as follows. For the cRac1-AAB and cRac1-BBA chimeras, a XhoI site at position 463 of the cDNA sequences of cRac1A and cRac1B was created by using degenerate oligonucleotides in combination with the QuickChange™ site-directed mutagenesis kit (Stratagene GmbH, Heidelberg, Germany), starting from the pFLAG-cRac1A and pFLAG-cRac1B plasmids, respectively. The creation of this site did not affect the amino acid composition of the encoded polypeptides. The resulting pFLAG-cRac1A/XhoI and pFLAG-cRac1B/XhoI plasmids were digested with XhoI. Each digestion resulted in the production of a 4.1-kb and a 1.15-kb fragment. Ligation of the 4.1-kb fragment from pFLAG-cRac1A/XhoI with the 1.15-kb fragment from pFLAG-cRac1B/XhoI resulted in the pFLAG-cRac1-AAB plasmid, while the pFLAG-cRac1-BBA plasmid was obtained by ligation of the other two fragments.

The pFLAG-cRac1-BAB plasmid was obtained by using a pair of degenerate oligonucleotides encompassing the nucleotide sequence corresponding to positions 425 to 463 of cRac1A. These oligonucleotides were used in combination with the QuickChange™ site-directed mutagenesis kit (Stratagene GmbH), starting from the pFLAG-cRac1B plasmid, to obtain the pFLAG-cRac1-BAB plasmid.

For the cRac1-ABB and cRac1-BAA chimeras we have used the unique NcoI site found at position 431 of the nucleotide sequences coding for cRac1A and cRac1B, which had been previously cloned into the BamHI + EcoRI-digested pRSET-A vector (Invitrogen). The pRSET-cRac1A and pRSET-cRac1B plasmids were digested with BamHI and NcoI. Each digestion resulted in the production of a 3-kb and a 0.44-kb fragment. Ligation of the 3-kb fragment from pRSET-cRac1A with the 0.44-kb fragment from pRSET-cRac1B resulted in the chimeric pRSET-cRac1-BAA plasmid, while the pRSET-cRac1-ABB plasmid was obtained by ligation of the other two fragments. The sequences coding for the two chimeric proteins were excised from the two pRSET chimeric constructs by digestion with BamHI and EcoRI, and inserted into the BglII site of the pFLAG vector by blunt ligation, to obtain the pFLAG-cRac1-BAA and pFLAG-cRac1-ABB plasmids, respectively.

The correctness of all the constructs was checked by sequencing, and the plasmids were used for transfection of primary retinal neural cells and CEFs.

### Expression of the GTPases in Eukaryotic Cells

For transfections of primary retinal neural cells, we used a protocol modified from Boussif et al. (1995). About 300,000 retinal cells obtained from E6 chick neural retinas were plated in each 1.5-cm-diam well containing a glass coverslip coated with 200  $\mu\text{g/ml}$  poly-D-lysine and 40  $\mu\text{g/ml}$  laminin. Cells were cultured overnight at 37°C, 5% CO<sub>2</sub> as described (de Curtis et al., 1991), to induce neurite extension. Cells were then incubated with 200  $\mu\text{l}$  of 150 mM NaCl containing 150 nmoles of polyethylenimine (PEI) 50 kD (Sigma-Aldrich), and 5  $\mu\text{g}$  of plasmid DNA, in 0.5 ml of transfection medium (50% retinal growth medium [RGM] and 50% DME, 5% FCS). After 3 h of culture, cells were washed once with serum-free RGM, and cultured for further 15–24 h in fresh serum-free RGM, before fixation for immunofluorescence. Quantitation of the effects of the expression of N17-cRac1A, N17-cRac1B, V12-cRac1B, and of the chimeric cRac1A/cRac1B constructs in retinal neurons were made by examining transfected, neurofilament-positive neurons in cultures. Neurites longer than three cell diameters were considered for quantitation. The values shown were obtained by analysis of several experiments.

For transfections of CEFs, cells were plated in 1.5-cm-diam wells containing an uncoated glass coverslip, and cultured at 37°C overnight. Cells were then incubated with new culture medium to which 3  $\mu\text{g}$  of plasmid DNA and 6  $\mu\text{g}$  of Dospoer (Boehringer Mannheim GmbH) were added. The Dospoer and the DNA were diluted separately in 20 mM Hepes, 150 mM NaCl, pH 7.4, to a final volume of 25  $\mu\text{l}$  each. The Dospoer was added to the DNA dropwise, and incubated 15 min at room temperature. The mixture was added to the wells containing cells with 1 ml of fresh medium with 5% FCS, 1% chicken serum. After 6 h cells were washed once with medium, and cultured for up to 24 h in fresh medium before further treatment.

To check for cell viability after expression of the N17-cRac1A and N17-cRac1B polypeptides, after transfection for 18 h with the respective constructs retinal cells were resuspended in fresh RGM containing 2.5  $\mu\text{g/ml}$  of propidium iodide (Belloc et al., 1994; Zamai et al., 1996; Darzynkiewicz et al., 1997), and incubated for 40 min at 37°C. After two washes in RGM, cells were fixed and processed for immunofluorescence, as described below. Transfected cells were identified by staining with the anti-cRac1B polyclonal antibody detected by FITC-conjugated sheep anti-rabbit IgG (Boehringer Mannheim GmbH), while the presence of propidium iodide in the same cells was identified by fluorescence in the rhodamine channel.

After transfection, the levels of expression of the cRac1A, cRac1B, N17-cRac1A, and N17-cRac1B polypeptides were evaluated by morphometric analysis. For each construct, 10 transfected cells (either CEFs, or neurofilament-positive retinal neurons) were selected and analyzed. The analysis was performed by analyzing optical sections including the cell body of neurons, excluding the neurites. Optical sections obtained under identical conditions using a DVC-250 confocal microscope (Bio-Rad Laboratories, Hercules, CA) were analyzed using the NIH Image software.

### Immunofluorescence

Transfected cells were fixed with 3% PFA and processed for indirect immunofluorescence, as described (Cattalino et al., 1995). Fixed cells were then incubated for 1 h at room temperature with the following dilution of primary antibodies: 1:500 anti-cRac1B polyclonal serum; 4  $\mu\text{g/ml}$  anti-Flag-M5 mAb; 1:200 anti-200-kD neurofilament protein polyclonal antibody; and 1:300 anti- $\beta$ -galactosidase mAb. Cells were subsequently incubated for 40 min with TRITC-conjugated sheep anti-mouse IgG together with FITC-conjugated sheep anti-rabbit IgG (Boehringer Mannheim GmbH), and observed using an Axiophot microscope (Carl Zeiss Inc., Thornwood, NY). When used, FITC-conjugated phalloidin (Sigma-Aldrich) was added during the incubation with a TRITC-conjugated secondary antibody.

### Western Blotting

Aliquots of the GST fusion proteins for the different GTPases were separated by SDS-PAGE (Laemmli, 1970), transferred to nitrocellulose filters, and probed with the polyclonal antibody against cRac1B. For the detection of the primary antibody, filters were incubated with 0.2  $\mu\text{Ci/ml}$  of <sup>125</sup>I-protein A (Amersham Corp., Arlington Heights, IL), washed, and then exposed to Amersham Hyperfilm-MP.

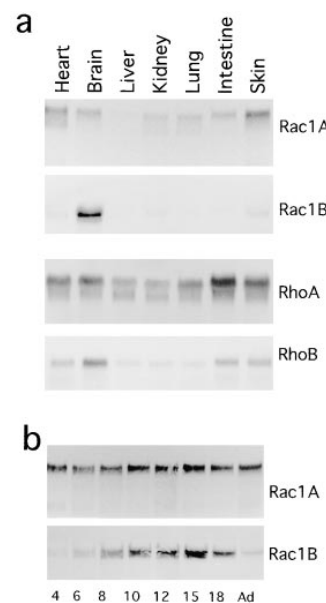
## Results

### Expression of Rho Family GTPases During Development

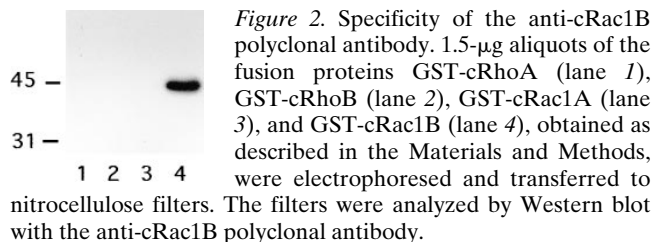
Northern blot analysis on mRNA prepared from different organs isolated from E10 chicken embryos showed a prominent expression of the *cRac1B* transcript in the brain compared with all other tissues examined (Fig. 1 *a*). The transcript for *cRhoB* showed also a high level of expression in the brain compared with other tissues, although its distribution was more widespread to different organs compared with *cRac1B*. Both *cRhoA* and *cRac1A* transcripts showed an ubiquitous distribution among the organs examined, with the exception of the liver, where only very low levels of *cRac1A* transcript could be detected. Analysis of the expression of the two *Rac* genes during brain development showed that the transcript for *cRac1A* was highly expressed throughout development, between E4 to E18, and in the adult chicken. In contrast, the expression of *cRac1B* was regulated during development: the expression of this transcript increased strongly between E4 and E15, to decrease afterwards, and being only weakly expressed in the adult (Fig. 1 *b*).

### Characterization of Antibodies Specific for the cRac1B Polypeptide

Our finding that the *cRac1B* gene is highly and specifically expressed in developing neural tissue prompted us to investigate the role of the cRac1B GTPase on the morphology and the cytoskeletal organization of neuronal cells. For this purpose, we raised a polyclonal antibody against a peptide corresponding to the COOH-terminal portion of the cRac1B protein, which corresponds to the most divergent peptide sequence from the highly homologous cRac1A polypeptide. When tested against the various Rho



**Figure 1.** Northern blot analysis of GTPase mRNA levels in different organs from E10 chick embryos (*a*) and during brain development (*b*). Equal amounts of RNA extracted from different organs from E10 chick embryos (*a*), or from E4, E6, E8, E10, E12, E15, E18, and adult (*Ad*) chicken (*b*) were electrophoresed on 1% agarose gels and transferred to filters as described in Materials and Methods. Filters were incubated with <sup>32</sup>P-labeled probes specific for the different GTPases, as indicated on the right of the blots. In *a*, filters for the *cRac1A* and *cRac1B* transcripts were exposed for autoradiography for 6 and 48 h, respectively. In *b*, the filter was first incubated for the detection of the *cRac1A* transcript (7 h exposure), and then stripped and used for the detection of the *cRac1B* transcript (6 h exposure).



**Figure 2.** Specificity of the anti-cRac1B polyclonal antibody. 1.5- $\mu$ g aliquots of the fusion proteins GST-cRhoA (lane 1), GST-cRhoB (lane 2), GST-cRac1A (lane 3), and GST-cRac1B (lane 4), obtained as described in the Materials and Methods, were electrophoresed and transferred to nitrocellulose filters. The filters were analyzed by Western blot with the anti-cRac1B polyclonal antibody.

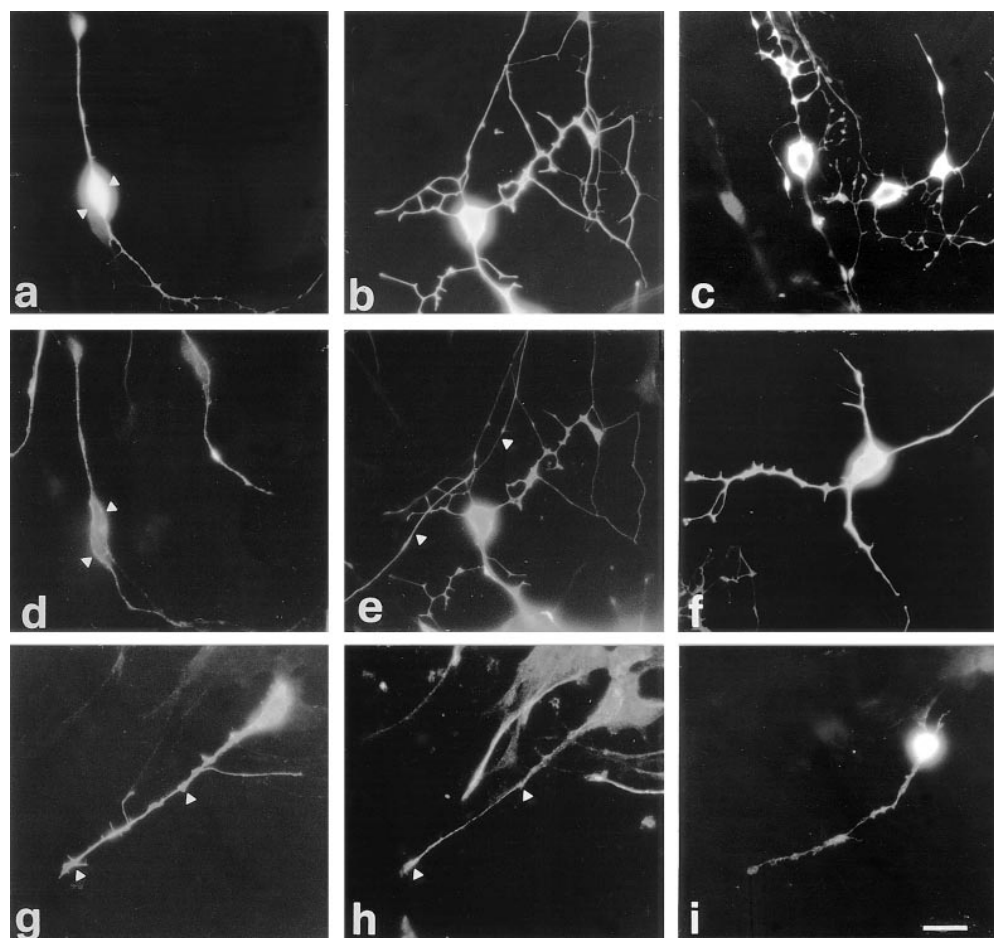
proteins investigated, the serum reacted only with cRac1B (Fig. 2).

### Overexpression of cRac1B into Retinal Neurons Affects Neurite Morphology

Previous work has shown that E6 retinal neurons respond well to laminin, by extending one or two neurites per cell. We have previously shown that at E6 very low levels of cRac1B transcript are present in the retina, as compared with the high levels of cRac1A transcript (Malosio et al., 1997), suggesting that comparatively different amounts of the two endogenous GTPases are present in E6 retinal neurons. We have used primary cultures of E6 retinal neurons to characterize the effects of the cRac1B GTPase on neuritogenesis. Different methods were tested to transfect these cells. The use of PEI 50 kD on cells cultured for 18 h on laminin allowed us to obtain transfection of retinal neu-

rons, identified in culture as neurofilament-positive cells. FACS<sup>®</sup> analysis revealed that under these conditions the efficiency of transfection of primary retinal cells ranged between 0.5 and 3% in different experiments (not shown).

The morphology of retinal neurons cultured on laminin was not affected by the expression of the  $\beta$ -galactosidase (Fig. 3, *a* and *d*). Transfected cells showed in general one long, poorly branched neurite, identified by staining with an antibody against the 200-kD neurofilament protein, similarly to non-transfected cells in the same preparations (Fig. 3 *d*). Overexpression of cRac1B by transfection of retinal neurons with the pFLAG-cRac1B plasmid induced a dramatic effect on neuritogenesis (Fig. 3, *b*, *c*, *e*, and *f*). Very often neurons showed an increased number of neurites per cell, and neurites were frequently highly branched compared with non-transfected cells. Furthermore, localization of filamentous actin (F-actin) and microtubules by incubation of transfected cells with fluorescent phalloidin, and with a mAb against tubulin, respectively, showed presence of F-actin in neurites and in all neuritic branches, and the presence of microtubules in all neurites and major neuritic branches (not shown). Transfection of retinal neurons with pFLAG-cRhoB-induced retraction of neurites and rounding up of the neurons (not shown). On the other hand, when retinal neurons were transfected with the pFLAG-cRac1A plasmid, no dramatic effect on neuronal morphology could be detected. cRac1A-transfected neurons showed a morphology



**Figure 3.** Effects of the expression of cRac1A and cRac1B GTPases on neuronal morphology. Retinal neurons cultured overnight on polylysine- and laminin-coated coverslips were transfected with pFLAG-LacZ (*a* and *d*), pFLAG-cRac1B (*b*, *c*, *e*, and *f*), or pFLAG-cRac1A (*g-i*). After 1 d more in culture, cells were processed for immunofluorescence using the anti- $\beta$ -galactosidase mAb (*a*), the anti-Flag-M5 mAb (*b*, *f*, *g*, and *i*), the anti-cRac1B polyclonal antibody (*c*), and the polyclonal antibody against the 200-kD neurofilament protein (*d*, *e*, and *h*). The same cells are shown in *a* and *d*; in *b* and *e*; and in *g* and *h*. Bar, 10  $\mu$ m.

**Table I. Comparison of the Levels of Expression of Rac GTPases After Transfection with Different Constructs**

Cell type	Protein expressed	Expression level*
CEF	wild-type Rac1A	96.6 ± 10.8
CEF	wild-type Rac1B	107.6 ± 13.9
Retinal neurons	wild-type Rac1A	136.5 ± 9.1
Retinal neurons	wild-type Rac1B	95.5 ± 5.4
Retinal neurons	N17-Rac1A	97.1 ± 8.2
Retinal neurons	N17-Rac1B	89.9 ± 4.1

CEFs or retinal neurons were transfected with the p-FLAG constructs for the expression of the proteins shown in the middle column, as described in the Materials and Methods. The proteins were detected by immunofluorescence, and expression levels quantified as described in the Materials and Methods.

\*Expression levels are in arbitrary units. Each value represents the mean ± SE of 10 different cells.

similar to that of non-transfected neurons, characterized by the presence of one long, poorly branched neurite per neuron (Fig. 3, *g-i*). The different effects observed upon overexpression of the two wild-type Rac proteins in retinal neurons was not due to differences in the levels of expression of the two polypeptides, since these effects were observed when neurons expressing similar levels of the two proteins were compared, as detected by immunofluorescence with the anti-Flag-M5 mAb (see examples in Figs. 3 and 10). Moreover, quantitation in neurons (and in non-neuronal cells) by morphometric analysis performed as described in the Materials and Methods, did not show significant differences between the levels of expression of the two wild-type polypeptides, as well as of the two dominant-negative N17-cRac1A and N17-cRac1B polypeptides (Table I).

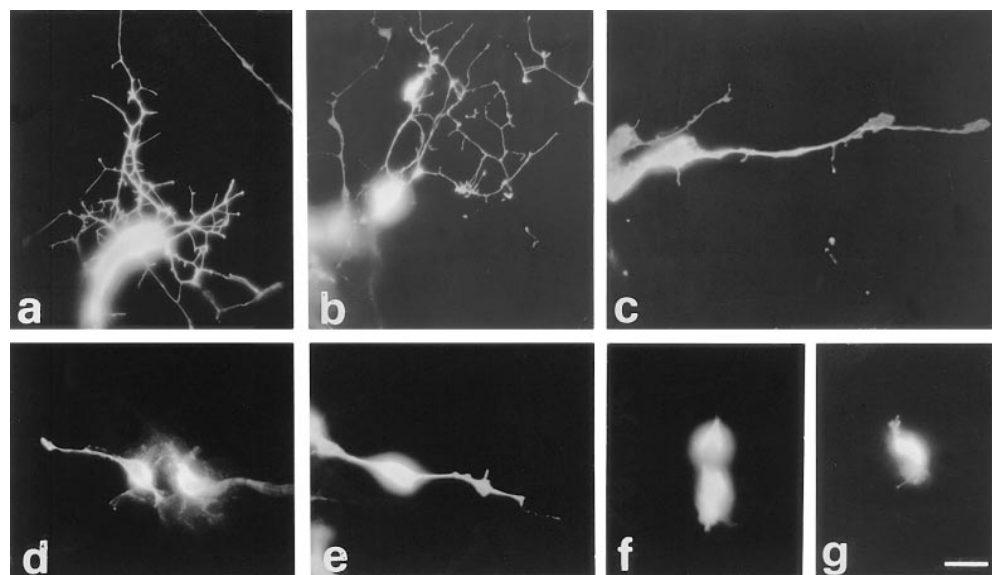
#### **Expression of a Dominant-Negative Form of cRac1B into Retinal Neurons Inhibits Neuriteogenesis**

To test whether the effect of cRac1B overexpression on neuriteogenesis was due to the expression of an active cRac1B GTPase, the plasmid p-FLAG-N17-cRac1B coding for a dominant-negative form of the GTPase was

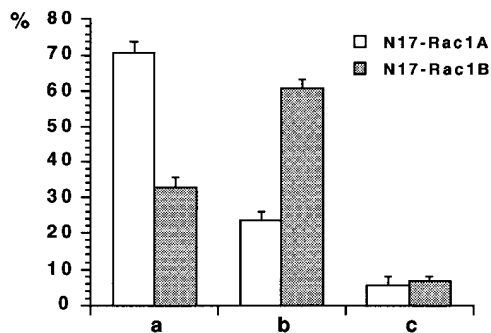
transfected into retinal neurons that had been previously cultured for 18 h on laminin (Fig. 4). Quantitation (Fig. 5) showed that about one-third ( $32.7\% \pm 3$ , SE) of retinal neurons expressing N17-cRac1B showed one or two long and poorly branched neurites (Fig. 4 *c*), typical of non-transfected cells and of cells transfected with the pFLAG-LacZ plasmid; a large fraction ( $60.8\% \pm 2.2$ , SE) of the transfected neurons had no, or very short (less than three cell body diameters in length) neurites (Fig. 4, *d-g*), whereas a minor fraction of N17-cRac1B-expressing cells ( $6.5\% \pm 1.2$ , SE) showed more than two neurites, and/or limited branching (not shown). These results did not only show that expression of N17-cRac1B was unable to induce the dramatic morphological modifications caused by the wild-type GTPase (compare Fig. 4, *c-g* with Fig. 4, *a* and *b*), but also indicated a negative effect of the inactive GTPase on neuriteogenesis. The inability of N17-cRac1B to prevent neuriteogenesis in all transfected cells was probably due to the fact that we had to transfect cells 18 h after plating on laminin, at which time several cells had already grown extensive neurites. We tried to transfect primary neurons at shorter times after plating on laminin, to prevent neurite formation before expression of the N17-cRac1B GTPase (not shown). This procedure was unsuccessful, since most cells detached from the substrate during the transfections.

A significantly different effect was obtained by expressing the dominant-negative form of the cRac1A GTPase (Fig. 5). More than two-thirds of the transfected neurons ( $70.6\% \pm 3.2$ , SE) had a morphology similar to that of non-transfected cells, with one or two long neurites, whereas only  $23.8\% (\pm 2.2, SE)$  of the neurons had no or very short neurites, and just  $5.6\% (\pm 2.3, SE)$  of them showed more than two neurites and/or limited branching.

To test for potential toxic effects of N17-cRac1B on neurons, transfected cells were stained with propidium iodide, which can only permeate through the membrane of damaged cells (see Materials and Methods). Retinal cells expressing N17-cRac1B and showing very short or no neurites (Fig. 6, *a* and *d*) were impermeable to propidium io-

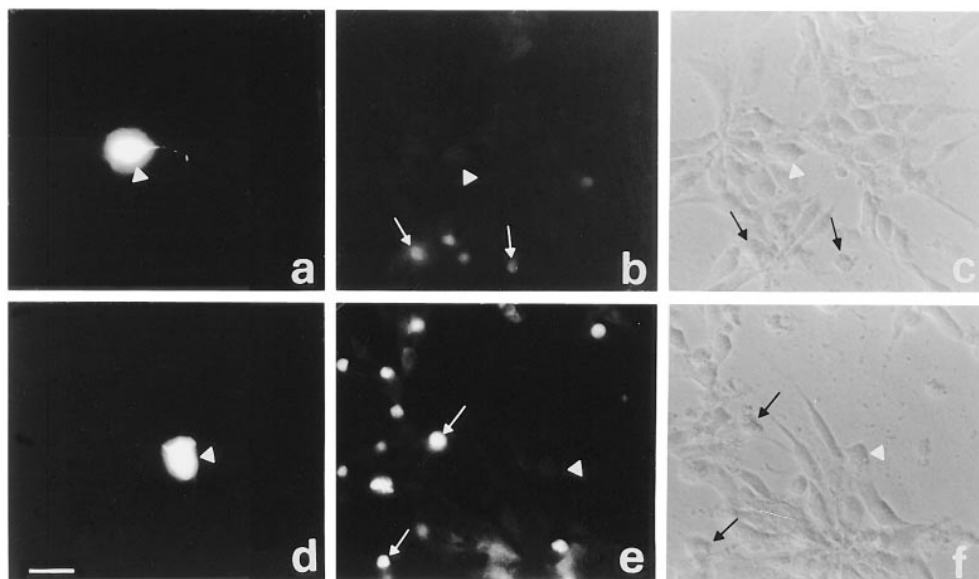


**Figure 4.** Effect of the expression of N17-cRac1B on neuronal morphology. Retinal neurons cultured overnight on polylysine- and laminin-coated coverslips were transfected with pFLAG-cRac1B (*a* and *b*), or pFLAG-N17-cRac1B (*c-g*). After 20 h in culture, cells were processed for immunofluorescence using the anti-Flag-M5 mAb. Bar, 10  $\mu$ m.



**Figure 5.** Quantitative analysis of the effects of the overexpression of the N17-cRac1A and N17-cRac1B proteins on neuritogenesis. Cultures of retinal neurons transfected as described in Fig. 4 were used for the quantitation of the effects of the expression of the dominant-negative forms of the cRac1A and cRac1B GTPases on neuritogenesis, as described in the Materials and Methods. (a) Percentage of neurons with one or two poorly branched neurites (more than three cell diameters in length); (b) neurons with no or short neurites (less than three cell diameters in length); (c) neurons with morphologies different from a and b (i.e., with more than two neurites and/or limited branching). Results are expressed as the mean percentage of cells ( $\pm$  SE) from four separate experiments. In a and b, the differences between N17-cRac1A (white bars) and N17-cRac1B (gray bars) are significant ( $P < 0.001$ ).

dide (Fig. 6, b and e). On the other hand, in the same cultures a number of non-transfected, damaged cells could be detected by positive staining with propidium iodide (Fig. 6, b, c, e, and f, arrows). Also N17-cRac1A-transfected cells were not accessible to propidium iodide (not shown). These data support the idea that inhibition of neurite extension by dominant-negative N17-cRac1B was specific, and not due to a more general toxic effect on neurons.



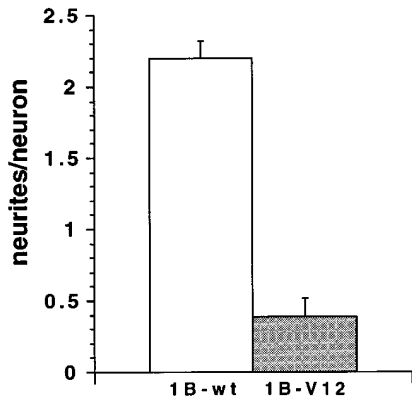
**Figure 6.** N17-cRac1B expression does not affect cell viability. Retinal neurons cultured overnight on polylysine- and laminin-coated coverslips were transfected with pFLAG-N17-cRac1B. After 18 h, cells were incubated with propidium iodide as described in the Materials and Methods, fixed, and then treated for immunofluorescence using the anti-Flag-M5 mAb. The same field is shown in a–c and in d–f. (a and d) Transfected neurons detected with the anti-Flag-M5 mAb; (b and e) propidium iodide staining; (c and f) phase contrast. Arrowheads show the position of the transfected neurons. Arrows in b and c, and in e and f point to propidium iodide-positive, non-transfected cells. Bar, 10  $\mu$ m.

### Expression of a Constitutively Active Form of the cRac1B GTPase Prevents Neuritogenesis

Transfection of E6 retinal neurons with the pFLAG-V12-cRac1B plasmid to express a constitutively active form of the cRac1B GTPase had an effect that was clearly distinct from that observed by expression of the wild-type cRac1B. In fact, most V12-cRac1B-expressing cells did not extend long, branched neurites, but only exhibited very short and narrow processes extending from the round neuronal cell body (not shown). The quantitative analysis shown in Fig. 7 reveals a dramatic negative effect on neuritogenesis upon expression of the constitutively active form of the cRac1B GTPase. These data suggest that the wild-type GTPase, able to cycle between an active (GTP-bound) and inactive (GDP-bound) form, is required for cRac1B-stimulated neurite extension.

### Expression of cRac1A and cRac1B Induces Similar Effects on the Organization of the Actin Cytoskeleton of Non-Neuronal Cells

Analysis of the expression of Rho family GTPase by Northern blot on mRNA isolated from E10 CEFs showed that while transcripts for *cRac1A* are abundantly expressed in these cells, the *cRac1B* mRNA is not detectable (not shown). The absence of the *cRac1B* transcript in CEFs makes them an ideal system to look at the effects of the exogenous expression of this GTPase on the organization of the cytoskeleton of non-neuronal cells. For this purpose, CEFs were transfected with the pFLAG-cRac1B plasmid coding for the wild-type GTPase. To optimize conditions, several agents were tested for transfection. Expression of pFLAG plasmids by using either Dosper or PEI to transfect CEFs produced similar results (not shown). However, because the use of Dosper resulted in better transfection efficiency for CEFs, the results pre-

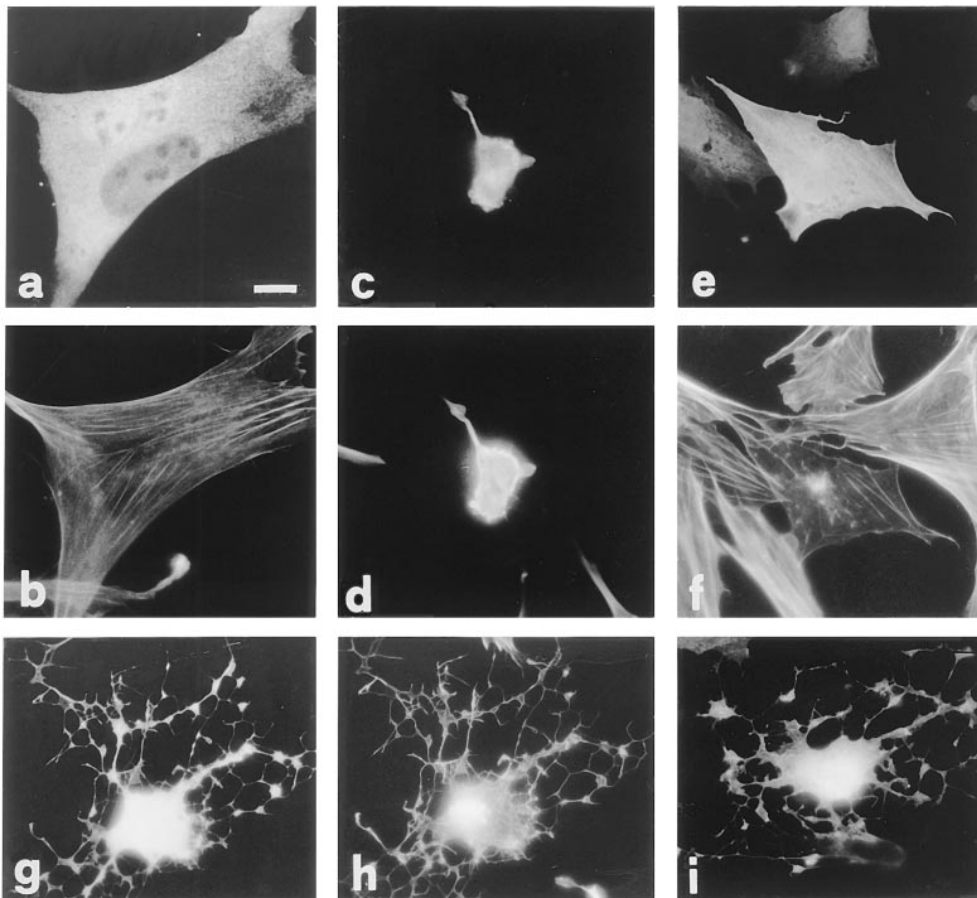


**Figure 7.** Analysis of the effect of the expression of wild-type and constitutively active cRac1B on neuritogenesis. Cultures of retinal neurons transfected with either pFLAG-cRac1B (1B-wt, white bar) or pFLAG-V12-cRac1B (1B-V12, gray bar) were used for the quantitation of the effects of the expression of the wild-type and constitutively active cRac1B GTPases on neuritogenesis, as described in the Materials and Methods. Columns represent the average number of neurites/neuron ( $\pm$ SE). The difference observed between cRac1B-wt and cRac1B-V12 is significant ( $P < 0.001$ ).

sented in this paper have been obtained by the latter technique. Expression of  $\beta$ -galactosidase by use of the pFLAG-LacZ plasmid did affect neither the morphology of CEFs (Fig. 8 a), nor the organization of their actin cy-

toskeleton, which was characterized by the presence of abundant stress fibers (Fig. 8 b), similarly to non-transfected cells (not shown). Expression of cRhoB by using the pFLAG-cRhoB plasmid induced rounding up of several cells already after 8 h of transfection (Fig. 8, c and d). Transfected, still spread CEFs showed an increase in F-actin density as revealed by phalloidin staining (not shown). On the other hand an increase of stress fibers was not evident, probably resulting from the presence of numerous pre-existing stress fibers in non-transfected CEFs. The changes in cell morphology induced by cRhoB were caused by actomyosin-induced contraction, since this effect could be prevented by incubation of the cells with 2,3-butanedione monoxime during transfection (not shown).

Transfection with pFLAG-cRac1B for up to 11 h induced expression of cRac1B without major changes in cell shape in the majority of CEFs (Fig. 8 e), although the transfected cells were often showing a strong decrease in the number of stress fibers and density of F-actin compared with non-transfected cells (Fig. 8 f). After 18–24 h of transfection, the morphology of most of the transfected cells was dramatically affected (Fig. 8 g), because of a complete reorganization of the actin cytoskeleton, with several cRac1B-transfected cells showing numerous, highly branched membrane protrusions rich in F-actin (Fig. 8 h) and in microtubules (not shown). The abundance of F-actin and microtubules in the processes produced by transfection with pFLAG-cRac1B suggests that the membrane protrusions originate as a consequence of



**Figure 8.** Effects of the expression of Rho GTPases on CEF morphology. CEFs cultured overnight on glass coverslips were transfected with pFLAG-LacZ (a and b), pFLAG-cRhoB (c and d), pFLAG-cRac1B (e–h), or pFLAG-cRac1A (i) plasmid. After 8 h (c and d), 11 h (e and f), or 1 d in culture (a, b, g–i), cells were processed for immunofluorescence. First antibodies used were: mAb against  $\beta$ -galactosidase (a), mAb anti-Flag-M5 (c, e, g, and i); in b, d, f, h, cells were stained with fluoresceinated phalloidin. The same cells are shown in a and b; in c and d; in e and f; and in g and h. Bar, 10  $\mu$ m.



the reorganization of the actin and tubulin cytoskeleton, rather than representing retraction fibers with remnants of F-actin. Moreover, F-actin- and microtubule-rich protrusions became more evident after longer transfection periods, with a number of transfected cells showing processes extending well beyond the usual length of non-transfected fibroblasts, and with some processes extending on the surface of the non-transfected cells (not shown).

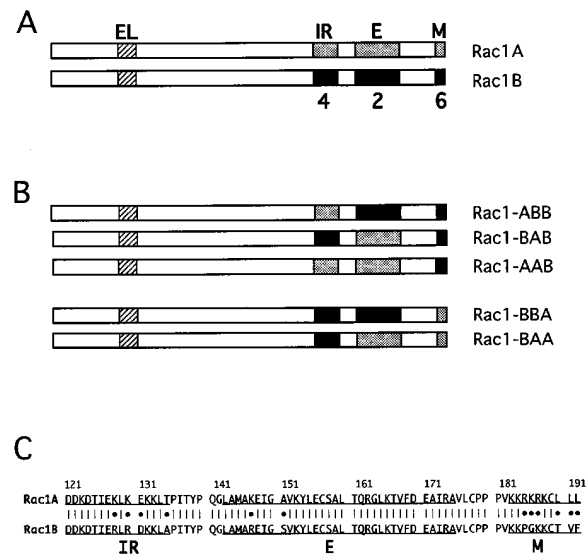
The overexpression of cRac1A in CEFs produced effects similar to those obtained by the expression of cRac1B. It induced the reduction of stress fibers in cells that were still keeping a flat morphology (not shown), and caused deep morphological alterations in several cells, which were characterized by the presence of numerous plasma membrane protrusions (Fig. 8*i*) rich in F-actin (not shown).

### The COOH-Terminal Portion of the cRac1B GTPase Is Sufficient to Confer Functional Specificity

With the aim of identifying the portion(s) of the polypeptide responsible for the specific effects of cRac1B on neuritogenesis, we have prepared five different constructs corresponding to the cRac1A/cRac1B chimeras obtained by different combinations of the three polypeptide stretches that contain all 12 amino acid differences existing between the cRac1A and cRac1B polypeptides. The cRac1A and cRac1B GTPases differ in four amino acid residues included in the Rho's insert region (Fig. 9*A*, *IR*), in two amino acid residues of a region (Fig. 9*A*, *E*) known to be required for the interaction of Rac with some effectors (Diekmann et al., 1995), and in six of the last eight COOH-terminal amino acid residues that contain the CAAX sequence involved in membrane anchoring (Fig. 9*A*, *M*). The five chimeras indicated in Fig. 8*B* were obtained as described in the Materials and Methods.

Transfection of E6 retinal neurons and immunofluorescence were used to evaluate the effects of the expression of these chimeras on neuritogenesis. Expression of either the cRac1-ABB or the cRac1-BAB chimera induced a pattern similar to that obtained by the overexpression of the wild-type cRac1B protein (Fig. 10, compare *b* and *c* with *a*). Several neurons showed more neurites that were often highly branched, suggesting that the respective substitution of the *IR* or *F* region of the cRac1B protein with the corresponding regions from cRac1A did not abrogate the cRac1B-specific effect on neuronal morphology. On the other hand, expression of the cRac1-BAA or cRac1-BBA chimeric GTPases resulted in a phenotype similar to that observed by overexpressing the wild-type cRac1A protein, with neurons generally expressing one poorly branched neurite (Fig. 10, compare *e* and *f* with *d*). Finally, expression of the cRac1-AAB chimera resulted in a phenotype similar to that induced by the overexpression of the wild-type cRac1B (Fig. 10, *g* and *h*), indicating that the substitution of the *M* region of cRac1A with the corresponding region from cRac1B is sufficient to confer the functional specificity characteristic of cRac1B.

Fig. 11 shows the quantitative analysis of the effects of the overexpression of wild-type and chimeric constructs on neuritogenesis from E6 retinal neurons. The average number of neurites per neuron in cells expressing either



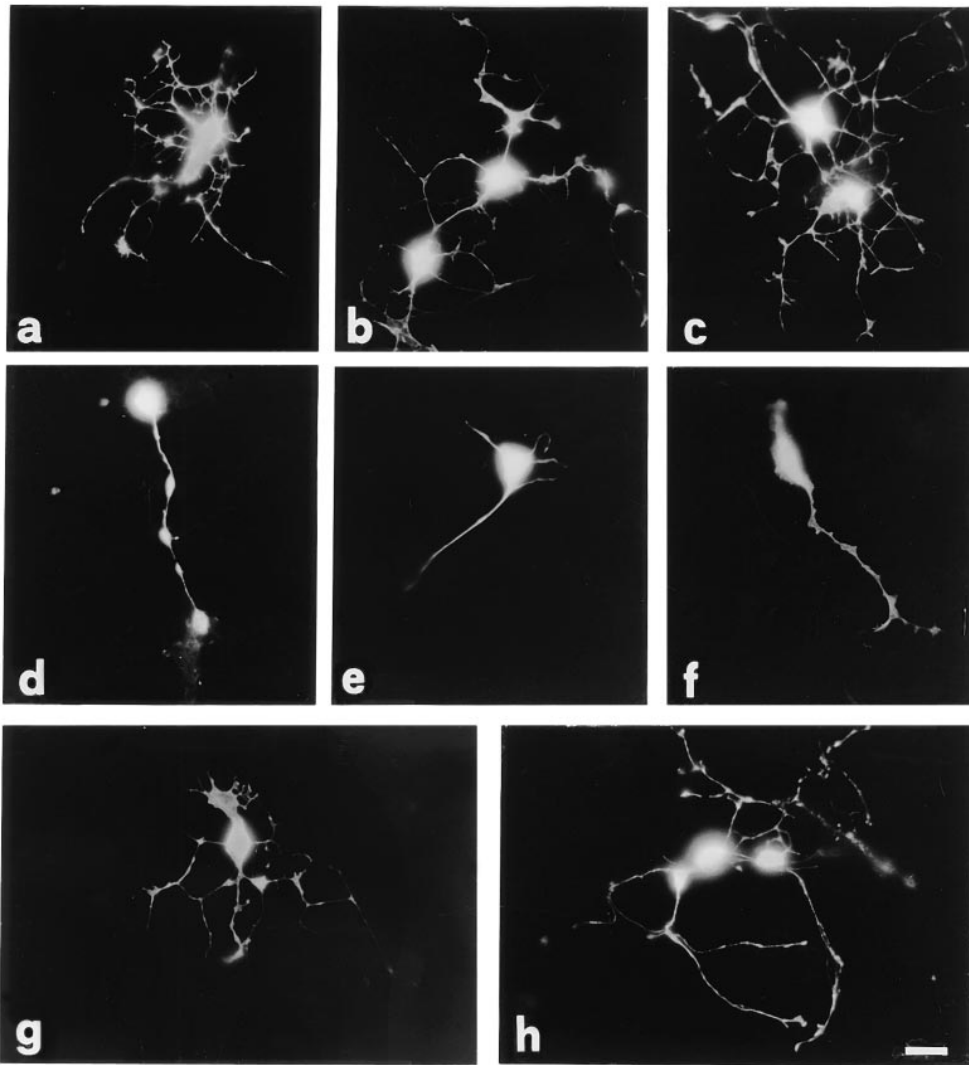
**Figure 9.** Schematic diagram of the wild-type cRac1A and cRac1B polypeptides (*A*), of the cRac1A/cRac1B chimeric proteins expressed in retinal neurons (*B*), and comparison between the COOH-terminal portions of cRac1A and cRac1B (*C*). In *A*, the three regions containing all 12 amino acid differences between the cRac1A and cRac1B polypeptides are indicated. The numbers under the areas indicated as *IR* (Rho's insert region), *E* (a region required for the interaction with some of the known Rac effectors), and *M* (the COOH-terminal region known to be involved in membrane targeting) indicate the differences in amino acid residues between the two GTPases. *EL*, the classical effector loop. In *B* are shown the five chimeric constructs obtained by changing one or two of the *IR*, *E*, and *M* regions between cRac1A and cRac1B. See the Materials and Methods for details on the preparation of these constructs. In *C*, the amino acid sequences of the COOH-terminal portion of cRac1A and cRac1B are compared. The black dots indicate amino acid differences between the two GTPases.

the wild-type cRac1B, or one of the three chimeric constructs with the COOH-terminal portion from cRac1B (cRac1-ABB, cRac1-BAB, and cRac1-AAB) was significantly higher when compared with neurons expressing either the wild-type cRac1A, or one of the two chimeric cRac1-BBA and cRac1-BAA GTPases. Over 50% of the neurons expressing one of the constructs including the *M* region of cRac1B showed branched neurites, which in the majority of cases included highly branched neurites. In contrast, poor neuritic branching was present in <15% of the neurons expressing any of the constructs containing the *M* region of cRac1A.

### Discussion

The molecular mechanisms regulating the cytoskeletal changes necessary for neuritogenesis and neurite maturation during the development of the nervous system are still largely obscure. Recent work has pointed out the role of Rho GTPases in neuritogenesis, including a number of studies that implicate the Rac1 GTPase (corresponding to the chicken cRac1A protein considered in this paper) in a number of different aspects of growth cone function (Jin



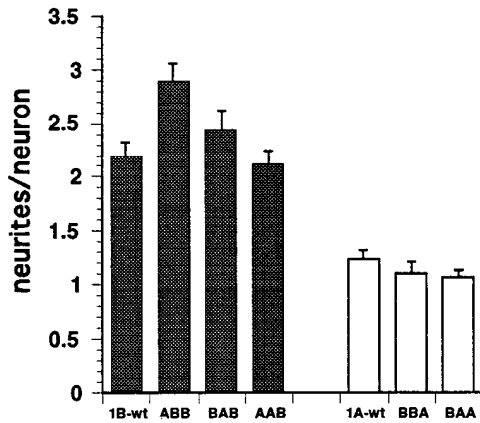


**Figure 10.** Effects of the expression of chimeric cRac1A/cRac1B GTPases on neuronal morphology. Retinal neurons cultured overnight on polylysine- and laminin-coated coverslips were transfected with pFLAG-cRac1B (a), pFLAG-cRac1-ABB (b), pFLAG-cRac1-BAB (c), pFLAG-cRac1A (d), pFLAG-cRac1-BAA (e), pFLAG-cRac1-BBA (f), and pFLAG-cRac1-AAB (g and h). After 1 d more in culture, cells were processed for immunofluorescence using the anti-Flag-M5 mAb. Neurons were identified by double staining with an anti-neurofilament antibody (not shown). Bar, 10  $\mu$ m.

and Strittmatter, 1997; Kozma et al., 1997; Lamoureux et al., 1997; Threadgill et al., 1997). Moreover, we have recently identified a novel member of the Rho family of small GTPases called cRac1B, which is highly and specifically expressed in the neural tissue of developing chick embryos (Malosio et al., 1997). In the present study we have analyzed the function of this GTPase in cultured primary neurons. One major finding from our study is that cRac1B specifically affects the morphology of retinal neurons, by inducing the formation of an increased number of neurites, and by dramatically increasing neurite branching. These effects were not observed by overexpressing the highly similar cRac1A GTPase. A second major finding is the strong inhibition of neuritogenesis upon expression of an inactive form of cRac1B. Finally, by expressing chimeric cRac1A/cRac1B constructs we have shown that the region containing the last eight COOH-terminal amino acid residues of cRac1B is necessary for cRac1B-mediated enhancement of neuritogenesis and neurite branching. Our results show for the first time that two highly related Rac proteins have very distinct effects on the development of the neuronal phenotype, while the dissection of cRac1B

function has uncovered new mechanisms underlying the functional specificity of different Rho family GTPases.

The striking differences on neuritogenesis observed by separately overexpressing the two Rac proteins in primary neurons was not due to different levels of expression of the two wild-type GTPases, since these differences could be reproducibly observed between neurons showing similar levels of either exogenous protein, as detected by immunofluorescence analysis. Similar levels of expression of cRac1B and cRac1A after transfection with the corresponding constructs were indicated also by the quantitative analysis shown in Table I. Moreover, the analysis of the effects of the chimeric GTPases clearly showed that neuritogenesis, including neurite branching, was significantly enhanced whenever the COOH-terminal eight amino acid residues from cRac1B were present, whereas these effects were not observed with any of the chimeras containing the COOH-terminal portion of cRac1A. Interestingly, the expression of the wild-type cRac1B GTPase was essential to induce increased neuritogenesis, since expression of the constitutively active V12-cRac1B GTPase, as well as the expression of the inactive N17-cRac1B GTP-



**Figure 11.** Analysis of the effect of the expression of wild-type and chimeric GTPases on neuritogenesis. Cultures of retinal neurons were transfected with pFLAG-cRac1B (1B-wt), pFLAG-cRac1-ABB (ABB), pFLAG-cRac1-BAB (BAB), pFLAG-cRac1-AAB (AAB), pFLAG-cRac1A (1A-wt), pFLAG-cRac1-BBA (BBA), or pFLAG-cRac1-BAA (BAA). After 1 d, cultures were used to quantify the effects of the expression of the different constructs on neurite extension, as described in the Materials and Methods. Columns represent the average number of neurites/neuron ( $\pm$ SE). The value obtained for each of the constructs containing the COOH-terminal M segment (see Fig. 8) from cRac1B (gray columns to the left) is significantly different from each of the values obtained from transfected neurons expressing any of the constructs with the COOH-terminal M segment from cRac1A (white columns to the right) ( $P < 0.001$ ).

ase had drastic inhibitory effects on neuritogenesis. These results strongly suggest that a cycling, wild-type GTPase is required for cRac1B-mediated neuritogenesis.

In contrast to what observed in neurons, we have found that cRac1A and cRac1B have similar effects on the cytoskeletal organization of fibroblasts, implicating a cell type-dependent specificity of Rac proteins action. It has been recently found that the activity of Rac3 (the human orthologue of cRac1B) can be regulated in vitro by Bcr (Haataja et al., 1997), a GTPase-activating protein highly expressed in the brain (Heisterkamp et al., 1993). These data, together with the finding that Tiam1, a guanine nucleotide exchange factor highly expressed in the brain (Habets et al., 1995), affects neurite outgrowth in neuroblastoma cells in a Rac-dependent fashion, suggest that neuritogenesis may be modulated by specific Rac regulators during development (van Leeuwen et al., 1997). Moreover, the differences existing between the cRac1A and cRac1B polypeptides may be sufficient to allow the interaction with distinct sets of neuronal effectors and/or regulators, which may be responsible for the different effects observed on the neuronal cytoskeleton. We have preliminary biochemical evidence supporting this hypothesis, since distinct sets of polypeptides from brain lysates interact specifically with the two activated Rac proteins in vitro (Di Cesare, A., S. Paris, and I. de Curtis, unpublished results).

The striking difference observed in this study by overexpressing the two Rac proteins into primary neurons is particularly surprising considering the high degree of homology between the cRac1A and cRac1B polypeptides, which only differ in 12 amino acid residues. Interestingly, none of

these residues are included in the “effector loop” (residues 22–45 of Rac). Instead, they are contained in three distinct regions that have been implicated together with the classical effector loop in the recognition of downstream effectors (Kreck et al., 1994; Joseph and Pick, 1995; Diekmann et al., 1995; Westwick et al., 1997). One of the three regions corresponds to a stretch of amino acid residues at the COOH terminus of the polypeptides, which is important also for targeting of the GTPases to the membrane; 6 of the 12 amino acid differences between cRac1A and cRac1B are clustered in this region. The results obtained by expressing distinct cRac1A/cRac1B chimeras in primary neurons have clearly established that this COOH-terminal region is required for cRac1B-specific action, since its substitution with the corresponding sequence from cRac1A completely abolishes the effects on neuritogenesis. Furthermore, this sequence is sufficient to mediate enhanced neuritogenesis when exchanged with the corresponding region onto the cRac1A GTPase. These results point for the first time to an important role of the COOH-terminal region in the determination of the specificity of Rac proteins action during neuritogenesis. Although the manner in which this region mediates cRac1B-enhanced neuritogenesis remains to be established, one possibility is that it may be involved in the interaction with cRac1B neuronal effectors and/or regulators. Alternatively, a different subcellular localization of the GTPases may be responsible for the differences observed between cRac1A and cRac1B action in neurons, although studies in transfected fibroblasts have not shown differences in the subcellular distribution of the two polypeptides (Albertinazzi, C., and I. de Curtis, unpublished data).

Rac GTPases have been implicated in axonal outgrowth during neuronal development in *Drosophila* (Luo et al., 1994). Moreover, perturbation of Rac1 activity in mouse Purkinje cells leads to severe loss of presynaptic terminals (Luo et al., 1996). Our finding that cRac1B has a dramatic positive effect on neurite branching suggests that this GTPase may be involved in the development of the mature neuronal phenotype in vivo. This hypothesis is supported by our previous data showing that the expression of *cRac1B* is restricted to the nervous system during embryonic development (Malosio et al., 1997), and by the finding that the recently identified human Rac3 GTPase (Haataja et al., 1997), which shares 99% amino acid identity with cRac1B, shows the highest expression in the brain. Furthermore, the expression of *cRac1A* and *cRac1B* is differentially regulated during retina (Malosio et al., 1997), and brain (this paper) development. In the retina, at E6 cRac1B shows levels of expression up to sevenfold lower than at later stages (E10–E12; Malosio et al., 1997), whereas the expression levels of cRac1A remain quite constant between E6 and E12. We have observed a similar situation in the brain, where *cRac1A* expression is constantly high at the stages analyzed, between E4 and adulthood, whereas the expression of *cRac1B* is developmentally regulated, with low levels of transcript up to E6, highest expression around E15, and low levels again in the adult. It is interesting to note that in the chick retino-tectal system, ganglion cell neurites reach the surface of the tectum at E8, and start to arbor through the tectum layers to reach their target around E12. At E18, arbors are rela-

tively mature, and they continue to increase in complexity at least until postnatal day 2. From E11 on, once they have reached the final target, retinal ganglion cell axons form organized synapses (Acheson et al., 1980; McLoon, 1985; Thanos and Bonhoeffer, 1987; Nakamura and O'Leary, 1989; Yamagata and Sanes, 1995). In chicken, many other neuronal types are branching their neurites and forming synapses during the same period. Considering the correlation between the high levels of expression of cRac1B after E8, and the timing of neurite branching during neuronal development, a dose-dependent effect of the cRac1B GTPase on neurite branching *in vivo* may be envisaged. According to this hypothesis, the increase in neurite branching observed in transfected E6 retinal neurons, which normally express low levels of endogenous cRac1B (Malosio et al., 1997), may be explained by the induced increase of cRac1B activity.

In conclusion, our finding that the neural-specific cRac1B GTPase has dramatic effects on the development of the neuronal phenotype strengthens the idea that Rho GTPases play important roles during neuronal development, and raises interesting questions on the nature of the molecular mechanisms that mediate the effects of this GTPase on the neuronal cytoskeleton. The finding that a short COOH-terminal sequence is essential for cRac1B-induced reorganization of the neuronal cytoskeleton represents an important new indication towards the understanding of the mechanisms underlying Rho proteins' functional specificity. Future work will be aimed at the identification of the molecular machinery responsible for the specificity of action of Rac GTPases during neuritogenesis and neuronal maturation.

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