Neuron, Vol. 19, 625–634, September, 1997, Copyright ©1997 by Cell Press

Regulation of Dendritic Growth and Remodeling by Rho, Rac, and Cdc42

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Summary

The acquisition of cell type-specific morphologies is a central feature of neuronal differentiation and has important consequences for nervous system function. To begin to identify the underlying molecular mechanisms, we have explored the role of Rho-related GTPases in the dendritic development of cortical neurons. Expression of dominant negative mutants of Rac or Cdc42, the Rho-inhibitory molecule C3 transferase, or the GTPase-activating protein RhoGAP p190 causes a marked reduction in the number of primary dendrites in nonpyramidal (multipolar) neurons and in the number of basal dendrites in neurons with pyramidal morphologies. Conversely, the expression of constitutively active mutants of Rho, Rac, or Cdc42 leads to an increase in the number of primary and basal dendrites. In cortical cultures, as in vivo, dendritic remodeling leads to an apparent transformation from pyramidal to nonpyramidal morphologies over time. Strikingly, this shift in favor of nonpyramidal morphologies is also inhibited by the expression of dominant negative mutants of Cdc42 and Rac and by RhoGAP p190. These observations indicate that Rho, Rac, and Cdc42 play a central role in dendritic development and suggest that differential activation of Rho-related GTPases may contribute to the generation of morphological diversity in the developing cortex.

Introduction

The diversity of cell types in the developing cerebral cortex is most strikingly apparent in terms of the dendritic morphology of neurons. Whereas layers 2, 3, 5, and 6 are dominated by pyramidal neurons, the neurons of layer 4 are predominantly nonpyramidal (reviewed by Gilbert, 1983). The pyramidal and nonpyramidal neurons can be further classified morphologically based on the extent of the apical dendrite or the number and branching pattern of the primary dendrites. This morphological diversity has several important functional consequences. For example, the particular branching pattern of a dendritic tree can influence the electrophysiological integrative properties of a neuron (Rall, 1964). In addition, the dendritic morphology determines the afferent populations with which a neuron may interact. Whereas many of the deep layer pyramidal neurons can receive afferent innervation from all of the superficial cortical layers by virtue of an apical dendrite that extends to layer 1, the innervation of nonpyramidal neurons is restricted owing to the limited extent of their dendritic arbors (Gilbert, 1983).

Studies over the past several years have indicated that the development of dendritic morphology is a highly dynamic process that is characterized by a great deal of growth and remodeling (Wise et al., 1979; Harris and Woolsey, 1981; Greenough and Chang, 1988; Ramoa et al., 1988; Dalva et al., 1994; McAllister et al., 1995). One major form of dendritic remodeling in the cortex appears to involve the withdrawal of the apical dendrite from a subset of pyramidal neurons. Koester and O'Leary (1992) examined the issue of dendritic remodeling in the development of layer 5 neurons, which contain subpopulations that either project to the tectum and spinal cord or to the contralateral hemisphere. Whereas the subcortically projecting neurons are characterized by an apical dendrite that terminates in layer 1, the callosally projecting neurons have a shorter apical dendrite that terminates in layer 4 (Hubener and Bolz, 1988; Hallman et al., 1988; Hubener et al., 1990). By examining the dendritic morphology of these two cell populations during development, Koester and O'Leary found that both populations start out with an apical dendrite that extends to layer 1. However, the apical dendrites of the callosally projecting neurons withdraw between postnatal day 4 (P4) and P7, until the apical process extends only to layer 4. They also reported that layer 6 neurons undergo a similar withdrawal of the apical dendrite. Dendritic remodeling involving withdrawal of an apical process seems not to be restricted to the pyramidal layers of the cortex. Peinado and Katz (1990, Soc. Neurosci., abstract) reported that between P2 and P7, 90% of the layer 4 neurons have an apical dendrite that reaches layer 1. Beyond P10, $<$30% of the neurons extend such an apical process, suggesting that a majority of neurons that start out with pyramidal morphologies undergo a morphological transformation.

Although these observations indicate that developing cortical neurons undergo extensive dendritic remodeling, the underlying regulatory mechanisms have not yet been identified. It is likely that the molecules involved would have the ability to influence the actin cytoskeleton, since dendritic growth and remodeling involve major cytoskeletal reorganization. Recent work from a number of laboratories indicates that the Rho family of small GTP-binding proteins are centrally involved in cytoskeletal reorganization in nonneural cells (reviewed by Hall, 1994; Chant and Stowers, 1995; Mackay et al., 1995). Notably, Hall and colleagues have used microinjection experiments to demonstrate that the Rho-related proteins RhoA, Rac-1, and Cdc42 function coordinately to regulate cytoskeletal reorganization associated with cell motility (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995). Since cell motility and growth cone movements share several cellular characteristics (Bray and Chapman, 1985), and since growth cones are believed to regulate the growth of axonal and dendritic processes, we decided to investigate the role of Rho-related GTPases in regulating dendritic growth and remodeling in developing cortical neurons. Our observations indicate that Rho (RhoA), Rac (Rac-1), and Cdc42

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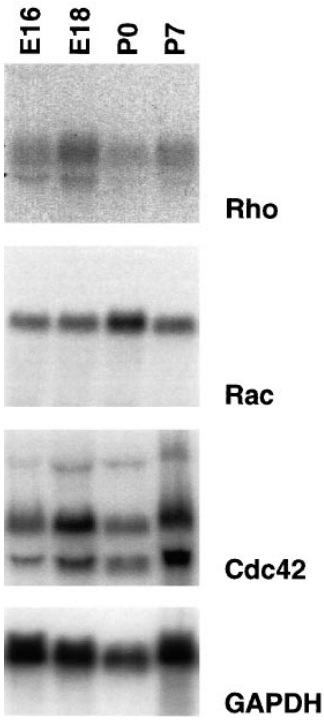

Figure 1. Northern Blot Analysis of the Expression of Rho, Rac, and Cdc42 during Cortical Development

15 μg of total cellular RNA isolated from rat neocortex at the ages indicated was separated on an agarose/formaldehyde gel, transferred onto nylon membranes, and hybridized with ^{32}P-labeled cDNA probes to the indicated GTPases or GAPDH.

play a central role in the specification of dendritic morphologies.

Results

To determine if Rho-related GTPases are expressed in the cortex during periods of neuronal growth and differentiation, we examined the expression of Rho, Rac, and Cdc42 in the neocortex by Northern blot analysis. As indicated in Figure 1, all three of the GTPases were expressed at all ages between embryonic day 16 (E16) and postnatal day 7 (P7) This observation suggests that these GTPases are likely to function in the cortex throughout development and are present in the cortex during periods of axonal and dendritic growth.

Our first goal was to determine whether the dendritic development of cortical cells in culture allowed morphologically distinct cell types to be identified. To reveal the morphology of individual cortical neurons, cultures were transfected with a β-galactosidase expression vector using a modified calcium phosphate transfection procedure. One to five days posttransfection, the expression of the β-galactosidase protein was detected using an antibody to β-galactosidase. As shown in Figure 2, the β-galactosidase protein product diffuses throughout the cell and allows the details of the cellular morphology to be visualized. These cultures have <1% glial cells, and accordingly >99% of the transfected cells had typical neuronal morphologies. Importantly, these initial transfections revealed that morphologically distinct cell types could be clearly distinguished in culture. Many of the cells had a pyramidal cell body, a gradually tapering major "apical" dendrite that terminated in a branched tuft, and a number of basal dendrites typical of pyramidal neurons (Figure 2C). At the same time, there were transfected neurons that were multipolar and had several equivalent primary branches. In this study, we refer to these neurons as nonpyramidal (Figure 2D). The observation that neuronal morphologies in vitro are remarkably similar to that seen in vivo suggests that the major cellular and molecular mechanisms involved in the specification of neuronal morphology continue to function effectively in these cultures. Because of their distinctive morphological characteristics, axonal and dendritic processes could also be clearly distinguished in the transfected cells. The accuracy of identifying processes as axonal or dendritic based on morphology was also confirmed by double immunofluorescence with anti-β-galactosidase and anti-Tau (an axonal protein) or anti-MAP2 (a dendritic protein; data not shown).

Since we were interested in transfecting multiple expression plasmids in most of the experiments, we performed a series of transfections with pairs of mammalian expression plasmids (β-galactosidase together with chloramphenicol acetyl transferase [CAT], E1A, or au5-tagged dominant negative forms of Rho-related GTPases) to determine cotransfection efficiency. At a molar ratio of 1:2 or greater (RSV-β-galactosidase:second plasmid), >95% of the β-galactosidase-positive neurons were also CAT-, E1A-, or au5-positive, indicating a very high degree of cotransfection efficiency. Examples of neurons cotransfected with RSV-β-galactosidase and RSV-CAT are shown in Figures 2A and 2B (see also Figure 4).

To characterize the development of cortical neurons in culture, cells were transfected with β-galactosidase at 2 days in vitro (2 DIV), and sister plates were fixed at 3, 5, and 7 DIV before being processed for β-galactosidase immunocytochemistry. As shown in Figure 3A, there is a small decrease in the total number of cells in culture over time. To determine whether the transfection procedure might itself induce a significant amount of cell death, the number of β-galactosidase-positive cells were also scored over the same period. Our analysis indicates that the reduction in the number of β-galactosidase-positive neurons is virtually identical to the reduction of total cell number, suggesting that the transfection does not induce additional cell death (Figure 3B). This was also apparent by visual inspection of the transfected cultures, since in general the transfected neurons had robust dendritic growth.

In the next series of experiments, we examined the effects of inhibiting the function of Rho-related GTPases on dendritic development. As shown in Figures 3C and 3E, there is a gradual increase in the number of primary dendrites in nonpyramidal neurons and in the number of basal dendrites in pyramidal neurons over time in culture. To directly evaluate the role of Rho, Rac, and Cdc42 in this process, we transfected the cultures with dominant negative (asparagine to threonine replacement at the position analogous to codon 17 of Ras)

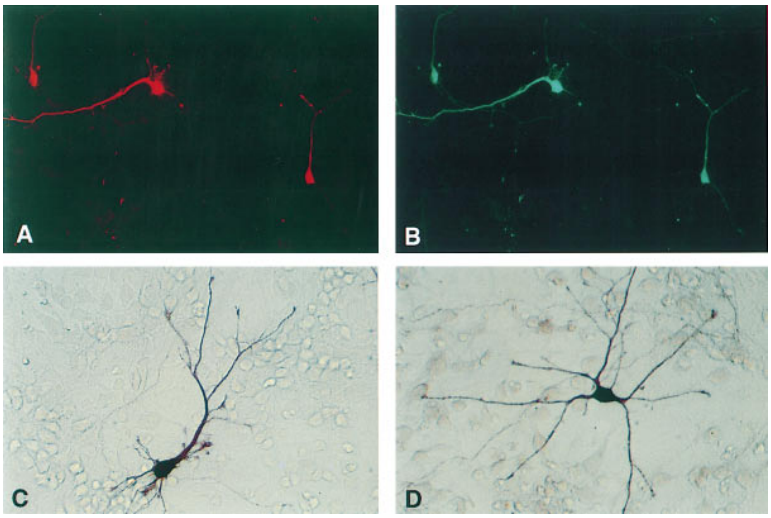

Figure 2. Examples of Cortical Neurons in Culture Transfected with Reporter Expression Plasmids

(A and B) Cortical neurons were cotransfected with expression plasmids encoding chloramphenicol acetyl transferase (CAT) and β-galactosidase at 1 day in vitro (1 DIV), fixed at 3 DIV, and processed for double immunofluorescence using rabbit anti-CAT and mouse anti-β-galactosidase antibodies. The anti-CAT antibody was detected using a Texas red-conjugated secondary (A) and the anti-β-galactosidase antibody was detected using a fluorescene-conjugated secondary (B). Comparison of fields (A) and (B) indicate that the transfected neurons express both the transfected CAT and β-galactosidase genes.

(C and D) Morphologically distinct cell types can be identified in culture. Cortical neurons were transfected with a β-galactosidase expression plasmid and visualized using anti-β-galactosidase.

(C) Example of a neuron with pyramidal morphology, characterized by a pyramidal cell body, one major primary dendrite that gradually tapers away from the cell body, and several minor basal dendrites.

(D) Example of a nonpyramidal neuron, characterized by several primary dendrites that emerge from the cell body and no apparent polarity in the orientation of the dendrites.

forms of these molecules (Coso et al., 1995). These dominant negative (DN) mutants have been shown to specifically inhibit the activation of downstream kinases by the corresponding GTPases and do not affect the function of other Ras superfamily members (Coso et al., 1995; Minden et al., 1995). The morphology of the transfected cells was revealed by cotransfecting the cells with β-galactosidase and immunostaing the cultures with a β-galactosidase antibody. As shown in Figures 3D and 3F, the expression of dominant negative mutants of Rac and Cdc42 led to a marked decrease in the number of primary dendrites in nonpyramidal neurons and in the number of basal dendrites in neurons with pyramidal morphologies, indicating that the functions of Rac and Cdc42 are required for the elaboration of dendritic processes. Dominant negative Rac had the most marked effects in both cell populations and led to a 40%–60% decrease in the number of primary dendrites (Figures 3D and 3F).

To determine whether the distinct effects of the various dominant negatives on dendritic development were related to differences in levels of expression of the transfected constructs, au5 epitope-tagged forms of dominant negative Rho, Rac, and Cdc42 were transfected into cortical neurons together with β-galactosidase. As shown in Figure 4, immunofluorescence using anti-β-galactosidase and anti-au5 indicated that the levels of expression of the three constructs were indistinguishable, suggesting that the distinct effects of dominant negative Rho, Rac, and Cdc42 are not related to differences in expression. As would be expected from a short six amino acid tag, the morphological effects of au5-tagged constructs were indistinguishable from the untagged forms (data not shown).

The dominant negative mutants of Rho-related GTPases are believed to act by competing with the endogenous proteins for exchange factor. To further confirm the involvement of these GTPases in the regulation of dendritic development, we used overexpression of the catalytic domain of RhoGAP p190 and expression of C3 transferase as alternate methods of inhibiting the function of Rho-related GTPases. RhoGAP p190 is a GTPase-activating protein that inhibits the function of all three members of the Rho family, but not members of the Ras, Rab, or Ral families of GTPases (Settleman et al., 1992; Ridley et al., 1993; Coso et al., 1995). C3 transferase has been widely used as an inhibitor of Rho and does not affect the function of Rac and Cdc42. As shown in Figure 5, expression of both RhoGAP p190 and C3 transferase led to a marked decrease in the number of primary and basal dendrites. Importantly, a myristoylated form of RhoGAP p190 was much more effective in inhibiting dendritic elaboration than the unmodified form, suggesting that the relevant target of RhoGAP p190 is most likely associated with the membrane. The inhibitory effects of C3 suggest the involvement of Rho in the process of dendritic elaboration. The failure of dominant negative Rho to inhibit dendritic growth to the same extent as C3 transferase may indicate that the dominant negative is not as completely inhibitory as C3, a possibility that has been noted previously (Qiu et al., 1995).

The above experiments suggest that the normal elaboration of primary and basal dendrites requires the function of Rho-related GTPases. We were therefore interested in determining whether the activation of these GTPases was sufficient to induce elaboration of dendritic processes. For these experiments, cultures were transfected with constitutively active mutants of Rho, Rac, and Cdc42 (glutamine substituted for leucine [QL mutants] at the position analogous to codon 61 of Ras; Coso et al., 1995) together with β-galactosidase, and the consequences were examined 2 days posttransfection. As shown in Figure 6, expression of individual constitutively active mutants led to an increase in the number of primary dendrites in nonpyramidal neurons. These observations indicate that the activation of Rho-related GTPases is sufficient to induce dendritic elaboration and suggest that the initiation and growth of dendritic

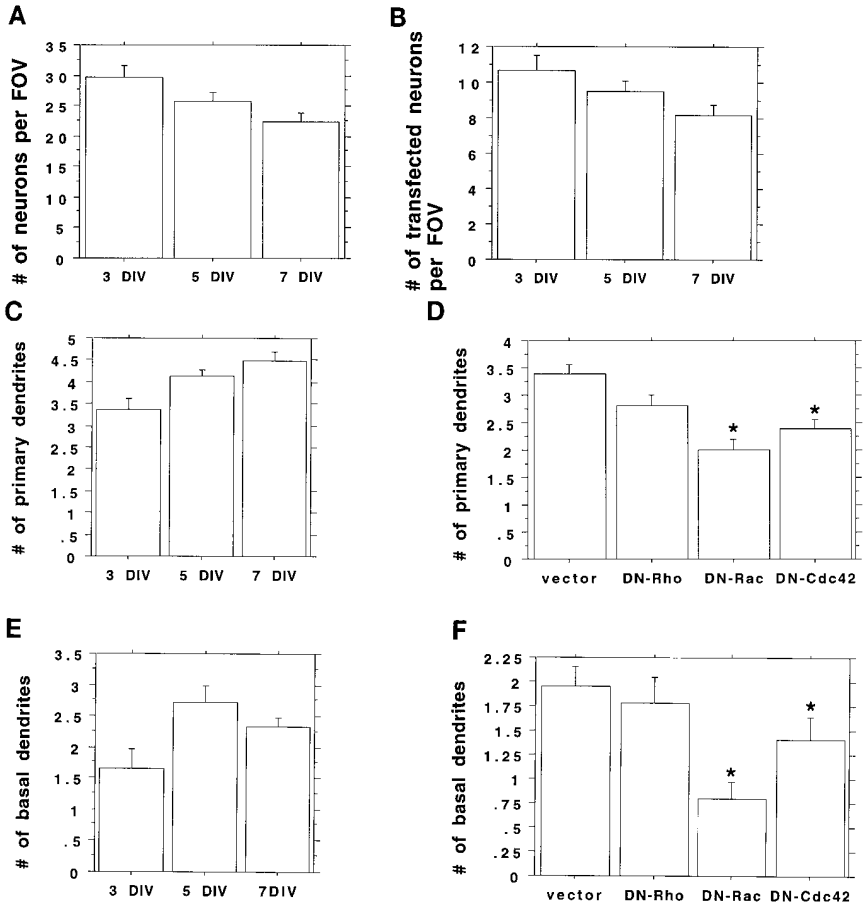

Figure 3. Development of Cortical Neurons in Culture and the Effects of Perturbing the Activity of Rho-Related GTPases on the Elaboration of Dendrites

(A and B) Change in the total number of neurons and the total number of transfected neurons as a function of time in vitro. Cells were transfected with a β-galactosidase expression plasmid at 2 DIV and fixed at 3, 5, or 7 DIV for analysis.

(A) Total number of neurons per field of view (FOV = 25,000 μm^2).

(B) Total number of transfected neurons per FOV (FOV = 1 mm^2).

(C and E) Change in the number of primary dendrites in neurons with nonpyramidal morphologies (C) and the number of basal dendrites in neurons with pyramidal morphologies (E) over time in vitro.

(D and F) Effect of expressing dominant negative mutants of Rho, Rac, and Cdc42 on the number of primary dendrites in neurons with nonpyramidal morphologies (D) and the number of basal dendrites in neurons with pyramidal morphologies (F). For (C) and (E), cells were transfected at 2 DIV and scored at 3, 5, and 7 DIV. For (D) and (F), cells were transfected at 2 DIV and scored at 5 DIV.

processes may normally be regulated by the activation of specific Rho-related GTPases.

It has been reported previously that axonal growth in Drosophila neurons and cerebellar Purkinje cells can be regulated by Rac (Luo et al, 1994, 1996). We were therefore interested in determining whether inhibition of Rho-related GTPases affects the growth of all neuritic processes in cortical neurons. This issue was investigated by examining the effects of transfecting dominant negative mutants of Rho-related GTPases or RhoGAP p190 on the growth of axons and the apical dendrite. Unfortunately, it was very difficult to reconstruct individual axons, which, although clearly identifiable, grew to great distances, frequently intersected other axons, and often had several branches. To get a quantitative sense of axonal growth in these cultures, we instead defined an axonal growth index which serves as a rough measure of total axonal growth per neuron (see Experimental Procedures). As shown in Figure 7, transfection of dominant negative mutants of each of the Rho-related GTPases but not the parent vector led to a >50% decrease in the axonal growth index, indicating that this family of proteins is critically involved in axonal growth in cortical neurons. In contrast, the length of the apical dendrite of pyramidal neurons was not at all affected by the expression of either the dominant negative mutants or RhoGAP p190 (Figure 7B). These observations indicate that whereas the growth of the axon is acutely sensitive to the inhibition of Rho-related GTPases, the growth of the apical dendrite is quite resistant to such perturbations.

Given the striking effects the perturbation of the Rho-related signaling pathways had on dendritic development, we were interested in evaluating the role of Rho-related GTPases in the specification of morphologically distinct cell types. Since the elaboration of primary dendrites in the cortex is associated with a transition from a pyramidal to a nonpyramidal morphology, we examined whether such a transition might also take place in vitro. As shown in Figure 8A, many of the transfected neurons

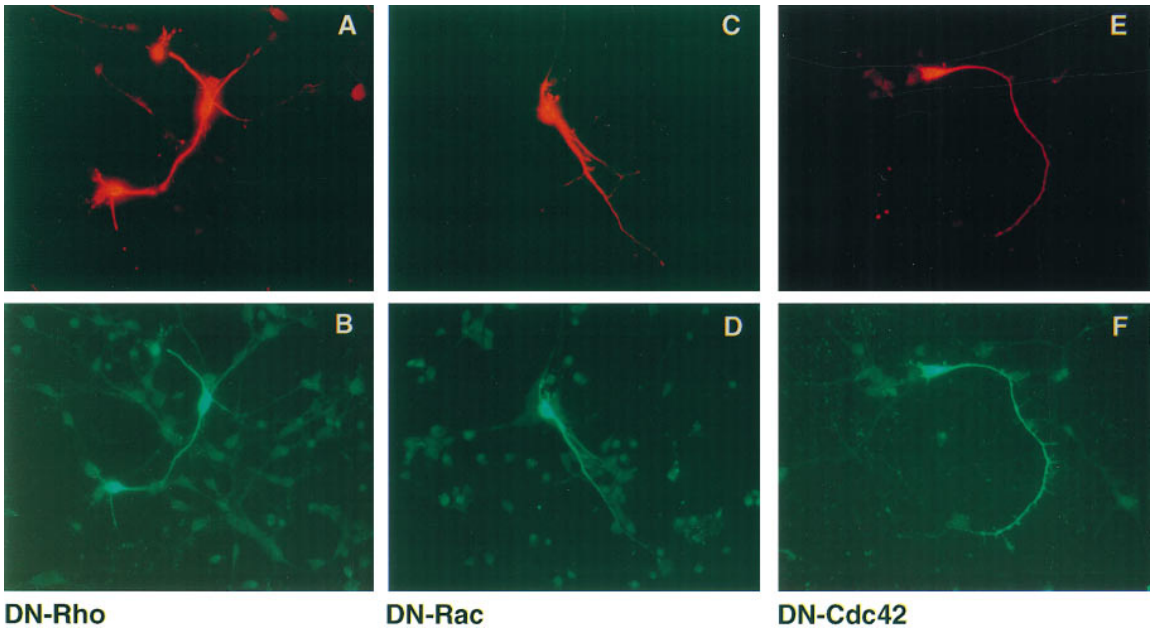

Figure 4. Relative Levels of Expression of Dominant Negative Mutants of Rho, Rac, and Cdc42 in Transfected Cortical Neurons

Cortical cultures were cotransfected with expression plasmids encoding β-galactosidase together with au5-tagged dominant negative Rho ([A] and [B]), dominant negative Rac ([C] and [D]), or dominant negative Cdc42 ([E] and [F]) at 2 DIV, fixed at 3 DIV, and processed for immunofluorescence. Expression of transfected plasmids was detected using polyclonal anti-β-galactosidase ([A], [C], and [E]) and monoclonal anti-au5 ([B], [D], and [F]).

have fairly simple dendritic morphologies at 3 DIV and are often characterized by one major dendrite that resembles the apical dendrite of a pyramidal neuron. By 5 DIV, however, the dendritic morphologies are much more complex, and the majority of neurons have nonpyramidal morphologies (Figure 8B). To determine if there was a systematic change in cell morphology in vitro, we transfected cortical cultures with β-galactosidase at 2 DIV and scored the transfected neurons at 3, 5, and 7 DIV in terms of their overall morphology. As shown in Figures 9A and 9B, there was a marked decrease in the fraction of neurons that had pyramidal morphologies during the first week in culture. There was a corresponding increase in the number of neurons with nonpyramidal morphologies during the same period. This change in distribution is much more dramatic than the relatively small change in the number of transfected neurons over time (Figure 3), indicating that it cannot be accounted for by a differential survival effect. Instead, it appears that a majority of neurons that initially have a pyramidal

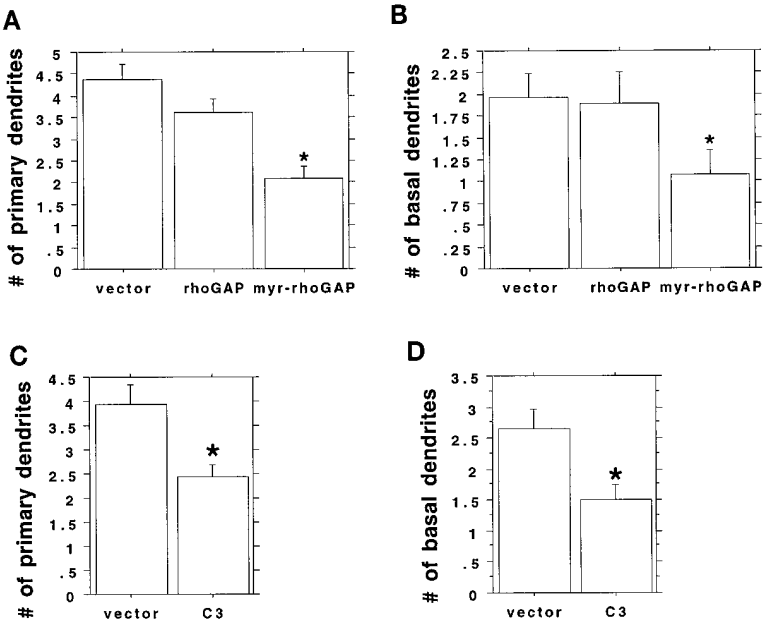

Figure 5. Effects of Expressing RhoGAP p190 and C3 Transferase on Dendritic Development

Cells were transfected with the indicated constructs at 2 DIV and scored at 4 DIV.
(A and C) Number of primary dendrites in neurons with nonpyramidal morphologies.
(B and D) Number of basal dendrites in neurons with pyramidal morphologies.

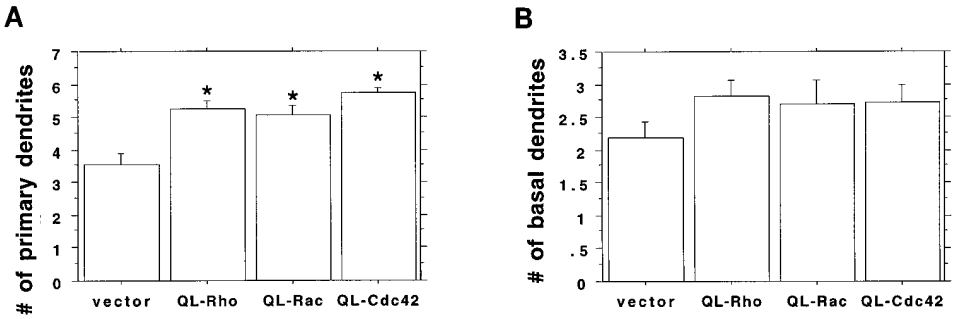

Figure 6. Effects of Expressing Constitutively Active Mutants of Rho-Related GTPases on Dendritic Development

Cells were transfected with indicated constructs at 2 DIV and scored at 4 DIV.

(A) Number of primary dendrites in neurons with nonpyramidal morphologies.

(B) Number of basal dendrites in neurons with pyramidal morphologies.

morphology undergo extensive dendritic remodeling to acquire nonpyramidal morphologies.

To determine whether this remodeling might involve the function of Rho-related GTPases, we examined the morphological consequences of inhibiting this family of proteins. At 1 day posttransfection, ~45% of the transfected neurons have pyramidal morphologies (Figure 9A). In neurons transfected with the parent vector, at 5 days posttransfection, ~15% of the cells have pyramidal morphologies, consistent with their undergoing a remodeling from pyramidal to nonpyramidal morphologies (Figure 9C). In contrast, transfection of dominant negative mutants of Cdc42 and, to a lesser extent, Rac led to a significant increase in the fraction of neurons that maintained their pyramidal morphologies (Figures 8C and 9C). In DN-Cdc42 transfected cultures, >35% of the transfected cells had pyramidal morphologies at 5 days posttransfection (Figure 9C), indicating that in the absence of Cdc42 function most of the neurons fail to undergo a morphological remodeling. Surprisingly, the expression of C3 transferase, which did have a marked effect on the elaboration of dendrites (Figure 5), did not affect the fraction of cells that acquired pyramidal morphologies (data not shown), suggesting that this morphological remodeling may not require Rho function. As an alternate way to examine the requirement of Rho-related GTPases in the acquisition of nonpyramidal morphologies, neurons were transfected with RhoGAP p190. As in the case of dominant negative Cdc42 and Rac, the expression of Rho-GAP p190 led to a marked increase in the fraction of neurons that maintained pyramidal morphologies (Figure 9C). Since these perturbations did not lead to a change in the total number of transfected cells, these observations suggest that the activity of Cdc42, and perhaps Rac, is required for the remodeling of cortical neurons from pyramidal to nonpyramidal morphologies. Expression of constitutively active mutants of Rho-related GTPases, however, did not lead to a significant change in the distribution of pyramidal and nonpyramidal neurons, suggesting that the transformation of neuronal morphology must involve other biochemical events in addition the activation of individual Rho-related GTPases (Figure 9D).

Previous studies have indicated that there is a correla-

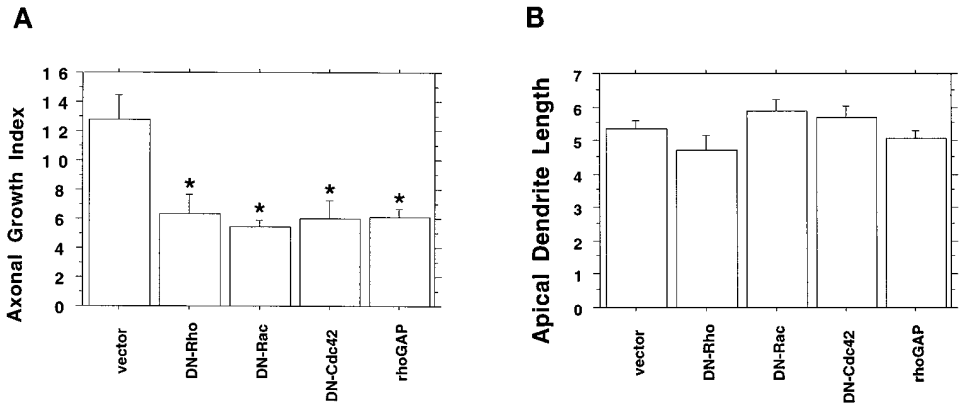

Figure 7. Effect of Inhibiting Rho-Related GTPases on Axonal and Dendritic Growth

(A and B) Effects of expressing dominant negative mutants of Rho-related GTPases and RhoGAP p190 on the Axonal Growth Index (see Experimental Procedures) (A) and the length of the apical dendrite in neurons with pyramidal morphologies (each unit coresponds to 25 μm) (B). Cells were transfected with the indicated constructs at 2 DIV and scored at 5 DIV.

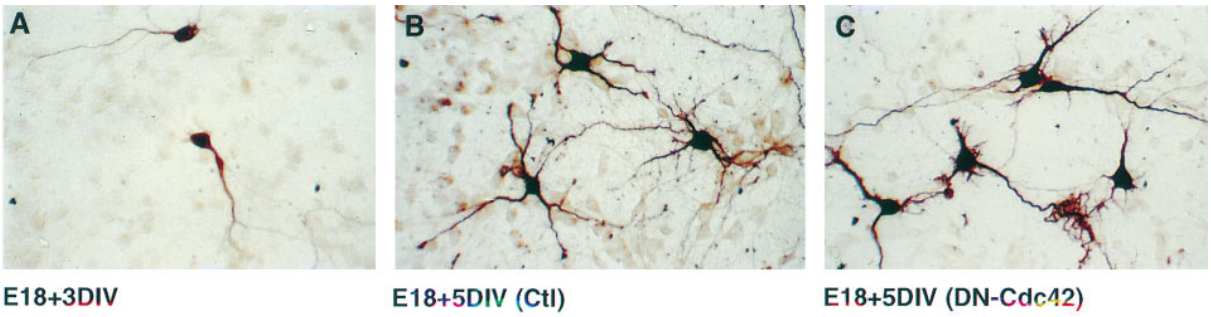

Figure 8. Effects of Inhibiting Cdc42 on Neuronal Morphology

(A) Example of neurons transfected with the parent vector at 2 DIV and visualized at 3 DIV. Many of these cells have one major dendritic process, which terminates in an apical tuft and resembles the apical process of a developing pyramidal neuron.

(B) Example of neurons transfected with the parent vector at 2 DIV and visualized at 5 DIV that are predominantly nonpyramidal in morphology and are characterized by muliple primary dendrites.

(C) Example of neurons transfected with DN-Cdc42 at 2 DIV and visualized at 5 DIV. Many of these neurons have pyramidal morphologies and have reduced basal or primary dendrites.

tion between neurotransmitter phenotype and neuronal morphology in the adult cortex. Whereas pyramidal neurons tend to be excitatory, nonpyramidal neurons can be either excitatory or inhibitory. Since the inhibition of Cdc42 and Rac causes a shift in favor of pyramidal morphologies, we wondered whether this was accompanied by a change in the neurotransmitters expressed by the cells. This possibility was evaluated by examining the distribution of GABA-positive (i.e., inhibitory) neurons in the transfected population by double immunofluorescent labeling of GABA and β-galactosidase. Approximately 10% of the neurons in these cultures were GABA-positive at 5 DIV. Transfection of dominant negative mutants of Rho-related GTPases had no apparent effect on the distribution of GABA-positive neurons (data not shown). This result suggests that at least at early

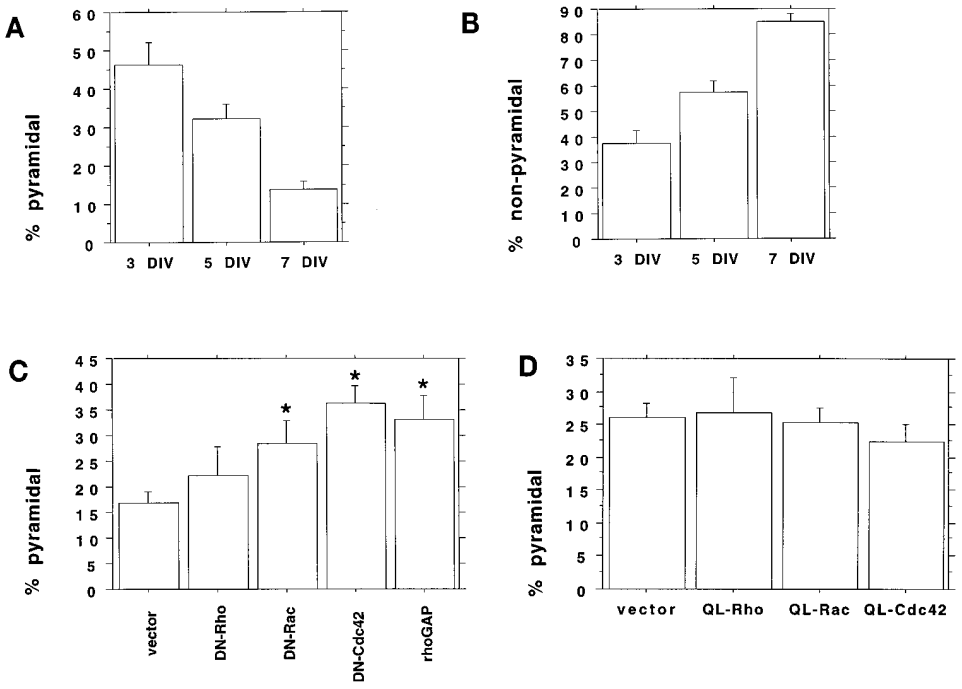

Figure 9. Effects of Perturbing the Activity of Rho-Related GTPases on the Distribution of Neurons with Pyramidal and Nonpyramidal Morphologies

(A) Percentage of cells with pyramidal morphologies at various times in vitro.

(B) Percentage of cells with nonpyramidal morphologies at various times in vitro.

(C) Effects of expressing dominant negative mutants of Rho-related GTPases or RhoGAP p190 on the percentage of neurons with pyramidal morphologies in the transfected cell population. Cultures were transfected at 2 DIV and scored at 7 DIV.

(D) Effects of expressing constitutively active mutants of Rho-related GTPases on the percentage of neurons with pyramidal morphologies in the transfected cell population. Cultures were transfected at 2 DIV and scored at 4 DIV.

developmental stages Rho-related GTPases do not regulate neurotransmitter phenotypes and that the acquisition of a cell type-specific morphology and expression of a neurotransmitter phenotype can be independently regulated.

Discussion

The focus of this study has been an analysis of the role of Rho-related GTPases in regulating dendritic development. We find that expression of dominant negative mutants of Rac and Cdc42, the Rho-inhibitory molecule C3 transferase, or RhoGAP p190 in cultured cortical neurons markedly reduces the number of primary dendrites in nonpyramidal neurons and the number of basal dendrites in neurons with pyramidal morphologies. Conversely, expression of constitutively active forms of Rho, Rac, or Cdc42 leads to the elaboration of dendritic processes. Our findings suggest that the Rho-related GTPases play a central role in dendritic development, and add to the growing body of evidence that supports a role for Rho, Rac, and Cdc42 in regulating cytoskeletal rearrangements.

Recent studies on the extracellular control of dendritic development indicate that the elaboration of primary dendrites in cortical neurons can be regulated by neurotrophins (McAllister et al., 1995, 1997). Since neurotrophins signal through receptor tyrosine kinases, and since Rho-related GTPases have been implicated in receptor tyrosine kinase signaling (reviewed by Hunter, 1997), it is tempting to speculate that the phenotypic consequences of neurotrophins with regard to dendritic development may be mediated via the activation of Rho, Rac, or Cdc42. We have begun to explore this possibility by asking whether neurotrophins can regulate the dendritic development of cultured cortical neurons and whether this involves the action of Rho-related GTPases. Our preliminary findings suggest that BDNF, NT-3, and NT-4 can affect dendritic development in this system (R. Threadgill and A. Ghosh, unpublished data). Interestingly it appears that dominant negative mutants of Rho, Rac, and Cdc42 do not equivalently affect dendritic growth induced by the various neurotrophins. Although we are in the initial stages of this series of experiments, these results are of interest in light of recent reports that suggest that different neurotrophins may promote the growth of distinct subsets of cortical neurons (McAllister et al., 1995, 1997) and raise the intriguing possibility that the specificity of the cellular response to a particular neurotrophin might be conferred by the differential activation of Rho-related GTPases.

The development of cortical neurons in vivo is characterized by dendritic growth and branching as well as extensive remodeling. One of the more striking aspects of this remodeling is the loss of the apical process from a subset of pyramidal neurons, which leads the cells to acquire nonpyramidal morphologies during development (Peinado and Katz, 1990, Soc. Neurosci., abstract). We find that cortical neurons in cultures are capable of the same kind of dendritic growth and remodeling; not only is there an increase in the number of dendrites, there is a population shift from pyramidal to nonpyramidal morphologies over time in culture. Regarding this observation, two points ought to be noted. First, we classify neurons as pyramidal based on the cell body shape and the presence of one thick dendritic process, which gradually tapers from the cell body and ends in an apical tuft. These cells look like pyramidal neurons in vivo, but it is impossible to say with certainty whether the pyramidal neurons in vitro and vivo are exactly the same population. One must therefore consider the term "pyramidal" an operational definition in the context of this study, and we have avoided calling neurons with pyramidal morphologies in vitro "pyramidal neurons," since this term denotes a specific class of neurons in vivo. Second, it should be noted that we do not have any direct evidence that the transformation from pyramidal to nonpyramidal morphologies we observe involves the withdrawal of an apical dendrite. It is, in principle, possible that neurons that start out with pyramidal morphologies acquire nonpyramidal forms by thickening and extending their basal dendrites, without major remodeling of the apical dendrite. It is unlikely, however, that all of the observed nonpyramidal neurons come about this way, since this scenario would predict that virtually all nonpyramidal neurons should have at least one dendrite the length of a typical apical dendrite. This is clearly not the case, since many of the nonpyramidal neurons in culture have relatively short dendrites. A definitive resolution of the cellular basis of the observed dendritic remodeling will require real-time imaging of individual transfected neurons, a goal we are actively pursuing.

One of the more striking results of our perturbation experiments is the finding that the shift in favor of nonpyramidal morphologies can be regulated by Cdc42 and Rac. Whereas a simple interpretation might hold that the activation of Cdc42 or Rac could induce this transformation by causing the withdrawal of the apical process and the elaboration of primary dendrites, such an inference is unlikely to be correct. First, we find no effect of expressing constitutively active mutants of Rho-related proteins on the distribution of neurons with pyramidal and nonpyramidal morphologies, although they are quite effective in promoting the elaboration of primary and basal dendrites. We have also not seen any effect of expressing individual constitutively active mutants on the length of the apical dendrite (R. Threadgill and A. Ghosh, unpublished data). Thus, the mechanism by which Cdc42 and Rac contribute to the transformation in favor of nonpyramidal morphologies is likely to be more complicated than the mechanism involved in the initiation of dendritic processes and will require further investigation. It will also be important to explore the role of Cdc42 and Rac in dendritic remodeling in slice cultures and in vivo, where distinct cell types can be definitively identified.

Although our results suggest that the activation of Cdc42 and Rac is involved in a remodeling from pyramidal to nonpyramidal morphologies, the extracellular signals that may specifically activate these molecules to initiate the remodeling in cortical neurons have not yet been identified. Since the remodeling of pyramidal neurons in vivo is layer specific, one possibility is that layer-specific afferents may provide the extracellular cue. For example, the remodeling of layer 4 neurons may be

triggered by thalamic or cortical afferents that terminate in layer 4. Since our cultures do not include thalamic neurons, and since dendritic remodeling in cortical slices can take place in the absence of cocultured thalamus (Bolz et al., 1990), a cortex-derived signal is perhaps more likely to be involved in triggering the remodeling. It should also be noted that we cannot rule out the alternate possibility that all cortical neurons undergo a Cdc42- or Rac-mediated remodeling from pyramidal to nonpyramidal morphologies as a default, but local cues in layers 2, 3, 5, and 6 inhibit these molecules and thereby prevent the remodeling. Although these are important issues that remain to be addressed, they do not detract from the central observation that Rho-related GTPases play an important role in the specification of dendritic morphologies.

Finally, it is worth considering the role of Rho-related GTPases in the larger context of neuronal differentiation. Recent evidence suggests that neuronal differentiation in the cortex is regulated, at least in part, by the action of neurotrophins, which signal via the activation of receptor tyrosine kinases (Ghosh and Greenberg, 1995; McAllister et al., 1995, 1997). Based on observations in other systems, it is likely that the effects of neurotrophins involve the activation of the Ras signaling pathway. Whereas perturbation of Ras affects differentiation quite globally (Hagag et al., 1986; Szeberenyi et al., 1990), we find that inhibition of Rho-related GTPases affects morphological differentiation without affecting other aspects of differentiation, such as cell cycle arrest or the expression of neuronal proteins such as Tau or MAP2 (A. Ghosh, unpublished data). Recent reports that the cerebellar expression of activated Rac in transgenic mice affects spine formation and axonal growth in Purkinje cells (Luo et al., 1996) and that DRac1 expression affects axonal growth in Drosophila (Luo et al., 1994) indicate that Rho-related proteins can influence various aspects of morphological development. Given that Rho-related GTPases are known to act downstream of Ras in certain cells (reviewed by Hunter, 1997), it is reasonable to propose that neuronal differentiation may involve the sequential action of GTPases of the Ras superfamily. Whereas Ras may function as a master switch to regulate the transition of an undifferentiated precursor cell to a postmitotic neuron, the action of Rho-related GTPases may be specifically involved in defining the morphological phenotype of the neuron. If that is indeed the case, it will be of great interest to explore the possibility that the differential expression or activation of Rho-related GTPases may underlie the generation of morphological diversity in the developing brain.

Experimental Procedures

Northern Blots

Total cellular RNA was isolated from neocortex at the indicated ages using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). RNA (15 μg) from each sample was loaded onto a 1.4% agarose/formaldehyde gel, transferred to nylon membranes, and hybridized with ^{32}P-labeled cDNA probes to RhoA, Rac-1, or Cdc42. Equivalence of relative RNA levels in the various lanes was confirmed by hybridizing the same blots with a probe to GAPDH.

Primary Cell Cultures

E18/19 cortical cells from Long-Evans rats were cultured as previously described (Ghosh and Greenberg, 1995), with the following minor modifications. The cortex was dissected in ice-cold HBSS (6.5 g/l glucose), digested in 10 U/ml Papain in dissociation media (2 × 20 min), and dissociated in culture media. The dissociated neurons were plated on polylysine laminin-coated 24 well plates at 3×10^5 cells per well in glutamine-free Basal Media Eagle (Sigma) supplemented with glutamine (to 1 mM), N_2 (to 1%; Gibco), and fetal bovine serum (5%).

Transfections

Cells were transfected by a modified calcium phosphate transfection procedure, developed in collaboration with M. E. Greenberg and H. Dudek (Harvard Medical School). Briefly, the culture media was removed and replaced with Dulbecco's Modified Eagle Medium (DMEM) 1 hr prior to transfection. The calcium phosphate/DNA precipitate was formed in HEPES buffered saline (pH 7.07) for 15–20 min, at which time the formation of the precipitate was observed as an increase in light scattering. The precipitate (30 μl) was added dropwise to the cells in 500 μl of DMEM. Following a 20–30 min transfection, during which a fine sandy precipitate covered the cells, the cultures were washed in DMEM and returned to the original culture media. The efficiency was typically between 1% and 5%, and there was no apparent toxicity to the cells. The product of transfected gene could be detected immunocytochemically as early as 12 hr posttransfection. In the cotransfection experiments, each well was transfected with 1 μg of RSV-β-galactosidase along with 3 μg of the vector (control) or an expression plasmid for the relevant Rho-related GTPase. In all cases, the DNA was prepared by cesium chloride double-banded maxi-preps. The various plamids used to perturb the function of Rho-related GTPases have been previously described (Coso et al., 1995).

Immunocytochemistry and Immunofluorescence

Cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS; 37°C) for 15 min, washed twice for 5 min with PBS (room temperature), blocked for 2 hr with 3% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS (room temperature), and incubated in the primary antibody (diluted in the blocking solution) overnight at 4°C. The secondary antibody incubation was performed using reagents from Calbiochem (fluorescence) and Vector Labs (peroxidase-based detections). The following antibodies were used: rabbit anti-CAT (5′-3′; 1:500 for immunofluorescence), mouse anti-au5 (Babco; 1:300 for immunofluorescence), rabbit anti-β-galactosidase (5′-3′; 1:1000 for immunofluorescence, 1:5000 for peroxidase), and mouse anti-β-galactosidase (Promega; 1:1000 for immunofluorescence, 1:5000 for peroxidase).

Analysis

In each experiment, transfections were carried out in duplicate wells, and each set of transfections was repeated in multiple experiments. For quantitative analysis of dendritic morphology, between 10 and 15 randomly selected fields and at least 100 transfected cells were scored per transfection condition. Pyramidal neurons were defined as cells that had a pyramidal cell body with one major dendrite that gradually tapered off from the cell body and terminated in a branched tuft (typical of apical dendrites in vivo), with an overall morphology typical of cortical pyramidal neurons (e.g., Koester and O'Leary, 1992). The axonal growth index (AGI) used in the analysis of axonal growth was calculated as follows. Each field of transfected neurons was framed by a 10 × 10 (200 μm × 200 μm) ocular grid (100 squares). For each field, the number of squares on the grid that included an axonal process was counted and divided by the number of cell bodies in the field of view to yield the AGI. (For example, if 60 of the 100 squares had an axon running through it, and if there were 5 cell bodies in the 10 × 10 grid area, then the AGI would equal 12). At least 10 fields scored in this way were used to calculate means and standard errors in the AGI for each transfection condition. This measure is roughly proportional to the total axonal length per neuron. Statistical analysis was performed using the Staview program from Abacus Concepts. Data are shown as mean ± SEM. Statistically significant differences between control and experimental conditions (Student's t test, $p < 0.05$) are indicated by asterisks in the bar graphs.

Acknowledgments

We thank J. Silvio Gulkind for generously providing us with expression plasmids for various Rho-related GTPases and RhoGAP p190; A. Hall and R. Treisman for providing us with the expression plasmid for C3 transferase; G. Redgrave, T. Moeller, and J. Gaare for help in the initial stages of this project; M. Greenberg, S. McConnell, D. Linden, A. Kolodkin, and D. Ginty for comments on the manuscript; and L. Ghosh for assistance in the preparation of the manuscript. This work was supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation, Scholar Award DRS-02 (A. G.).

Received March 24, 1997; revised August 20, 1997.

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