The Role of RhoA Protein in Neuronal Differentiation

Ph.D. Thesis

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I. Introduction

The first Rho protein was found by (and named after) its homology to Ras proteins; (1), members of the Rho family, however, have a much weaker transforming potential, and in contrast to the *ras* gene, the *rho* gene is rarely found mutated in human tumors (2). Almost forgotten for decades, during the early 90's the Rho proteins gained new attention through the findings of Alan Hall and his coworkers, who recognized the pivotal role of these proteins in the regulation of the actin cytoskeleton (3, 4). Ironically, the association of Rho proteins with proliferation, differentiation or apoptotic processes, and nuclear signaling in general, lately became the topic of many research papers (5) and Rho proteins are now believed to be involved in two distinct processes: cytoskeletal regulation and nuclear signaling. However, the connection between the Rho-governed cytoskeletal and nuclear pathways, which bears both theoretical and practical importance, is still to be elucidated.

The PC12 phaeochromocytoma cell line seems to be an especially suitable model system for studying the role of Rho proteins. These small, roundish cells, which proliferate in serum-containing media, stop dividing and extend long, branching neurites on various stimuli, including on treatment with nerve growth factor (NGF). NGF-induced neuronal differentiation includes changes in gene expression and an extensive rearrangement of the cytoskeleton (6). Inhibition of Rho proteins by *Clostridium botulinum* C3 toxin also induces neuronal differentiation in PC12 cells (7). Thus, neuronal differentiation of PC12 cells is an event where nuclear and cytoskeletal events are both involved and interconnected; and Rho is likely to participate in both of them.

This thesis describes the initial phase of our Rho project, when we focused on the role of RhoA – the best known of the three Rho proteins – in neuronal morphological differentiation of PC12 cells. To study the role of RhoA in PC12 cells, we generated and characterized PC12 clonal cell lines expressing various RhoA mutants. We established that RhoA has a role – although an opposite one – both in initiation and elongation of PC12 neurites during neuronal differentiation and regeneration as well (8). Since clonal cell lines stably expressing mutant proteins offer an especially useful model system for biochemical studies, as an extension of this project we plan to study the RhoA-initiated nuclear pathways and try to determine the role of RhoA in the NGF-induced differentiation process.

II. Theoretical background

1. The Rho family of small molecular weight GTPases

The Rho proteins belong to the Ras superfamily of small molecular weight GTPases. This superfamily has more than 80 mammalian members, and can be categorized into at least nine distinct branches (5, 9). All these proteins work as molecular switches and govern different signal transduction pathways. The best studied of them, the members of the Ras family regulate, cell proliferation, differentiation, and survival, and can cause cell transformation (10). Others are involved in as diverse mechanisms as nuclear transport processes (the Ran family), intracellular vesicle formation, and trafficking (the Rab and Arf families) and oxidative burst (Rac proteins, a subfamily of the Rho branch).

Presently, at least 14 mammalian Rho family proteins have been identified, which share significant (ranging from 50 to 90%) sequence identity to each other (Fig. 1). Sequence and functional similarities allow the definition of at least five distinct groups in this family. The members of the groups represented by Rac1, RhoA and Cdc42 – much of our knowledge of Rho family proteins has been derived primarily from the studies of these three proteins – all induce the assembly of actin microfilaments (11), though into different formations, while RhoE and the related proteins (12), as well as RhoD (13), cause the disruption of the actin cytoskeleton. Beside their effect on the cytoskeleton, several Rho family proteins, including Rac1, RhoA, Cdc42 and RhoG, were shown to have a role in cell proliferation and transformation (14-18).

The closest relatives of RhoA are RhoB and C, with a sequence homology of 85% (19). These three proteins are often referred to as "*Rho proteins*," partly because all three are specifically inactivated by *Clostridium botulinum* C3 toxin, a widely used research tool in Rho-related experiments (20, 21). They show, however, significant differences. While rhoA (and C) are constitutively expressed, rhoB is an early response gene inducible by different growth factors as well as cellular stess, e.g. UV irradiation (19, 22-24). Rho proteins also differ in their subcellular localisation: ~75% of cellular RhoA is found in the cytosol, (the inactive pool), and the active form is bound to the cell membrane (25). RhoB, however, is mostly associated with the vesicular compartment (26). Small GTPases use various lipid moieties to bind to the membrane; it includes a geranylgeranyl group for RhoA and C, but either a farnesyl or geranylgeranyl group for RhoB (27). Rho proteins also have different upstream regulation: although Dbl activates all Rho proteins (RhoA, B and C, as well as Rac1 and Cdc42) (28), Ost reacts only with RhoA and Cdc42, not with RhoB (29). Based on the above mentioned differences, as well as on the fact that the sequence differences are concentrated in the C-terminal part of the proteins, in the putative effector region, it should be assumed that RhoA, RhoB and the little-studied RhoC must have considerably different functions. Among the three Rho proteins, RhoA has enjoyed most of the attention and often has served - not quite justifiably - as a representatitve of the group. RhoB, being the putative target of a new family of anticancer drugs, the farnesyl-transferase inhibitors, has recently been under close scrutiny (30). The specific function of RhoC, however, is still elusive.

2. Regulation of Rho activity

The GTPase cycle

Small molecular weight GTPases funtion like binary switches: they are "on" and active when GTP-bound, and inactive when assotiated with GDP (Fig. 2). Three different classes of regulatory molecules influence the relative amount of GTP- and GDP-bound Rho protein in the cell: GTPase activating proteins (GAPs) increase the low intrinsic rate of GTP hydrolysis, which increases the amount of GDP-bound inactive molecules in the cell. Guanine nucleotide exchange factors (GEFs), also called guanine nucleotide dissotiation stimulators (GDS), catalyse the release of bound GDP, thus allowing GTP to bind. The third class of proteins that regulate the activity – the GTP/GDP cycle and the membrane association – of Rho are the guanine nucleotide dissociation inhibitors, the GDIs. GDIs assotiate both with GTP-bound, and with lower affinity, with GDP-bound Rho. GDIs maintain Rho proteins in their existing nucleotide-bound form, but remove them from the membrane, thus inactivate them.

Rho GEFs

Rho GEFs belong to the Dbl family of proteins (28, 31). These molecules share the common DH (Dbl homology) domain, that is responsible for the Rho-GEF activity of the protein. The Dbl family is currently comprised of more than 20 members, and more than half of them were identified on the basis of their ability to induce transformed foci when expressed in NIH3T3 fibroblast. These molecules are strong oncogenes, especially when compared to the Rho proteins, however, activation of the respective Rho protein is required for their oncogenic activity.

The substrate specificity as well as tissue distibution of Rho-GEFs is quite varied, e.g. Dbl activates almost all Rho family members, FGD1 and TIAM (5, 32) are specific for Cdc42 and for Rac1, respectively, Lbc reacts only with RhoA, B and C (33), while Ost activates Cdc42 and RhoA, but neither Rac1, nor RhoB or C (29). This promiscuity of the Rho GEFs (especially compared with the specificity of Ras-GEFs) causes a specific experimental problem. The widely used dominant negative mutants of the small GTPases (e.g. N17Ras or N19RhoA) exert their dominant effect by sequestering the specific activator GEFs (34). Thus, a dominat negative RhoA mutant might theoretically supress all Rho family proteins in one cell type, or only RhoA and Cdc42 in the other, depending on the specificities and relative amounts of the different GEFs present in the cell. This might explain the lack of effect when using a dominant negative Rho mutant, especially when compared to the effect of C3 exoenzyme, which modifies and thus inactivates all available RhoA, B and C molecules, but not Rac or Cdc42. Likewise, C3 and a dominant negative RhoA or RhoB mutant might all have different effect in a specific cell.

RhoGAPs

At least 16 Rho-specific GAPs have been identified to date (5, 35). They serve as negative regulators of Rho, although they are often mentioned as putative Rho-effectors, an assumption supported by the presence of specific functional domains in some of the family members.

An especially exciting example for a GAP serving as an effector could be the newly recognised molecule, named Graf (GAP for Rho Associated with Focal Adhesion Kinase) (36). Its central GAP domain is specific for RhoA and Cdc42, but not for Rac, and it's carboxy-terminal SH3 domain can bind FAK (Focal Adhesion Kinase). What makes it even more interesting is that it is present in PC12 cells; moreover, it is phophorylated upon NGF (or EGF) treatment, and this phosphorylation induces a conformational change in the SH3 domain. Thus, Graf might have a role in the morphological differentiation of PC12 cells, linking NGF signalling, RhoA and Cdc42 to the formation of focal adhesions and neurite outgrowth.

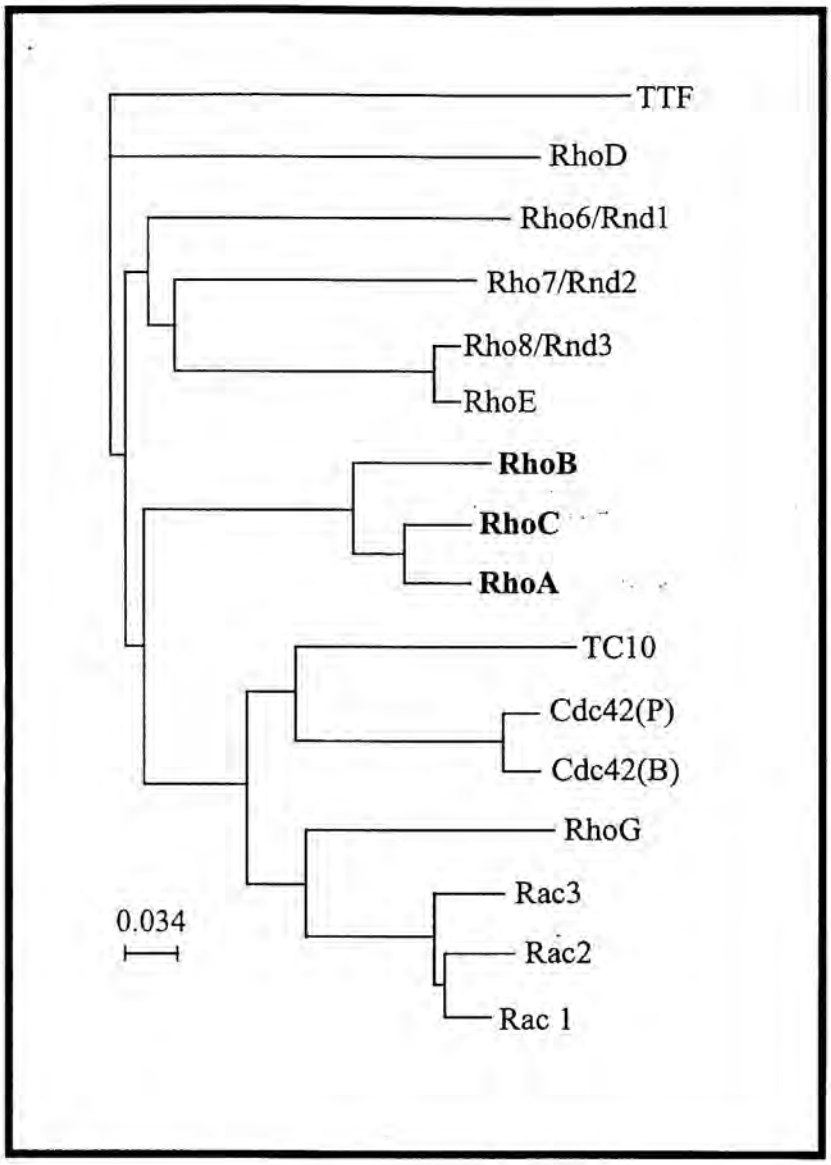

Fig. 1. Rho family dendrogram. The branch lenghts are proportional to the estimated difference along each branch.
(Based on J.C.B. 141. 187, 1998)

Myr5 (fifth unconventional myosin from rat) has a tail region with GAP activity for members of the Rho family, namely for RhoA, Cdc42Hs, and with lower activity for Rac1. Myr5 is able to bind actin filaments in an ATP-regulated manner, so it might provide a direct link between Rho GTPases and the actin cytoskeleton (37).

RhoGDIs

Compared to Rho GAPs and GEFs, only a few RhoGDIs have been discovered (5). The three known molecules are designated RhoGDIα, β and γ. The first discovered RhoGDIα is ubiquitously expressed, and reacts with all Rho-family proteins tested, while RhoGDIβ (or LY GDI) is specific for hematopoetic cells, and RhoGDIγ is preferentially expressed in a small number of tissues, including brain.

The inactive, cytosolic RhoA is associated with GDI, and this complex must be disrupted in order to activate RhoA. Inactivation of RhoA can be achieved through association with Rho-GDI, moreover, phosphorylation of RhoA (e.g. by protein kinase A) increases the affinity of GDI for the protein independent from the bound nucleotide (38).

Although RhoGDIα can associate also with RhoB in vitro, almost all RhoB molecules in the cell are membrane-associated (19, 25), thus the role of RhoGDI in regulation of RhoB is questionable.

The link between cell membrane receptors and Rho

Rho proteins are regulated by several soluble growth factors (including PDGF, EGF, NGF and LPA) (25), as well as by cell-matrix association through integrin and other receptors (39-42). Little is known, however, about the pathways linking these receptors to the inmediate Rho regulators. One possible pathway would include Ras, but growth factors activating a G-protein coupled receptor (e.g. LPA) seem to use Ras-independent pathways to reach Rho (43) possibly involving $G_{\alpha 12}$ and/or $G_{\alpha 13}$ (44, 45). Crk and other adaptor proteins might link integrin and tyrosine kinase receptors to Rho proteins (46), providing another Ras-independent pathway. Compared to the growing knowledge about Rho effectors, new information available about upstream Rho-regulators is rare, but an information burst must be coming.

The adaptor protein Crk seems to have an interesting role in NGF-induced differentiation of PC12 cell. Cellular Crk-II was shown to be phophorylated upon NGF-treatment (47), although Crk did not appear to be necessary for NGF-induced differentiation (48). However, ectopic expression of the activated v-*crk* potentiated NGF-induced neurite outhgrowth (49) when cells were kept in a serum-free medium. This effect might be expalined by Ras-, or Rap1-mediated activation of the Erks (50-52). On the other hand, v-*crk*-expressing PC12 cells show hyperactivation of the Rho-effector $p160^{ROCK}$, and can not extend neurites in the presence of serum or LPA, which keep RhoA in an active state (46). Hence, both the Ras- and RhoA-mediated signalling pathways seem to be potentiated by Crk, and those signalling mechanisms seem to counteract each other. The apparent contradiction might be resolved by assuming a tight spatial and temporal coordination of these pathway, but the details of these regulation remain to be experimentally proven. It is similarly unclear wether Crk is activated primarly by an NGF-receptor mediated pathway and (among others) leads to formation of focal adhesions, or wether Crk is activated primarly by binding to focal adhesions and NGF serves as a secondary modulator of it's activity. The second theory would put Crk in a pathway that can modify the process of neuronal differentiation by cell-matrix or cell-cell interactions.

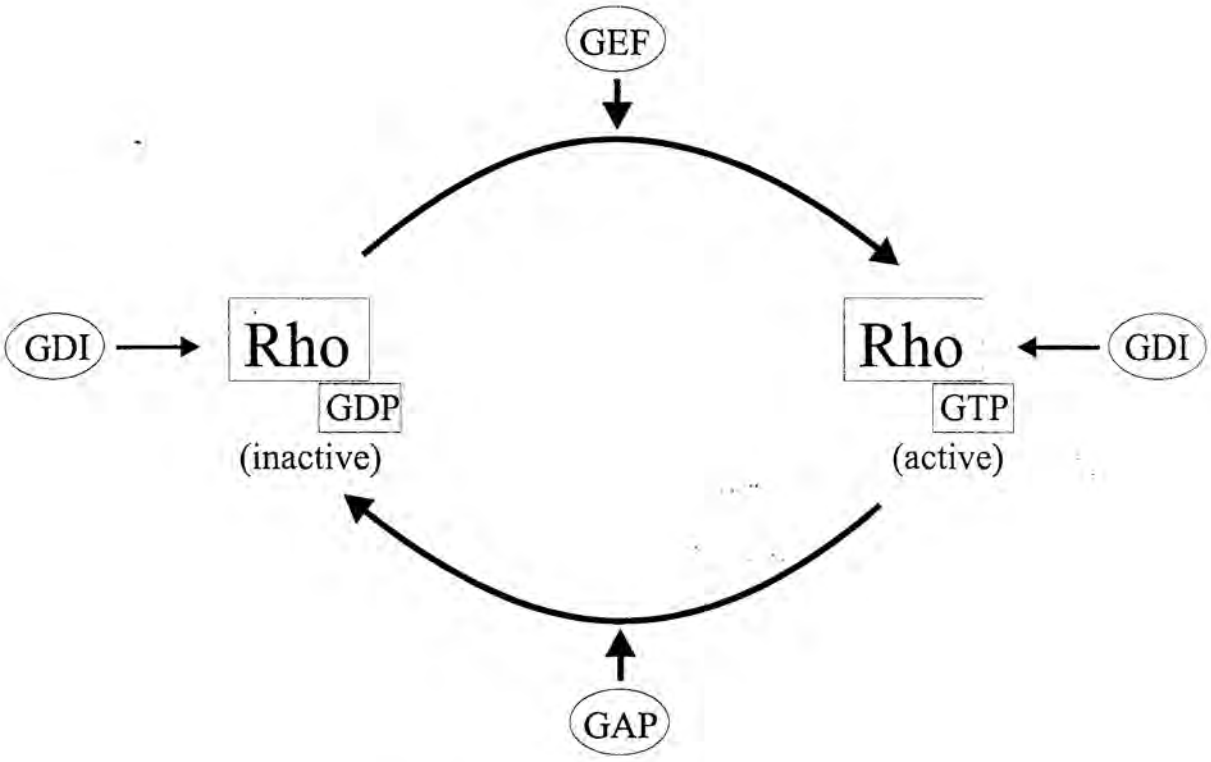

Fig. 2. The GTPase cycle of Rho

3. Rho effectors

Rho protein-effector interactions

Ras and Rho family proteins share only a 30% overall sequence similarity, but show a much higher rate of identity in their core GTP binding sequences, and diverge primarily in their C-termini (5). Mutations affecting the GTP cycle of Rho proteins could be made on basis of the respective Ras mutations, and proved to function identically; V12Ras, V12Rac, V12Cdc42 and V14Rho mutanst are consitutively active, while N17Ras, N17Rac, N17Cdc42 and N19Rho mutants are dominant negative (3, 4).

As with Ras, the conformation of the so called switch I (amino acids 32-40) and switch II domains (amino acids 62-78) of Rho is sensitive to GTP versus GDP binding. Switch I involves the primary effector site of Ras (binding of Raf-1, PI3K and GAP), and clearly indicated in effector binding to Rho (53). Both Ras and Rho family members contain additional putative effector domains: Mutations in these additional effector domains could abrogate specific effector functions (53), more importantly, chimeras involving these domains can switch effector specificity; e.g. the $Rac^{73}Rho$ chimera (which contains the first 73 amino acids of Rac combined with the C-terminal part of Rho) induced stress fiber formation instead of lamellipodia (54). Because these additional effector sites do not seem to be involved in conformational changes upon GTP/GDP binding, a hypothesis that some GTPase-effector interactions (e.g. PI5K (55)) are independent of GTP binding is emerging (5).

Consensus binding motifs in different classes of Rho effectors

In case of the effectors of Rho family proteins, unlike with Ras, at least three clear consensus binding sequences have been identified. The CRIB (for Cdc42/Rac Interactive Binding) motif is found in most but not all Cdc42/Rac effectors, but not in Rho effectors (56). For Rho binding proteins, two distinct sequence motifs, named REM-1 and REM-2 (Rho effector Motif) , have been described (5). Both classes of proteins also contain coiled coil (CC) motifs, which might provide the basis for multimerization of these proeteins with other signalling molecules. Several putative Rho effectors, however, do not contain any of these consensus binding sites, and in some cases (e.g. PI3K) only the Rho-dependent regulation, but no direct Rho-binding was experimentally demonstrated. The different binding motifs seem to recognize different effector sites in Rho. Among proteins containing REM-1, rhophillin was shown to bind the aa region 75-92 that partially overlaps with the switch II region and extends over its C-terminus. RhoGAP and PRK-1 also need this region for binding. Citron, however, associated with the aa region 24-40, while ROCK, a REM-2 motif-containing protein, needed both binding sites for assotiation to Rho (53).

REM-1 motif-containing proteins

The REM-1 motif is shared by two kinases (designated PRK-1 and -2, PKC-Related Kinase) bearing strong sequence similarity to PKC, and two novel proteins lacking kinase domains, named Rhotekin and Rhophilin (57). PRK-1 (originally named p120 PKN, the change also aims to avoid confusion with the earlier described NGF activated kinase) was found to be activated by LPA in a RhoA dependent manner by Watanabe and his team (57). However, Mellor et al. (58) reported that PRK-1 is activated by RhoB rather than RhoA. Moreover, in their experiments, overexpression of PRK-1 failed to induce two well-established RhoA pathways, stress fibers or SRF activation, but they found that PRK-1 was colocalised with RhoB in the endosomal compartment. Additionally, PRK-1 has been shown to phosphorylate neurofilament proteins (59), suggesting that Rho — through PRK-1/PKN — can play a role in the regulation of intermediate filament assembly.

PRK-2 was originally found as an Nck-interacting protein, which binds RhoA but not Rac or Cdc42 (60). Vincent and Settleman (61), however, reported that p140PRK-2 interacts with both Rac1 and RhoA; moreover, the interaction with Rac *in vitro* is completely GTP-

dependent, whereas the interaction with RhoA is nucleotide independent. Among Rho functions, in vitro SRF activation by PRK-2 was described by Quillam et al. (60), while Vincent and Settleman (61) reported that expression of the kinase deficient form of PRK-2 caused disruption of the actin cytoskeleton.

Rhotekin and Rhophilin lack any catalytic activity, but both contain putative protein-protein or protein-lipid association domains (57). Based on this, they might be involved in translocation of Rho to various components of the cytoskeleton, serve as scaffolding proteins, or even negatively regulate Rho function; however, essentially nothing has been described regarding their function (5).

REM-2 motif-containing proteins

REM-2 is found in p 160 ROCK and in its isozymes, ROCK II/ROKα/Rho kinase (5). Among the identified Rho effectors, p160ROCK has received much of the attention to date. Its role as the Rho effector responsible for cytoskelatal rearrangement seems to be firmly established. This involves induction of focal adhesions and the fomation of stress fibers (62), as well as neurite retraction (63). p160ROCK was also suggested to be involved in activation of SRF and focus formation (64). Mutational analysis of the Rho effector region, however, clearly indicates the involvement of additional Rho effectors in both of these processes (53).

Other Rho effectors

Several Rho-binding proteins lack either REM-1 or REM-2 sequences. p140mDia can interact with both RhoA-GTP and profilin, thus it maight also be involved in regulation of actin assembly (65). p116RIP is another example of proteins that bind equally to GTP- and GDP-Rho *in vitro*. However, when expressed in N1E-115 neuroblastoma cell, p116RIP showed the same activity as C3 toxin or dominant negative RhoA (66). Diacylglycerol kinase Θ is likely to bind to the primary effector region or RhoA in a strictly GTP-dependent manner, however, the kinase is negatively regulated by RhoA. Inhibition of this enzyme might lead to accumulation of DAG, thus to enhanced PKC activity (67). Citron is a protein of unknown function, although it has also a kinase-like domain (5). The myelin binding subunit (MBS) of myelin phosphatase has C-terminal sequences sharing a structural similarity to PRK-1; however, no detectable REM motif was observed (68). The concerted action of p160ROCK and myelin phosphatase in regulating myosin light chaine phosphorylation, thus myosin activation, was demonstrated by several research groups (68, 69).

In addition to the molecules for which direct binding to Rho was shown, some other molecules were reported to be activated by GTP-Rho in crude membrane or cell lysates. They include PI5K (phosphatidyl-inositol 4-phosphate-5-kinase) (55), PI3K (70) and phospholipase D (PLD) (71). PIP2, the product formed by PI5K, binds to a varieaty of actin-binding proteins and is thought to regulate actin filament assembly by uncapping barbed ends of actin filaments and relasing actin monomers. Both PI5K and PI3K were found to be activated also by Rac; moreover, the cytoskeletal effects induced by them are more Rac- than Rho-specific. PLD, or its different isozymes can be activated either by Rho or Arf proteins, or by both of them. It is not clear yet, however, in which Rho function PLD is involved.

4. Rho proteins and cytoskelatal regulation

Rho proteins have been implicated in a variety of cellular processes, including regulation of cell shape, cell attachment, cell motility and invasion, cell-cell interactions and cytokinesis (For rewiev see 11, 72). In all the above mentioned processes, reorganization of the actin microfilament structure plays a crucial role, and the different Rho family members have been linked to specific types of actin rearrangement.

In Swiss 3T3 fibroblasts, RhoA regulates the formation of actin stress fibers, which are made up of filament bundles that abut the plasma membrane at focal adhesions, a structure whose formation is also regulated by this protein (3). Rac controls the dynamics of lamellipodia – pleat-shaped protrusions at the cell periphery that have a role in cell migration (4) – while Cdc42 is involved in the formation of thin, finger-like cytoplasmic extensions called filopodia, which might be involved in recognition of the environment (73). In addition, both Rac and Cdc42 regulate the assembly of focal complexes, an adhesion structure clearly distinct from the ones induced by RhoA (73).

Each GTPase can be activated independently of each other by extracellular signals. Bradykinin directly induces filopodia through Cdc42 (74), while Rac is involved in the generation of lamellipodia by PDGF (4), and LPA activates Rho-dependent stress-fiber formation (3). In fibroblast, however, they can act in concert and form a cascade, in which Cdc42 induces filopodia and also activates Rac. Rac in turn causes the induction of lamellipodia and activates RhoA, leading to stress fiber formation (Fig. 3). Although the Cdc42->Rac->Rho cascade is far from being specific for fibroblasts, it is not ubiquitous either: e.g., in MDCK epithelial cells, Rac failed to activate the Rho pathway (75). There is also a very special relationship among these proteins in neuronal cell wich will be discussed later in detail.

At focal adhesions stress fibers are linked to integrins through a multiple protein complex. Several protein kinases, including FAK (focal adhesion kinase) and Src are assotiated to focal adhesions. Rho activation not merely induces the appearance of focal adhesions, but also incresases the tyrosin phosphorylation at this complex, hence activating signal transduction pathways originating from them. The pathway leading from cell-matrix assotiation through integrin activation to Ras activation, thus cell cycle progress, would be an example for this (76).

Members of the ERM (Ezrin-Radixin-Moesin) family of proteins also serve as general crosslinkers between the plasma membrane (especially the membrane protein CD44) and actin filaments. Evidence is accumulating that Rho has a role in regulating this connection in addition to regulating focal adhesions. ERM family proteins are concentrated at specific sites of the plasma membrane such as cell-cell adhesion sites or cleavage furrows. RhoA was shown to colocalise with ERM proteins at these sites (77), and participates in regulating them. Supression of radixin and moesin also altered growth cone motility and neurite formation in neurons (78), that are well-known Rho-assotiated processes.

After identification of a number of targets for Rho proteins, the mechanism underlying their ability to regulate actin organization remains unclear. One model involves Rho kinase and myosin light chain phosphatase which would increase the amount of active, phosphorylated , thus activate myosin, and induce sliding of the actin filaments. (68, 69, 79). This model might underlie the involvment of Rho in the regulation of cytokinesis, cell motility, and smooth muscle contraction.

Rho family members also seem to be directly involved in actin polymerization. This process, when monomeric G-actin is rapidly turned into polymeric F-actin, is determined by two classes of actin-binding proteins: sequestring proteins, which inhibit actin polimeryzation by binding to G-actin, and capping proteins, which attain the same effect by binding to the fast-polymerizing barbed end of the filament. Both sequestring proteins (e.g. profilin) and capping proteins (e.g. gelsolin) were shown to be regulated by Rho family proteins, both

probably through polyphosphoinositides (65, 80, 81). Thus the Rho-regulated phosphatidylinositol 4-phosphate 5-kinase (PI5K) (55) could be the link between Rho and the reorganisation of the cytoskelton. Other Rho effectors could regulate actin polymerization by association to different actin-binding proteins, like the Cdc42 effector WASP (Wiskott-Aldrich syndrome protein).

It has been suggested that Rho also stimulates the formation of focal adhesions through p160ROCK by a mechanism in which the mechanical tension generated by stress fibers induces the aggregation of integrins (62, 82-85).

It is still an open question how Rho family members regulate each other's activity. Dbl family members, however, are logical candidates for this function. In this regard, it is especially interesting that Ost can be bound to activated Rac, while it can activate Cdc42 and RhoA, suggesting that Ost might regulate a cascade totally different from that in fibroblasts (29). Lipid second messengers (e.g. arachidonic acid) were also implicated in linking Rho GTPases (86).

Although among cytoskeletal proteins, actin microfilaments are considered the primary targets of Rho proteins, several recent studies suggested, that Rho might be directly involved in regulation of the intermediate network. Two direct Rho effectors, ROKα (87) and PRK1 (also named PKN) (59) were reported to phophorylate neurofilament (NF) proteins, and thus regulate neurofilament assembly. A ROK-induced NF phosphorylation that results in NF disassembly would nicely fit into the mechanism of neurite retraction, but has not supported by any experimental proofs as yet.

5. The role of Rho proteins in regulation of neuronal morphology

History

The first indication of the role of Rho proteins in regulation of neuronal cell morphology was provided by experiments with thrombin (88) or lysophospahtidic acid (LPA) (89, 90) a component of serum. The reports stated that administration of these molecules caused the rapid retraction of extended neurites in several neuronal cell lines. LPA also induced the formation of stress fibers in fibroblasts (3), a Rho-dependent phenomenon; additionally, *Clostridium botulinum* C3 toxin, which specifically inhibits RhoA, B and C, suppressed the LPA or thrombin induced neurite retraction (43, 91) The inactivation of Rho by C3 toxin also induces neuronal differentiation, thus cessation of proliferation and extension of neurites in PC12 and neuroblastoma cells (7), further supporting the idea that activation of Rho is counteracting, and inactivation is promoting, neurite extension.

The first implication of other Rho family proteins, namely Cdc42 and Rac, came from a theoretical consideration (92). Filopodia and lamellipodia play an important role in growth cone function, thus pathfinding and extension of neurites. Because in fibroblast the formation of filopodia and lamellipodia were found to be regulated by Cdc42 (93) and Rac (4) proteins respectively, this prompted the suggestion that Cdc42 and Rac also regulate actin dynamics in the growth cone.

Lastly, developmental studies, in which activated or dominant negative forms of these proteins are expressed *in vivo,* provided further evidence for the involvement of Rho-family proteins in the regulation of neurite outgrowth and neuronal morphology in general. Alterations of Rac1 and Cdc42 activity lead to failure of extension of many neurites in *Drosophila* (94), influenced neuronal cell migration and axonal growth in *C. elegans* (95), and expression of constitutively active Rac1 in mouse Purkinje cells caused alterations in dendritic morphology (96).

Terms and model systems

The initial achievements in this field induced a plethora of papers aiming to dissect the role of Rho family proteins in regulation of neuronal cell morphology, thus in neuronal differentiation, neurite initiation, growth cone function, neurite extension and pathfinding, as

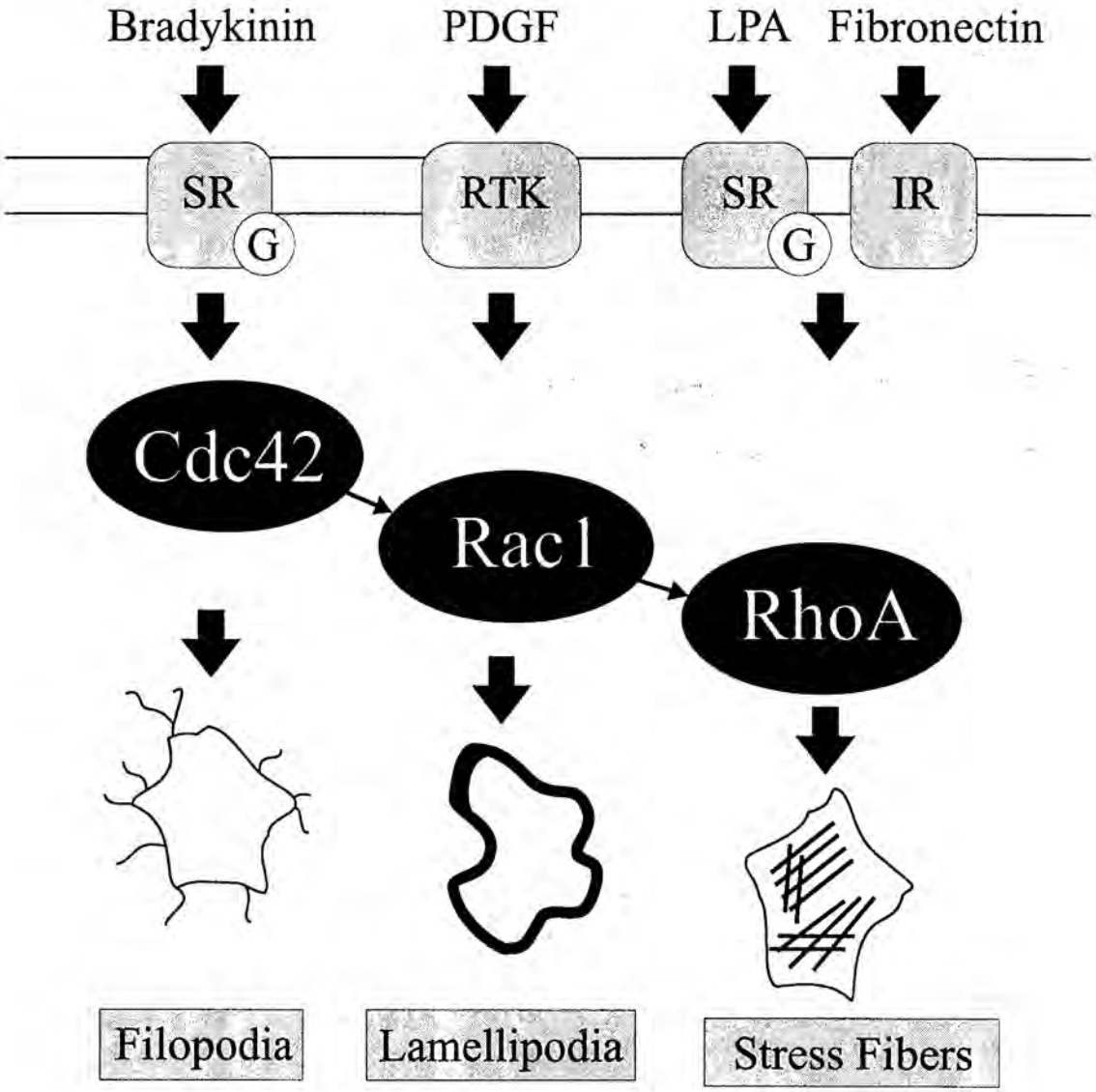

Fig. 3. Cytoskeletal effects of Rho family proteins in fibroblast. (Based on: Oncogene, 17. 1415, 1998)

Abbreviations:
SR - Seven Transmembrane Receptor/Heterotrimer G-protein Coupled Receptor
G - GTP
RTK - Receptor Tyrosine Kinase
LPA - Lysophosphatydic Acid
IR - Integrin Receptor

well as in neurite retraction and regeneration. The terminology used in different papers describing the series of events during neurite formation is quite varied and thus seems to be somewhat confusing. Therefore, a definition of terms necessery for describing neuronal morphological differentiation follows. Henceforth these will be used accordingly, even if the paper cytied applied a different terminology.

Neuronal differentiation describes a process when non-neuronal cells become committed to neuronal lineage. It involves a change in the gene expression pattern of the cell resulting in cessation of proliferation and the expression of neuronal markers which is followed by the appearance of neurites and the characteristic neuronal morphology in general. Growth cone advance, i.e. the formation of new filopodia and lamellipodia at the leading edge of the growth cone, involves mainly the rearrangement of the actin cytoskeleton. Neurite initiation is the beginning of the development of neurites, the "budding" of a new neurite from the cell body. It is likely to involve similar actin cytoskeletal rearrangement as is described for growth cone advance. The number of neurites per cell is often used to characterize the activity of this process, although the final number of neurites also depends on later events, such as stabilization of the neuritic shaft or neurite retraction. Neurite extension refers to the process of lengthening of the neuritic shaft, including actin reorganization and extension microtubules that stabilize the axonal shaft. Neurite extension follows the advance of the growth cone, and the adhesion of the growth cone to the substratum provides the necessary tension for the assembly of the neurite. Nevertheless, the term neurite extension is often used to describe the full process of neurite formation, including neurite initiation, growth cone advance and the extension of the neuritic shaft. Neurite outgrowth can also refer to this whole process. Neurite retraction is the rapid disappearance of a neurite, caused by a rapid increase of actomyosin contractility, accompanied by microtubule and intermediate filament disassembly. The collapse of the growth cone, associated with actin depolymerization, is often the first step of this process; but like growth cone advance and neurite extension, growth cone collapse and neurite retraction can be separated during special experimental conditions. Neurite regeneration can follow the retraction of neurites caused by chemical repellants as well mechanical disruption of the protrusions, as after trituration of cells or isolation of primary neurons. Neurite regeneration involves the initiation and extension of neurites, but does not require a change in the gene expression pattern of the cell.

Despite the large number of papers on the topic of regulation of neuronal morphology, there still is no clear picture of these events. The results are somewhat confusing, and often seem contradictory. To clarify the situation one must consider at least three important differences in the model systems used.

First, the experiments included at least three different cell types: primary neuronal cells of different origin, neuroblastoma cell lines and PC12 cells. These cells differ considerably in regard of their need of differentiation signals and the signal transduction pathways initiated by these signals. Primary neurons are postmitotic, terminally differentiated. They might need specific factors, e.g. NGF, for survival. After their isolation or trituration followed by replating, the mechanism of neurite regeneration can be studied. PC12 cells, on the other hand, proliferate in presence of serum, and need specific signals, such as NGF for induction neuronal differentiation. NGF induced differentiation of PC12 cells requires the activation of the Ras/MAPK pathway (6) and presumably the involvement of Rho family proteins in the process of neurite extension. Neuroblastoma cells used in these type of experiments, e.g. the N1E-115 cell line, also proliferate in the presence of serum, but leave the cell cycle and extend neurites on removal of the serum – thus inactivation of RhoA – or on expression of dominant negative RhoA (66). Consequently, findings in one model system tend not to be always transferable to other cell lines.

Second, different methods were used to modify Rho function; thus, different aspects of regulation and different phases of neurite growth could be studied. After replating of primary neurons, or triturating different proteins into cells, the processes of neurite regeneration, including neurite initiation and neurite extension can be studied. Microinjection

of proteins into differentiated neurons, or induced expression of a mutant in an already differentiated cell, makes possible the separate study of neurite extension. Transfection of proliferating cell lines, e.g PC12 cells, enables the study of neuronal commitment, followed by the initiation and elongation of neurites. Because neurite initiation and neurite elongation involve the rearrangement of different cytoskeletal elements, they are likely to be differently regulated. Thus, one must consider whether a specific experimental design targets one ore more of these processes.

Third, neuronal morphological differentiation depends not just on soluble growth factors and chemical repellants, but it is also profusely influenced by cell-matrix and cell to cell interactions. The different matrices induce different sets of receptors, and also have different effects on neurite behavior. For example, PC12 cells growing on N-CAM-expressing fibrolasts extend neurites even without NGF treatment, but myelin strongly inhibits neurite formation; Rac1 seems to be involved in growth cone advance of primary motor neurons growing on laminin or fibronectin (known activators of the β_1 integrin receptor), but not on poly-D-lysin (97). In our experiments we always used non-specific matrices (e.g. poly-D-lysin or Matrigel, as opposed to laminin); this aspect of differentiation is not discussed in this summary.

Mechanism of neurite retraction

Among the mechanisms involved in regulation of neuronal morphology, neurite retraction seems to be the one best understood. It can be caused by several chemorepulsive factors, such as thrombin (88), lysophospahtidic acid (LPA) (43, 91), sphingosine-1-phosphate (98), bradykinin (99) or prostaglandin E2 (PGE_2) (100). Shortly (seconds in neuroblastoma cells (99), 5-15 minutes in PC12 cells (43)) after the application of these agents, filopodia of the growth cone retract, growth cones collapse, neurites shorten and can even completely disappear during the following hour. The cell body, which usually flattens on neurite induction, also rounds up. Contraction of the actin-cytoskeleton is apparently involved in these events, driven by the motor protein myosin.

An elegant demonstration of the contraction of the neurite on the local application of LPA was designed by Jalink et al (91). In this assay they mechanically detached the shaft of a long neurite from the dish, and then subjected the loose part of the neurite to a continuous flow of medium in such a manner as to bend and thus stretch it. Application of LPA caused the rapid shortening of the neurite, indicative of contractile force generation. Cells treated with the actin-disrupting agent cytochalasin D failed to shorten their neurites, similarly to those treated with KT5926, a selective inhibitor of the myosin light chain kinase (MLCK), an important regulator of myosin activity.

C3 toxin, which inactivates RhoA (as well as RhoB and C), prevents neurite retraction and growth cone collapse caused by LPA and the previously mentioned agents (43, 91). Moreover, microinjection of activated V14RhoA induces neurite retraction in the model systems tested (101). Thus, the involvement of RhoA in their signal transduction pathway is well supported. Rho, however, is not necessarily involved in growth cone collapse induced by every agent. Collapsin-1, for example, induces growth cone collapse accompanied by cessation of neurite outgrowth, but not neurite retraction, and the signal transduction pathway goes through Rac (102).

Among several targets of Rho, p160ROKα was shown to participate in Rho-dependent contractile events, such as formation of stress fibers and the regulation of cytokinesis (69). p160ROKα is enriched in brain, and expressed in NGF-differentiated PC12 cells. Katoh et al. (101) demonstrated that microinjection of activated V14RhoA or the constitutively active form of p160ROKα into differentiated PC12 cells induced a rapid neurite retraction. On the other hand, microinjection of the kinase deficient mutant of ROKα had no effect; moreover, the dominant negative form prevented the PGE induced neurite retraction (100).

Hirose at al. (103) used a different model system, the N1E-115 neuroblastoma cell line. These cells cease to proliferate and extend neurites on serum removal, thus inactivation of RhoA. Hirose at al. have shown that Y-27632, a specific inhibitor of p160ROKα, prevented LPA-induced neurite retraction, while unlike normal cells, those expressing activated V14 RhoA or overexpressing the wild–type p160ROKα failed to extend neurite in absence of serum.

The contraction of the actomyosin system in smooth muscle and non-muscle cells is thought to be regulated by two mechanisms: one is dependent on increase in free Ca^{++} ion in the cytoplasm in the cell and is mediated by myosin light chain kinase (MLCK), and the other is a Ca^{++} sensitization mechanism (103). Both mechanisms lead to increased myosin light chain phosphorylation. Several lines of evidence indicate a role for the ROCK family in the latter mechanism. The myosin binding subunit of myosin phosphatase (68) is a known substrates of ROCK, moreover, Amano et al. (69). demonstrated that the Rho kinase, the p60ROCKα isozyme, might directly phosphorylate MLC. Thus, activation of ROKα leads to phosphorylation and activation of myosin. Hirose at al. (68) have also shown that MLC phosphorylation was elevated in N1E-115 cells during neurite retraction caused by LPA, and this elevation was reduced by Y-27632, a specific p160ROKα inhibitor.

Taken together, these data suggest a model of signal transduction in neurite retraction for both PC12 and neuroblastoma cells: LPA or PGE_2 stimulate their cognate seven-transmemebrane receptors, which in turn activates RhoA through an as yet unknown mechanism. The direct RhoA substrate, p160ROKα phosphorylates, thus inactivates myosin phosphatase, as well as phosphorylating MLC. These two mechanisms together increase the amount of phosphorylated, active myosin, and induce the contraction of the actin cytoskeleton, which leads to neurite retraction (Fig 4).

The mechanism of neurite initiation and growth cone advance

As described earlier, the growth cones guide the extending neurite by constantly protruding and retracting filopodia and lamellipodia. Kozma et al. (93) demonstrated that microinjection of activated V12Cdc42 or V12Rac1 into serum-starved N1E-115 cells promotes the formation of filopodia and lamellipodia at growth cones and along neurites (increasing the size of these processes). Although PC12 cells do not extend neurites on transient expression of activated V12Rac1 or V12Cdc42, the dominant negative (N19) mutants of these proteins inhibit neurite formation in the presence of NGF, as was demonstrated by the decreased number of differentiated, neurite bearing cells expressing these mutants. Moreover, membrane targeting, thus activating the Rac/Cdc42 effector PAK1 (p21 activated kinase 1), did induce neurite outgrowth in PC12 cells (104). Taken together, these results suggest that activation of Cdc42 and Rac1 is necessary for neurite formation in neuroblastoma and PC12 cells.

The Rho-inactivating C3 toxin or expression of the dominant negative N19RhoA also induced filopodia and lamellipodia formation in N1E-115 cells even in the presence of serum (141). The C3 response was inhibited by coinjection of dominant negative N17Cdc42 or N17Rac1, while the Cdc42 response could be competed by coinjection with dominant negative RhoA, suggesting that there is a competion between the Rac/Cdc42 and Rho mediated pathways (93). This is further supported by the results of Leeuwen et al (105), who used N1E-115 cells transiently expressing Tiam1, a guanine nucleotide exchange factor for Rac1. Tiam1 or V12Rac1 expressing N1E-115 cells rapidly produced neurite even in presence of serum, showing that activation of Rac1 leads to neurite formation even in presence of activated Rho. Moreover, if plated in absence of serum, these cells no longer responded to LPA by neurite retraction, suggesting that the Rho proteins could not be activated in presence of activated Rac1. Further support for the opposite effects of Rac and Rho pathways comes from experiments with fibroblast, where the Cdc42/Rac1 effector PAK1 was found to phosphorylate thus inactivate MLC kinase, consequently, decrease the amount

of phosphorylated, active MLC (106). Rho, however, increases the phosphorylation of MLC through activation of Rho kinase (p160ROCK) (69) and inhibition of MLC phosphatase (68).

Because microinjection of the dominant negative N17Cdc42 into N1E-115 cells blocked both the formation of filopodia and lamellipodia, induced by serum removal; while the dominant negative N17Rac1 blocked lamellipodial activity only (93), it can be concluded that inactivation of RhoA leads to sequential activation of Cdc42 and Rac1 in neuroblastoma and probably also in PC12 cells during neurite initiation and growth cone advance.

Based on these data, a model for neurite initiation and growth cone advance in neuroblastoma cells can be formed. In these cells removal of the serum, and thus inactivation of RhoA leads to cessation of proliferation and neuronal differentiation. Inactivation of RhoA, leads in turn to a sequential activation of Cdc42 and Rac1. These activated GTPases recruit PAK1 to the plasma membrane, which might serve as a scaffolding protein for proteins mediating actin polymerization, and formation of filopodia and lamellipodia, leading to neurite initiation and growth cone advance. (Fig. 5.) This model is probably appropriate also for PC12 cells, especially in case of the C3-mediated differentiation. NGF, however, might or might not activate Cdc42 and Rac1 through inactivation of RhoA in PC12 cells. Yet, inactivation of RhoA is very likely part of the NGF initiated signal transduction pathways. Indirect evidence for this was provided by Takahashi et al. (82), who demonstrated that NGF treatment in PC12 cells reduces the amount of Rho available for ADP-ribosylation, thus, the amount of active Rho.

Unfortunately, this model is not likely to work in primary neuronal cells. Although there were no experiments designed specifically to study the role of Rho proteins in neuronal initiation or growth cone advance in these cells, data from related experiments suggest a different relationship between these proteins in primary neurons. In primary cortical neurons, for example, expression of the dominant negative Cdc42, Rac1 or RhoA all decreased the number of primary dendrites and the length of the axon (107). Because the mutated genes were transfected into regenerating neurons with growing neurites, the length of the neurites measures the compound effect of growth cone advance and neurite extension, while the number of neurites mainly indicates the activity of neurite initiation. In primary sensory neurons (chicken dorsal root ganglion/DRG neurons) trituration of C3 toxin increased the length of the regenerating neurite (again, this experimental design measures the compound effect of neurite initiation and neurite extension) (102). The effect of C3 was not influenced by the dominant negative N17Rac1 suggesting that inactivation of Rho does not lead to activation of Rac in these cells. Similarly, no evidence was found for counteraction of Cdc42/Rac and Rho in primary motor neurons during neurite initiation or growth cone advance (97).

The mechanism of neurite extension

The two major cytoskelatal components of the growth cone are filamentous actin (F-actin), which is located predominantly in the peripheral cytoplasmic domain, and microtubules, which are distributed in the central cytoplasmic domain. Microtubules are also the prominent cytoskeletal component of the neurite shaft. Axonal advance, lengthening the neuritic shaft, requires the function of microtubules, and actin filaments appear to play a role in interpreting environmental clues and in guiding microtubules during axonal steering; also the actomyosin network is likely to provide the necessary tension and pulling force for microtubule extension (108).

Cell surface receptors binding to the substrate make functional linkages – form focal complexes - with the underlying actin cytoskeleton. Anchoring of the distal actin network generates tension between the central and peripheral domains of the growth cone. Myosin might be the molecular linker between microtubules in the central domain and actin filaments in the leading edge and the transition zone, and generate pulling force if the distal region of the peripheral domain is restrained by adhesion. A second possibility for generating tension involves the localized contraction of the actomyosin network in the transition zone.

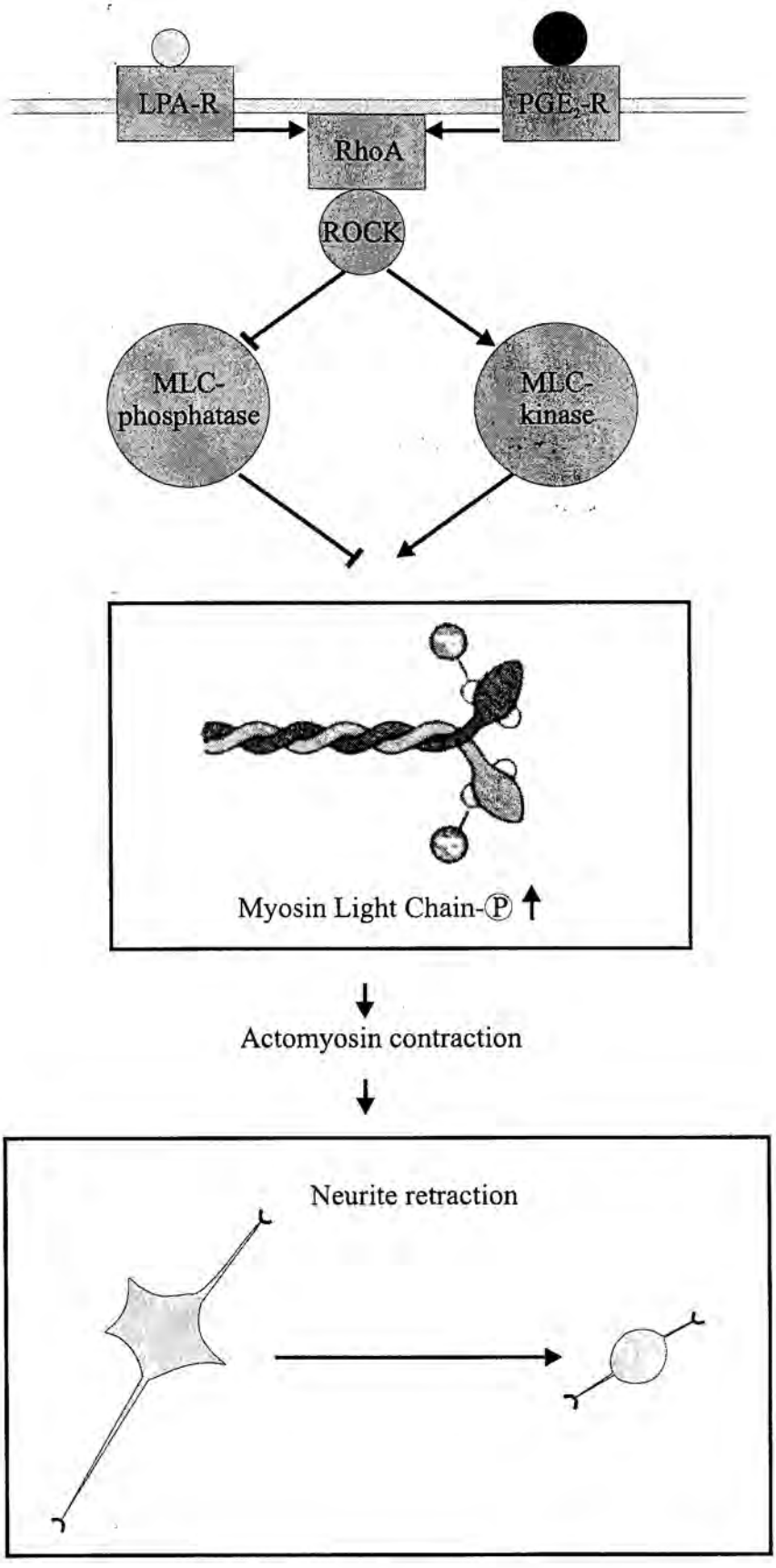

Fig. 4. Mechanism of neurite retraction
(Based on: JBC 273 2489, 1998)

Abbreviations:
MLC - Myosine Light Chain
LPA-R - Lysophosphatydic Acid Receptor
PGE_2 - R - Prostaglandyne E_2 Receptor
ROCK - Rho-Activated Coiled Coil-Containig Kinase

According to a third model, microtubule extension might be promoted by a mechanism not directly related to tension, but rather by microtubule advance into F-actin depleted regions (108).

Interaction between cell adhesion molecules and the cytoskeleton, the different focal complexes, and thus the tension seems to have an important role in neurite extension. PC12 cells expressing one fifth of the normal levels of vinculin, an important molecule of focal complexes, form filopodia and lamellipodia normally at the growth cone, but those have a much shorter lifespan and retract before advance of the growth cone can take place. As a result vinculin-depleted axons grow much slower than controls (109).

An elegant demonstration of the role of tension in neurite extension was provided by Lamoureux et al. (110). They have shown, as others previously, that Rac1 function is necessary for neurite initiation and thus for growth cone motility and advance in PC12 cells, as evidenced by the decreased number of neurites in dominant negative N17Rac1 expressing cells. They state, however, that Rac1 function does not have a role in neurite assembly *per se* thus in elongation of the neuritic shaft. In their experiments, they replaced the normal pulling function of the growth cone by experimentally applied tension. They have found that despite the lower neurite initiation capacity and decreased spontaneous growth-cone mediated neurite elongation, Rac1-deficient PC12 cells respond normally to the experimentally applied tension, thus having a normal linear relationship between the applied tension and elongation rate. Therefore they conclude that Rac1 is required for the motile and adhesive function of the growth cone, but not for neurite elongation.

Growth cone function, i.e. neurite initiation and neurite extension, proved independent in the experiments designed by Wylie et al. (111). They used antisense oligonucleotides to deplete myosin IIB, the predominant myosin isoform in mouse neuroblastoma (Neuro-2A) cells. Their measurements showed no significant difference in the number of neurites per cell body after antisense oligonucleotide treatment at any timepoint of the treatment, as compared with treatments by sense or scrambled oligonucleotides. Formation of short filopodial protrusions and membrane ruffling (considered to be regulated by Cdc42 and Rac1, respectively), remained also unaffected. However, neurite extension, and thus the length of the neurites, was diminished significantly on depletion of myosin IIB. Taken together, this suggests that myosin IIB function is not required for neurite initiation; rather, it is needed only after process formation has begun, while Rac1 (and probably Cdc42) are involved in growth cone advance but not in extension of the neuritic shaft.

In addition to a role in neurite outgrowth, myosin II was shown to have a role also in neurite retraction. In this process, increased myosin light chain phosphorylation (through RhoA and p160ROCKα (69)) as well as MLC phosphatase (68) is clearly indicated. Right now, there is no solution for this apparent contradiction, although Wylie et al. (111) suggested that phosphorylation of the myosin heavy chain by casein kinase II might have a role in regulation of neurite extension. Although depletion of casein kinase II also prevents neuritogenesis in neuroblastoma cells; moreover, casein kinase II was shown to phosphorylate myosin heavy chains *in vitro* (59), the functional role of phosphorylation of myosin heavy chains by casein kinase II remains to be elucidated. Furthermore, there are no reports about any connection between Rho family members and casein kinase II or myosin heavy chain phosphorylation.

Taken together, while activation of Rac1 (and most likely also that of Cdc42) is required for neurite initiation and growth cone advance, as well as for formation of the pulling force for neurite extension, it does not seem to be involved in neurite extension *per se*. Rho, on the other hand, seems to be inhibited during neurite initiation, or at least locally at the leading edge, at the site of filopodium and lamellipodium formation. The role of Rho in generation of the pulling force for neurite extension, which happens at a different location from filopodium and lamellipodium formation, however, has not been investigated as yet.

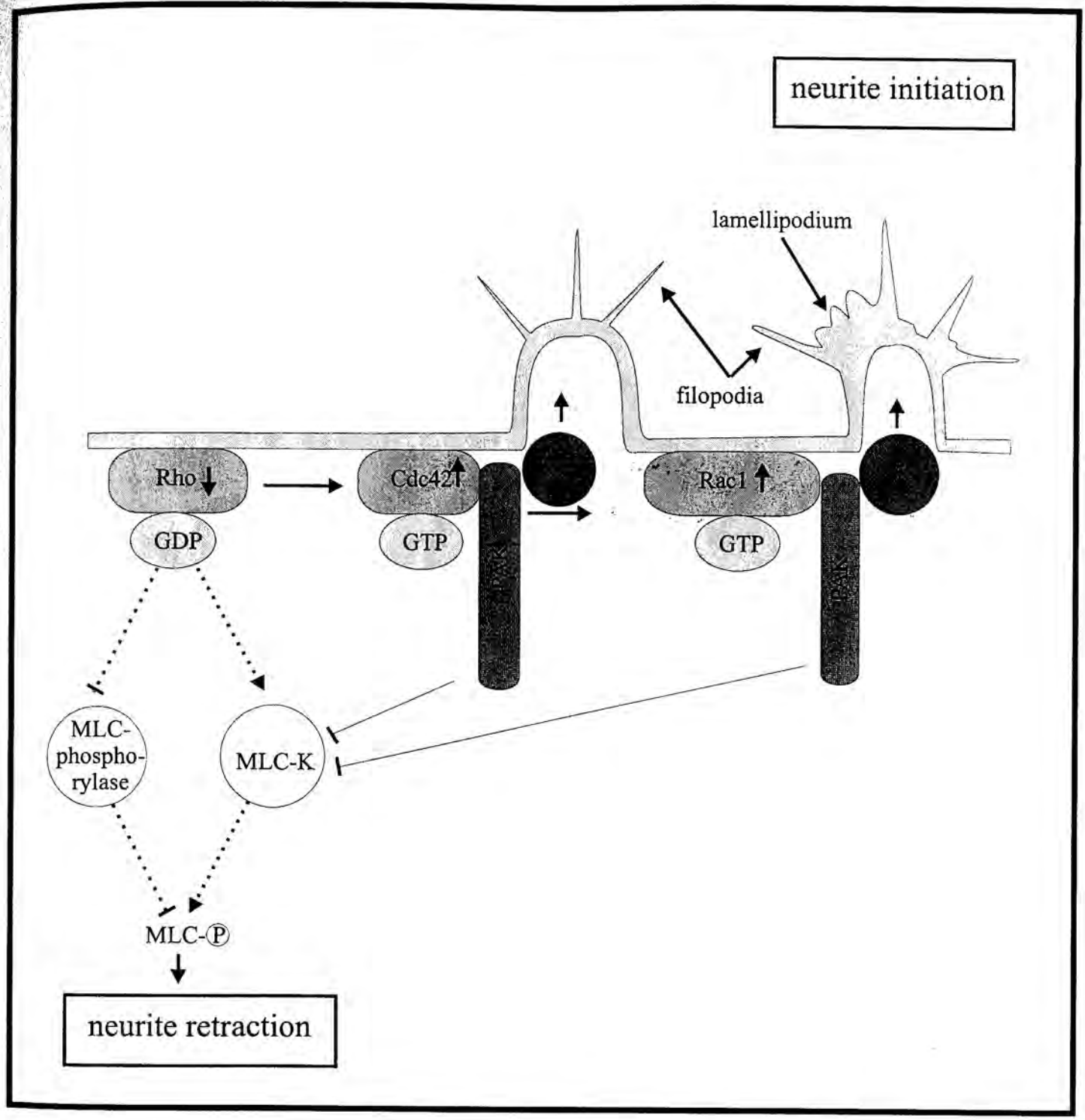

Fig. 5. Mechanism of neurite initiation.
(Based on: EMBO J. 17. 754, 1998)

Abbreviations:
PAK - p21 Activated Kinase
MLC - Myosine Light Chain
MLCK - Myosine Light Chain Kinase
MLC-P - Phosphorylated Myosine Light Chain

Similarly, little is known about the regulation of microtubuli during neurite extension, and there are no reports stating the involvement of any Rho-family protein in this process.

6. The role of Rho proteins in regulation of cell proliferation, transformation and cell cycle regulation

Transformation

A 1998 Oncogene review (5) alludes to Robert Frost's 1916 poem[1] in its title: "The RHOad less traveled gets congested" when describing the newly gained interest in the role of Rho proteins in transformation, proliferation and cell cycle regulation. This road was really almost not taken, or rather abandoned for sake of the other one (i.e. the cytoskeletal effects of Rho), during the first ten years of Rho's history, but has emerged as an important theme during the past five years. Despite our wealth of knowledge on this topic, we still have to learn wether Rho really makes all the difference in Ras and non-Ras-mediated transformation pathways or in regulation of cell cycle in general.

Avraham and Weinberg (112), who first described Rho, identified this protein by its sequence homology to Ras when looking for other Ras-related oncogenic proteins. Rho, however, proved to be only a weak oncogene according to their experiments; even more interesting, the transformation activity seemed to be associated with the wild type rather than activated (V14) RhoA. During the following years almost all Rho-family proteins (RhoA (15) RhoB (113), Rac1 (14), and Cdc42Hs (16)) proved to be more or less oncogenic, but often showed only a partial transforming capacity. For example, fibroblasts transformed with V12Cdc42 formed colonies in soft agar and tumors in nude mice but did not show reduced requirement for serum (16).

Despite extensive searches, hardly any Rho mutations were found in human tumors (2), contrary to the ~ 20% frequency of Ras mutations. However, a newly discovered Rho family member, TTF, was found rearranged by t(3;4) chromosome translocation in certain non-Hodgkin lymphomas (114) but the role of TTF in lymphoma development has not yet been addressed. Even more interesting is the possible involvement of Rho family in function of the BCR-Abl fusion protein in $Ph1^+$ chronic myelogenous leukemia cells, BCR being a bifunctional GAP and GEF for Rac and CDC42 (28). Although these cells might show deregulated Rho protein function, it is not clear, wether the BCR sequences present in p185 or p210BCR-Abl contribute to the oncogenic function (5).

Contrary to the lackluster performance of Rho proteins described above, several Rho-family GEFs, thus activators of Rho proteins, including Dbl (115), Ost (29), and Lbc (33) (for rewiev see (116)), have a strong oncogenic potential. Moreover, Lbc, which is specific for RhoA, B, and C, needs Rho activity for transformation (33). Since Rho seems to be in the crossroad of signaling pathways coming from soluble growth factors and matrix receptors, it is noteworthy that Lbc-transformed cells are matrix-independent, but still need soluble growth factors for proliferation.

Even more compelling evidence for the role of Rho proteins in transformation comes from experiments to delineate Ras effector pathways involving transformation of fibroblast. Although it is clear that the Raf-MAPK cascade is the essential mediator of these processes, several lines of evidence suggest a role for Rho proteins. The early observation that Ras transformed cells undergo morphologic alterations that include cytoskeletal changes, and that activated Ras causes increased membrane ruffling and motility (functions later assigned to

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[1] Two roads diverged in a yellow wood,
And sorry I could not travel both
...
Two roads diverged in a wood, and I-
I took the one less traveled by,
And that has made all the difference.
The Road not Taken by Robert Frost

Rac) provided the first clue for this assumption (117). Later, three experimental approaches supplied important pieces of evidence. First, co-expression of the dominant negative mutants of Rho-family proteins (RhoA (15), RhoB (113), Rac1 (14), Cdc42 (16), and RhoG (18)) caused a significant, although incomplete reduction in focus-forming activity of Ras. Second, co-expression of activated forms of these proteins with activated Raf-1 showed synergism. Third, among Ras effector mutants two (37G and 40C), that lost the ability to bind and activate Raf, retained the ability to cause full transformation, although the phenotype of these cells resembled more that of Rho- or Rac- than Ras transformed cells (118).

A possible link between Ras and Rho pathways was provided by the discovery of p190RhoGAP, a protein that directly associates with p120Ras GAP (119). However, whether this association leads to up– or downregulation of Rho activity is still not clear, nor is the real functional relevance of this pathway. Subsequently, several possible links between the Ras and Rho proteins were suggested, including PI3K, an established effector of Ras (120), candidate effector of Rho (70); arachidonic acid (86) or tyrosine phosphorylation of Rho-GEFs in a Ras-mediated manner, e. g. the Ras-dependent phosphorylation of the Rac-GEF Vav through Lck in T-lymphocytes. (121, 122). Another possibility is the Ral-link: activation of Ral-GDS, a direct Ras effector, leads to Ral activation. A Ral-binding protein, RIP1 contains a Rho-GAP domain, showing the strongest activity towards Cdc42, but hardly any towards RhoA (123). Despite all these possibilities, the exact mechanism that links these pathways still remains to be characterized. Recently, the Ras and Rho pathways have been shown to cooperate in regulation of cdk-inhibitors (namely p27Kip1 and p21Cip1) (124, 125), suggesting that whatever the links between these pathways is, it has a functional relevance.

In addition to the involvement in Ras transformation, Rho proteins also contribute to the transforming action of other oncoproteins. This includes the viral Abl and polyoma middle T (5). In both cases; coexpression of dominant negative Rac1 inhibited focus-formation activity. Several G-protein coupled receptors activate Rho proteins, and were shown to have transforming potential, thus the involvement of Rho proteins in their transforming pathways is quite likely. Among them, two newly recognized receptors, namely mas and XGR were found to cause transformation by activation of Rac and Rho respectively (5).

Among Rho effectors, p160ROCKα might have a role in Rho mediated transformation (126, 127); however, it does not seem to be sufficient for the task (64). Unfortunately, no other possible partners have been identified yet.

Regulation of the cell cycle and proliferation

The function of an oncoprotein by definition must be associated with regulation of cell proliferation. An early indication that Rho family proteins might be involved in regulation of growth comes from the observation that RhoB is an early response gene induced by growth factor stimulation (e.g. EGF, PDGF or NGF) and also by UV exposure (19, 23, 24). The amount of two other members of the family, the Rac-related RhoG and Rac2 in T-lymphocytes, was reported to increase on induction or proliferation. The RhoA and RhoC genes, however, as well as Rac1 and Cdc42, are expressed continuously (5).

Another link between Rho proteins and cell cycle regulation was provided by experiments with C3 toxin that caused Swiss 3T3 cells to leave the cell cycle and accumulate in G_1 phase (128). Inhibition of Rho proteins by C3 toxin in PC12 cells also induces cell cycle arrest; moreover, C3-treated PC12 cells undergo neuronal differentiation, accompanied by expression of neuron-specific genes and neurite extension (7). This is very similar to NGF induced neuronal differentiation; a direct proof, however, for the involvement of Rho proteins in NGF induced differentiation has not been provided to date. The observation that EDIN-induced ADP-ribosylation was increased on NGF treatment of PC12 cells, indicating an

increase in the amount of Rho proteins with non-masked effector regions, thus an increase in the amount of the supposedly inactive Rho proteins, might have served as an indication for this (82).

Similar to C3 treatment, microinjection of dominant negative mutants of Rho proteins, and also Rac1 and Cdc42 into Swiss 3T3 fibroblasts also inhibited cell cycle progression as evidenced by the block of serum-stimulated DNA synthesis, whereas microinjection of constitutively active Rho proteins caused G_1 progression and induction of DNA synthesis (129).

Further support for the association of Rho function and cell cycle regulation comes from the findings that both RhoB and RhoA activity are varying during the cell cycle. RhoB is the product of a growth-factor inducible early response gene, which has very short half-life (~2 hrs). RhoB is completely missing from G_0-phase cells (thus from serum starved and from terminally differentiated cells), but appears at the G_1-S transition, peaks during the S phase and disappears with the S-G_2 transition (19). In contrast, RhoA, as well as RhoC, is constitutively expressed both at the mRNA and at the protein level. Despite the constant amount of total RhoA proteins, the amount of the membrane associated, active RhoA, can be increased on various treatments, including LPA, the Rho-activating lipid growth factor (25). Noguchi et al. (130) has also shown that newly synthesized RhoA is translocated to the membrane of growth factor stimulated thyroid epithelial cells around mid-G1 phase, and the level declines coincident with S-phase entry.

The same research group (130, 131) also provided the first clues on the way RhoA might regulate the cell cycle. They have shown that the level of p27Kip1, a cyclin dependent kinase inhibitor, did not decline in C3-treated thyroid cells on growth induction, and therefore concluded that RhoA activity is necessary for the elimination of p27Kip1, and thus for cell cycle progression in these cells.

p27Kip1 is expressed at high levels in quiescent cells and in cells arrested by contact inhibition(132) or by treatment with cAMP (133) or lovastatin (134). At least the last two can be possibly linked to Rho proteins: the cAMP-activated PKA specifically phosphorylates, thus inactivates, RhoA (38). Although lovastatin, the farnesyl-transferase inhibitor inactivates Ras, some of its effects are more consistent with perturbation of RhoB function (30). Ras, however, is considered the main regulator of cell cycle progression, and several research groups linked Ras to regulation of p27Kip1 (135, 136). An interesting model was suggested by Weber et al. (125) who has shown that PDGF induces G1 progression in Chinese hamster (IIC9) fibroblasts through regulation of cyclinD1 and p27Kip1. They have found that Ras activity is necessary for activation of both cell cycle regulatory proteins. CyclinD1, however, is induced at the mRNA level by a signal tranduction pathway involving the Erks, while p27Kip1 is regulated by protein degradation through RhoA, independently of the Erks. This is consistent with the findings of Hirai et al. (131) in thyroid epithelial cells. Nevertheless, others reported that Erk activity is involved in regulation of p27Kip1 in other cells, including NIH 3T3 fibroblasts, and by this they question the involvement of RhoA in this (135, 136). In PC12 cells neuronal differentiation can be induced by NGF through the activation of the Ras-Raf-ERK pathway (137); moreover, NGF seems also to inactivate RhoA as part of its differentiation pathway (8, 82) An interesting suggestion based on this is that in PC12 cells Ras accomplishes its antiproliferative effect (partly) by upregulation of p27Kip1 through inhibition of RhoA. Right now, this suggestion lacks any experimental support, although p27Kip1 was shown to be involved neuronal differentiation of Neuro-2a neuroblastoma cells induced by thyroid hormones (138), moreover, overexpression of p27Kip1 in N1E-115 neuroblastoma cells induced neuronal differentiation *per se* (139).

Several research groups suggested that a too strong activation of Raf could lead to cell cycle arrest instead of cell growth because in this case p21Waf1 would also be induced leading to inactivation of cdks (140). Olson et al. (124) has suggested that RhoA might counteract this effect, thus signals from Ras and Rho interact to regulate p21Waf1 expression.

Summarizing the data it can concluded that inactivation of Rho proteins (RhoA, B and C) leads to cell cycle arrest also in PC12 cells. Whether inactivation of Rho is part of the NGF-induced differentiation pathways, and if so, how it is achieved and which Rho effector pathways it involves, remains to be elucidated.

7. Regulation of gene expression by Rho

Several lines of evidence suggest that Rho proteins are involved in proliferation and differentiation pathways, so they have to be also involved in gene regulation. Until now, however, only a few of Rho-regulated transcription factors have been identified. Even with those, it is not clear which Rho effector pathways lead to them or which Rho functions are associated with them.

SRF and the related, muscle-specific MEF2 are involved in Rho-mediated muscle differentiation

Because functional SREs are found in the promoters of genes involved in the early mitogenic response, it is surprising that SRF activation might not be involved in the transforming activity of RhoA. However, SREs are also found in the promoters of muscle-specific genes, and two independent research groups reported recently that RhoA signaling via SRF plays an important role in myogenic differentiation. Expression of RhoGDI, which inactivates Rho, Rac, and CDC42, completely suppressed muscle differentiation, but treatment with C3 toxin, which inactivates only RhoA, B and C, or expression of dominant negative N19RhoA, also inhibited the development of myotubes (141). RhoA was found to activate both the c-fos and the skeletal α-actin promoter in muscle cells, which both contain SREs, and dominant negative SRF blocked the V14RhoA mediated skeletal α-actin promoter activation. Neutralization of SRF activity by microinjection of SRF antibodies or blocking of SRF gene expression by SRF antisense oligonucleotides prevented terminal differentiation of myoblasts (142).

Takano et al. (141) also reported that expression of RhoGDI or dominant negative forms of Rho family proteins in myoblasts suppressed transcription of muscle specific genes, especially that of MEF2 myogenin and MRF4, three master regulatory proteins that activate many muscle specific genes. MEF2, like SRF, is a member of the MADs box family of transcription factors. Transcription of MEF2 mRNA as well as binding of MEF2 to the myogenin promoter is regulated by Rho proteins. Since MRF4 functions downstream of other myogenic transcription factors including myogenin, the Rho-mediated regulation of MEF2 was suggested to be the essential factor in Rho mediated muscle differentiation.

Although not activation but inactivation of Rho proteins induces neuronal differentiation of PC12 cells, based on the example of SRF and MEF2, MADs box family transcription factors are good candidates of Rho regulated pathways in PC12 cells too.

SRF is a nuclear target of RhoA as well as Rac1 and Cdc42

c-fos is one of the early response genes induced by serum. This effect is regulated in part by the serum response element (SRE) in the c-fos promoter region. SRE binds two transcription factors, SRF (serum response factor) and TCF (ternary complex factor). The latter needs SRF for its binding to SRE, hence the name. TCF is activated by the Ras-MAPK pathway, which is one of the most important proliferation pathways elicited by serum growth factors. c-fos promoter mutants that cannot bind TCF are not responsive to the Ras-MAPK pathway, but remain responsive to signals elicited by the whole serum, or the serum mitogen, lysophosphatidic acid (LPA). LPA activates multiple signaling pathways, including the Ras-MAPK and Rho pathways (143, 144). Thus, SRF was the first candidate for a Rho regulated transcription factor .

In 1995 Hill and coworkers (145) showed that SRF mediates transcriptional activation by serum or LPA, as well as AlF_4, a general heterotrimeric G protein activator. Activated

forms of the Rho family GTPases, RhoA, Rac1 or Cdc42 can also activate transcription via SRF; moreover, functional RhoA is required for signaling to SRF by LPA or AlF_4, but not by activated Rac1 or Cdc42. They also found that neither the PI3K-, p70S6K-, nor Ca^{2+}-mediated pathways have a role in LPA induced SRF activation, thus neither of these LPA activated pathways, but only the RhoA activated one, leads from the LPA receptor to SRF. They also suggested that neither the integrity of the actin cytoskeleton nor activation of focal adhesion kinase (FAK) - both believed to be downstream of Rho - is required for SRF-linked signaling. This work established SRF as a nuclear target of RhoA, but could not identify the Rho effector pathway leading to it.

This question seems to be still open due to contradictory reports. By using different effector mutants of RhoA, Sahai and coworkers (126) found that signaling to SRF from RhoA did not correlate with binding of Rho kinase (ROK) or protein kinase N (PKN). Therefore, they suggested that SRF activation might be mediated by a still unknown effector. Contrary to this, experiments with activated or dominant negative forms of ROK indicated that Rho kinase could serve as a mediator between RhoA and SRF (101) .

To identify the Rho effector pathway leading to activation of SRF and other nuclear targets would be significant, as it could help to clarify an other interesting question: What is the connection between the cytoskeletal and nuclear function of Rho? Are these pathways regulated independently; that is, do thus do these pathways bifurcate directly at Rho, or is the cytoskeletal rearrangement a prerequisite of the Rho-dependent transcriptional activation?

NFκB might be a link between Rho activation and apoptosis

NFκB (nuclear factor κB) is a member of the Rel family of transcription factors. There are at least seven members of this family, the prototypical DNA binding heterodimer is composed of p50 NFκB1 and p65 RelA. NFκB is normally retained in the cytosol in an inactive form through interaction with IκB inhibitory proteins. Signal induced phosphorylation and subsequent degradation of IκB releases NFκB from the complex, so the liberated NFκB translocates to the nucleus and activates transcription of its many target genes (146). Among them are genes involved in the immune response and inflammatory response (e.g. Igκ, Interleukin/IL-2, Il-1, Il-6, TNFα , (146)), cell-cell adhesion (I-CAM) (147, 148) and cell growth (p53, cyclin D1, c-Myc) (149).

NFκB is activated by a wide range of signals, many of which are thought to be related to injury and inflammation, and to cellular stress and apoptosis. Among them is TNFα (146, 150), the inflammatory cytokin that can induce apoptosis, bradykinin (151) and serotonin, which are rapidly generated following inflammation or injury, and the lipid mediator LPA, which is released from platelets upon activation (152). The role of NFκB in these processes was clearly indicated. NFκB activity is also induced upon N-CAM (Neural Cell Adhesion Molecule) binding to neurons and astrocytes (153). N-CAM mediates cell adhesion primarily through homophilic binding, and affects cell migration, neurite outgrowth and target recognition during development. In response to neural injury, N-CAM can inhibit the proliferative response of astrocytes and promote axonal regeneration.

NFκB is also involved in antiapoptotic signaling. Induction of NFκB is part of the survival pathway activated by $\alpha_v\beta_3$ integrin receptor in endothelial cells (148), and inactivation of NFκB induces apoptosis in H-ras trasformed fibroblast (154). Survival of sympathetic neurons in the presence of NGF also depends on NFκB activity, additionally, NGF can not rescue undifferentiated PC12 cells from apoptosis is NKκB if downregulated (155)

RhoA, Rac1 and Cdc42 were reported to activate the translocation of NFκB by inducing the phosphorylation IκB (156). These Rho proteins are known to be activated by several of the above-mentioned signals, including bradykinin, LPA and by cell-cell and cell-matrix adhesion. Bradykinin activates RhoA, and uses PI3K, a known Rho effector, to

induce NFκB (157). Perona et al. (158) suggested that RhoA and Cdc42 might mediate the activation of NFκB by TNFα, but PI3K is not involved in this pathway. Although there is no direct proof for the involvement of Rho in the LPA mediated NFκB activation, the antiapoptotic effect of LPA in neuronal cell lines, e.g. PC12 cells, where the $G_{i/0}$/Ras/MAPK pathway has a limited role in proliferation and survival, might very well go through the $G_{12/13}$ and Rho proteins to reach NFκB.

Of particular interest is the role of Rho proteins in apoptosis. Reports are contradictory on this; in epithelial cells and fibroblasts RhoA was found to both induce (159, 160) and prevent (161) apoptosis. This might be the case also with PC12 cells, where inactivation of RhoA, B, and C induces differentiation (7), so activation of Rho proteins should rather be involved in proliferative and antiapoptotic pathways. This is further supported by the observation that expression of the activated RhoA does not induce apoptosis in sympathetic neurons, as activated Rac and Cdc42 do (162). In contrast to fibroblasts, in neuronal cells RhoA and Cdc42/Rac proteins are often regulated opposingly; this is the case, for example, during neurite initiation (93, 105). Although not in neuronal cells, RhoA was suggested to regulate NFκB activation (158), which is also part of the survival pathway in PC12 cells and sympathetic neurons (155, 163), similarly to PI3K, a known Rho effector molecule (164). Thus, the role of a RhoA-PI3K-NFκB pathway in the survival of PC12 cells seems to be plausible, but lacks any experimental evidence at present.

DB1 is a RhoB regulated transcription factor

SRF is activated by both RhoA and B (145), but in the case of NFκB only the activation by RhoA is proven (158). DB1, however, is specifically associated with RhoB function, and RhoA or Ras has little or no effect on it. This is especially interesting, because RhoB effectors and functions, especially those that are different from the RhoA mediated ones, are poorly defined. RhoB regulates DB1 by direct binding to it, and as an effect of this, DB1 is unavailable for transcriptional activation (30). This is also a unique mechanism; none of the previously described Rho-regulated transcription factors has a similar way of activation.

DB1 was first identified as a regulator of the IL3 promoter (30, 145), but its expression seems to be ubiquitous. Very little is known, however, about is function. Vezf1, a closely related protein, whose expression is limited to endothelial cells, seems to have an important role in endothelial lineage determination (165). Based on the scarce information it is not easy to predict the role of DB1 in RhoB-mediated processes. However, given the unique properties of RhoB, e.g. its possible role in C3 mediated neuronal differentiation of PC12 cells, any data about it deserves attention.

Several problems remain to be solved regarding Rho. These include identification of both the upstream regulators and downstream effector pathways, however, the most interesting might be to match specific Rho effectors to specific Rho functions.

III. Experimental results

Methods and Protocols[2]

1. Cell Culture

List of Cell Lines Used:

- PC12 — rat phaeochromocytoma cell line
- M-M17-26 — dominant negative Ha-Ras (N17Ras) expressing PC12 cells
- 123.7 — PKA deficient PC12 cells (carrying a mutant PKA regulatory subunit, thus having greatly reduced PKA I and II activities)
- A126-1B2 — PKA deficient PC12 cells (carrying mutant PKA II)
- PGV14/N19/0 — PC12 cells expressing activated (V14) or dominant negative (N19) RhoA from a steroid inducible promoter, or containing the empty vector (PG0, mock transfected cells).
- PEV14/N19/0 — PC12 cells expressing activated (V14) or dominant negative (N19) RhoA from a constitutively active promoter, or containing the empty vector (PE0-mock transfected cells).

PC12 cells (and all clones deriving form this cell line) were grown in Dulbecco's modified Eagle Medium (DMEM) or in RPMI 1640 with 11.1 mM glucose, supplemented with 5% calf serum and 10% horse serum (normal medium).

Stable transfected clones were selected and maintained in the normal medium containing 400 mg/l Geneticin.

For differentiation experiments (with NGF, C3 toxin or Bt_2-cAMP, ionomycin plus/minus NGF) cells were plated on plastic dishes coated with 100x diluted Matrigel and exposed to the differentiation agent in DMEM supplemented with N1 additives. During NGF-differentiation experiments, cells were fed with fresh NGF-containing medium every 2 day.

N1 was replaced with 0.5% fetal calf serum for experiments with clones transfected with the steroid-inducible vector, because the N1 supplement contains progesterone. To induce the expression of this pGRE vector, either 0.5μM Dexamethasone or 0.1 μM Progesterone was added to the medium.

2. Site-directed Mutagenesis

Site-directed mutagenesis was used to generate the dominant negative RhoA (N19RhoA) mutant.

Site directed mutagenesis was done by the Chameleon Mutagenesis kit (Stratagene).

The *in vitro* DNA synthesis reaction included two primers: a mutation primer

[2] Only methods used by the applicant in this project are mentioned. For references and details about the source of materials and cells please see the accompanying papers (Appendix: Tigyi et al., 1996a and b, Sebok et al., 1999)

(5'GCCTGTGGAAAGCAATG-CTTGC-3') that was designed to modify the 19th codon of RhoA by replacing a threonine encoded by ACA, for asparagine encoded by CAA; and a selection primer (5'-CGCAGGAAAGAAGAAGATCTGAGCAAAGGCCCCA-3') that modified a unique Afl III site in the pEXV plasmid to a Bgl II site.

Plasmids containing modified sequences were first selected for the absence of the Afl III site by transformation after Afl III digestion, than the isolated plasmids were checked by Bgl II digestion.

Mutations were confirmed by sequencing the insert.

3. Generation of Plasmid Constructs

List of plasmid constructs generated and used:

- pGRE-N19RhoA,
- pGRE-V14RhoA,
- pGRE-wtRhoA,

List of other plasmids used:

- pEXV-wtRhoA,
- pEXV-N19RhoA,
- pEXV-V14RhoA,
- pEXV,
- pGRE
- pSV2neo,
- GAPDH

Human wild type and activated (V14) RhoA was provided subcloned in the mammalian expression vector pEXV-3. These constructs carry an N-terminal epitope sequence for the myc epitope tag. After generating the dominant negative (N19) RhoA from the wild type RhoA, the wild type, dominant negative and activated (V14) RhoA mutants were subcloned into the steroid-inducible pGRE-5 vector. For this, a 900 bp EcoRI-EcoRI insert from the pEXV vector, containing the full-length RhoA was subcloned into the EcoRI site of pGRE-5. The orientation of the insert was confirmed by restriction mapping.

4. Transfection

Stable transfections were used to generate PC12 cell lines expressing mutant RhoA proteins.

Cell line :	*Plasmids used for transfection:*
• PEV14	pEXV-V14RhoA,
• PEN19	pEXV-N19RhoA
• PE0	pEXV-3
• PGV14	pGRE-V14RhoA\
• PGN19	pGRE-N19RhoA
• PG0	pGRE-0

For transfections, plasmids were isolated by Quiagene Maxiprep Kit.

Stable transfections were done by the calcium-phosphate precipitation method. Each transfection was a cotransfection of 10-20 μg of DNA including the plasmid of interest , the plasmid pSV2neo to provide Geneticine resistance and calf thymus carrier DNA. To increase the transfection efficiency cells were exposed to a 3 minute glycerol shock 5 hrs after transfection.

5. PCR, asymmetric PCR

PCR was used for primary testing of Geneticin-resistant clones, asymmetric PCR was used for generating PCR-products for sequencing

For testing of the presence of the introduced rhoA-construct the primers 5'- AGGAGGACCTGAACC-3'and 5'-ACAAAGCCAACTCTACC-3' were used, complementary to the last five codons of the myc-tag and codons 53-57 of RhoA, respectively. A ~200 bp product was generated from the transfected cell lines, non-transfected cell (cells that has not taken up plasmid DNA) were negative with these primers.

For asymmetric PCR, DNA isolated from the clones was first amplified with the myc-rhoA53-57 primer pair (see above) used in 1 to 1 ratio, than the PCR product was purified to remove the unused nucleotides and reamplified with the same primer pair used at 1:100 asymmetric ratio.

6. Sequencing

Sequencing was used to verify the mutation introduced into rhoA and to verify the presence of the correct rhoA mutation in the clonal cell lines.

Sequencing was done by the dideoxy (enzymatic) sequencing method using a Stratagene kit. Primers included plasmid specific sequences (for sequencing the rhoA-insert) and PCR-primers (see above) (to check the presence of the correct mutation in the clonal cells).

7. Northern blot

Northern blot analysis was used to verify the expression and the streroid-inducibility of the introduced RhoA constructs.

The probes used were:

- a rhoA-specific probe
  A 900 bp EcoRI-EcoRI fragment from the pEXV-RhoA vector containing full-length rhoA cDNA that carried the myc-tag.
- GAPDH probe
  (This probe was used to verify even loading of RNA into the lanes.)

For Northern blots, total cellular RNA was isolated from PCR-positive clones using Trizol reagent (Gibco). According to photometric measurements the $A_{260/280}$ ratio of isolated RNA was generally between 1.6 and 1.9.

RNA (15μg/lane) was separated on 1% agarose-formaldehyde gels and blotted onto nitrocellulose membranes.

Membranes were hybridized at 65 °C, usually with both probes together.

The probes used were labeled with [α-^{32}P]-dCTP with random priming in vitro DNA synthesis reaction using the Ready-To-Go DNA Labeling Kit (Amersham).

Hybridization was visualized on X-ray films and/or by using a PhosphorImager, that made possible the quantitation of bands.

8. Western blots

Western blots were used to verify the expression of the introduced RhoA mutants and for membrane translocation assays.

The antibodies used were:

- anti-RhoA, antibody

mouse monoclonal antibody, against the epitope corresponding to aa 120-150 of RhoA, non cross-reactive with RhoB or RhoC (Santa Cruz, 26C4)

- anti-mouse secondary antibody
  HRP-labeled

For Western blots 3-5 x 10^6 cells were plated in 100 mm dishes.

Cells were washed with Dulbecco's phosphate-buffered saline, and homogenized in ice cold lysis buffer containing a protease inhibitor cocktail (phenylmethylsulfonyl flouride, leupeptine, aprotinin and soybean trypsin inhibitor). The homogenate was centrifuged to remove the debris, and the protein concentration of the supernatant was determined.

Proteins were separated in 15% SDS-PAGE and transferred to nitrocellulose membranes using a semidry blotter.

Nonspecific binding was blocked with 3% serum albumin.

Membranes were incubated overnight at 4 °C with the first antibody (anti RhoA) diluted 1000 to 4000 x in TBST containing 0.3 % serum albumin. Incubation with the secondary antibody was 1 hr at room temperature. The antibody was diluted 1000-2000 x in TBST containing 0.3 % serum albumin.

Membranes were developed with a chemiluminescence-based method.

9. Cell Proliferation Assays

Cell proliferation assay was used:

- to test the role of G_i-mediated Ras-activation in LPA-induced proliferation of PC12 cells (treatment with LPA, LPA+PTX and serum)
- to test the proliferation of mutant RhoA expressing cells. (PEV14, PEN19, PE0 and wt-PC12 cells).

Cells were plated at 0.5-1 x 10^5 cells/well in 6-well plates.

Triplicate or duplicate cultures were harvested resuspended in serum-containing normal medium by vigorous agitation to break up cell clumps and counted in a hemocytometer.

10. Neurite Retraction Assays

Neurite retraction assay was used:

- to study the signal-transduction pathway of LPA-elicited neurite retraction
  Molecules tested included: LPA, Bt_2-cAMP,.PTX, forskolin, , ionomycin, C3 toxin, *C. difficile* A and B toxins
  Cell lines used were: PC12, M-M17-26, A126-1B2, 123.7 (see above)

- to verify that expression of dominant negative RhoA in PEN19 cells was sufficient to counteract activation of RhoA by LPA.
  Cell lines used were: PEN19-1, 2, PE0-1 and wt-PC12 cells.

For neurite retraction assays cells were plated on collagen-coated 30 mm dishes and differentiated with NGF in DMEM supplemented with N1 additive. For some experiments, cells were differentiated with C3 toxin in the same medium. Typically, after 2-3 days of NGF-induced differentiation ~75-80% of cells developed neurites longer than two cell diameter, and neurite retraction was observed uniformly on practically all cells in these cultures. Wild type PC12 cells and clonal cell lines were treated equally and showed similar differentiation properties, with the exception of the dominant negative Ras expressing M-M17-26 cells. M-M17-26 cells do not differentiate on NGF, therefore they were exposed to NGF together with ionomycin (0.2 μM), and groups of neurite-bearing cells were chosen for analysis.

LPA and LPA-like phospholipids were applied in albumin-bound form. To study the signal transduction pathway elicited by LPA, cells were pretreated with various agents (e.g. Bt_2-cAMP, C3 toxin, see above) before addition of LPA.

Neurite retraction was typically followed for 30 to 60 minutes, because most changes in neurite length occur in the first 30 minutes of treatment. During this time, cells were kept at 37 °C and at 5% $C0_2$ levels. This was achieved by placing the inverted microscope fitted with a video camera into a cell culture incubator.

To allow the accurate measurement of neurite length microscopic fields containing at least 15 neurite-bearing cells were chosen, and images were collected from this field immediately before (t=0) and 15, 30, 45 or

60 minutes after addition of the test compound.

Neurite lengths were measured using the NIH Image software implemented on a Macintosh IICi computer. Mean neurite lengths were determined by dividing the combined lengths of all neurites in a field by the number of differentiated cells (Cells bearing at least one neurite longer than the cell body were defined as differentiated; and only processes meeting this criteria at (t=0) were included in neurite length measurements.)

Data are usually presented by using the ratio of mean neurite length at time = 30 min as a percentage of that at t=0 (In most graphs, $\Delta L\%$ is shown as a mean value with its SEM, which represents a mean of ΔL^n obtained in n independent cultures, where n was between 3 and 8.

11. Morphometric Analysis of Differentiated PC12 cells

Morphometric analysis was used to characterize the effect of expression of different RhoA mutants on NGF-induced differentiation of PC12 cells.

Cell lines used were:

- PEV14/N19/0
- PGV14/N19/0
- wt-PC12

Characteristics studied were:

- % Differentiated Cells
- Total neurite output
- Neurite initiation
- Neurite branching

All experiments were done on at least 3 different clones, plus on wild type and mock-transfected cells (2 PG0 and PE0) clones). Every experiment was done at least three times.

For these experiments, cells were plated on Matrigel-coated (100 x diluted) 24-well plates in 2.8-5.6 x 10^3 cells/cm^2. At these low densities, individual neurites could be traced. Three to ten fields containing a total of at least 100 cells were randomly chosen and a video-imaging system was used to collect the images.

Cells bearing at least one neurite longer than the cell body were defined as differentiated (% Differentiated Cells) ; and only processes meeting this criteria were included in neurite length measurements.

Total neurite output was determined by dividing the combined lengths of all neurites in a field by the number of differentiated cells.

Neurite initiation was defined by dividing the number of neurites per soma.

Neurite branching was calculated by dividing the number of growth cones by the number of neurites.

Quantitation of neurite initiation and neurite branching was based on measurements done on solitary cells whose entire neurite tree was traceable.

12. Membrane Translocation Assay for RhoA

Membrane translocation assay was used to study NGF-dependent inactivation of RhoA.

Cell lines used were:

- PEV14, PE0 (mock transfected) and wt-PC12.

5 x 10^6 cell were washed, transferred to microcentrifuge tubes and incubated in DMEM/N1 in a CO2 incubator for 1 hr. The cell suspension was than treated with NGF or Bt_2-cAMP. Following the incubation, cells were pelleted for 30 sec.

The pellet was resuspended in lysis buffer containing a protease inhibitor cocktail, homogenized and a crude membrane fraction was prepared by centrifugation at 50.000xg for 30 min at 4 °C. 40 μg samples of the membrane proteins were loaded per well and electrophoresed. Western blotting was performed as described above.

Results and Discussion

The work presented in this thesis stems from two sources: after being involved in studies concerning the role of Ras GTPase in NGF-induced neuronal differentiation of PC12 cells, I joined a project aimed to identify the molecular targets and signal transduction events that are required for lysophosphatidic acid (LPA) elicited neurite retraction in the same model system. Initially my aim was to study the role of Ras in LPA-mediated signal transduction events, later - based on the date collected - a new project focusing on the role of Rho in neuronal differentiation was initiated.

The role of Ras and Rho GTPases in lysophosphatidic acid -induced neurite retraction of PC12 cells

Our results concerning LPA-initiated signaling pathways were presented in two papers (Tigyi et al, 1996a and b, please see Appendix), here I summarized the experiments dealing with the role of Ras and Rho GTPases in LPA-induced neurite retraction, leading to the new project focusing on the role of RhoA in neuronal differentiation.

LPA is a normal constituent of the serum, generated during blood clotting. LPA elicits various cellular responses in many different cell types, which all appear to affect two elementary cellular functions: cell proliferation and cell shape regulation. Among others, LPA was shown to inhibit neurite outgrowth and cause rapid retraction of the established neurites of NGF-differentiated PC12 cells as well as of various neuroblastoma cell lines. LPA and LPA-like lipids have been identified in the posthemorrhagic cerebrospinal fluid, so LPA might have a role in stroke-related neuronal damage and might even inhibit regenerative sprouting.

The signal transduction mechanism mediating the proliferative response of LPA in fibroblast has been previously linked to the G_i-mediated activation of the Ras-Raf-Erk cascade. Much less was known, however, about the molecules mediating LPA-induced cell shape changes. The aim of this project was to systematically study the role of known LPA-mediated signal transduction pathways in neurite retraction, including the G_i-mediated cAMP-PKA and Ras activation, G_q-mediated phosphoinositide-Ca^{2+}-signaling, and Rho signaling, which was only much later linked to the activation of G_{12} and G_{13}.

Concerning the role of Ras GTPase in neurite retraction of PC12 cells we found that:

- The LPA-induced proliferation of PC12 cells could be attenuated by pertussis toxin (PTX) (J. Neurochem. 66. 537-548. 1996., Fig 3.), suggesting the role of a G_i-mediated pathway.
- LPA-induced neurite retraction, however, was independent of PTX treatment (J. Neurochem. 66. 537-48, 1996., Fig 2.).
- PC12 cells lacking Ras activity (the M-M17-26 clone, constitutively expressing dominant negative N17Ras, and induced for neurite extension by a combination treatment of ionomycin and NGF) developed neurite retraction and cell body spreading comparable to wild type PC12 cells (J. Neurochem. 66. 537-48, 1996., Fig 4.).

PTX has previously been shown to attenuate the mitogenic affect of LPA by disrupting the G_i-mediated activation of Ras (144) in fibroblast. G_i might not couple to Ras in PC12 cells, but based on the third experiment we could conclude that the LPA-induced neurite retraction in PC12 cells is independent of Ras.

Concerning the role of Rho GTPase in neurite retraction of PC12 cells we found that:

- *C. botulinum* C3 transferase, a specific inhibitor of Rho, blocks LPA-induced neurite retraction in PC12 cells (J. Neurochem. 66. 537-548, 1996., Fig 3.), similarly to what was reported on N1E-115 neuroblastoma cells, suggesting that Rho is involved in cell shape changes mediated by LPA.

- The study also analyzed in detail the neurite-protective effect of cAMP. cAMP, similar to C3, contributes to the neuronal differentiation of PC12 cells. We showed that cAMP inhibited LPA-induced neurite retraction (J. Neurochem. 66. 549-558, 1996., Fig 6.). When analyzing the connection between the cAMP system and Rho, we found that C3 still induced neurite outgrowth in PKA-deficient 123.7 cells (Fig 10.); and C3 differentiated 123.7 cells, just like C3-treated wild type PC12 cells, were resistant to LPA-induced neurite retraction (Fig 8 and 10.). Therefore, we concluded that either the PKA and Rho systems work independently of each other, or PKA is upstream of Rho in the LPA-induced morphoregulatory pathway.
  At that time (1996) there was no connection known between Rho and the cAMP system. However, in 1996 Lang et al. (38) reported that in lymphocytes PKA can phosphorylate and thus inactivate Rho independent of the nucleotide bound to it. In view of this, the neuroprotective effect of cAMP could be explained by the PKA-mediated inactivation counteracting the LPA-mediated activation of Rho.
  Following this line of thinking later we found that elevation of the cAMP level by Bt_2-cAMP in PC12 cells removed RhoA from the cell membrane, thus inactivated it (see J. Neurochem. 549-558 1999, Fig 5.). By this finding we proved the presence of a direct link between the cAMP system and RhoA in PC12 cells.

Based on these data we decided to focus on the role of Rho in regulation of neuronal morphology of PC12 cells.

The role of RhoA in regulation of neuronal differentiation of PC12 cells

Our own data and also several other observations suggested that RhoA is not only involved in cytoskeletal changes but might have a more substantial role in the NGF-induced differentiation process. Some of the direct clues include the findings of Nishiki et al. (7) who published in 1990 that C3 toxin, just like NGF, induces neuronal differentiation in PC12 cells. The *in vitro* results of Takahashi and his coworkers (82) linked the Rho pathway even more directly to NGF-induced differentiation: they have shown that pretreatment of PC12

cells with NGF reduces the amount of Rho available for modification by EDIN (Epidermal Cell Differentiation Inhibitor), an ADP-ribosylase purified from *S. aureus* E-1, that modifies the same aa residue in the effector region of Rho as C3 toxin, thus – they concluded – NGF reduces the amount of active Rho.

In order to study the role of Rho proteins in differentiation of PC12 cells we generated stable PC12 clones expressing dominant negative (N19) or activated (V14) mutants of RhoA, the best known of the three Rho proteins (RhoA, B or C). We used two different vectors, one expressing rhoA under the control of a consititutive (PE clones), the other under a steroid inducible (PG clones) promoter. This made it possible to investigate the involvement of RhoA in regulation of the morphological changes at different stages of NGF-induced differentiation. This model system also provides a possibility for biochemical investigations regarding the involvement of RhoA-mediated signal transduction pathways in proliferation and differentiation of PC12 cells. Several data from such experiments were published (8) and are included in this thesis, moreover, we consider this as a primary direction for our future investigations.

Here I summarize our results with the intent to highlight the context and relevance of these findings. These data were published in 1999 in Journal of Neurochemistry, for detailed description of the experiments please see this paper in Appendix (Sebok et al., J. Neurochem. 73. 949-960, 1999.). All Figures mentioned below refer to this paper.

Differentiation and proliferation of clones expressing activated V14RhoA

- Activated RhoA expressing PEV14 cells did not show signs of morphological differentiation in response to NGF. Similarly, PGV14 cells, expressing V14RhoA following steroid induction showed greatly diminished morphological response (Fig 2).
- Inactivation of the mutant V14RhoA either by C3 toxin (Fig.4A) or by Bt_2-cAMP-mediated activation of PKA (Fig.4B.) partially restored the differentiation of the clones, confirming that the lack of differentiation in these clones was due to RhoA activation.
- Induction of the activated V14RhoA also inhibited neurite regeneration (Fig 9.), but C3 treatment reverted the inhibition.

Based on these results we concluded that activated RhoA disrupts NGF-induced neuronal morphogenesis both during initial differentiation of NGF-naive cells and during regeneration of neurites.

- The proliferation of V14RhoA expressing cells (PEV14 clones) in normal, serum-containing medium did not differ significantly from that of control cells.
- Contrary to wild type and mock transfected cells, V14RhoA expressing cells did not cease proliferating but continued to divide in presence of NGF (Fig. 3).
- The activation of Erks which are part of the NGF signaling cascade and whose activation is necessary for NGF-induced differentiation of PC12 cells, was similar in wild type and V14RhoA expressing cells (Fig. 5A).

These data indicate that in addition to its role in neuronal morphogenesis, RhoA appears to affect NGF-induced cessation of cell proliferation without effecting proliferation in presence of serum growth factors. The former seems to be independent of the Ras-Erk pathway, the molecules involved in this mechanism, however, remain to be determined.

- NGF (and also Bt_2-cAMP) removed RhoA from the membrane of PC12 cells, and thus inactivated it (Fig. 5B).

This suggests that that inactivation of RhoA by removal from the plasma membrane is part of the NGF signaling. The signal transduction pathways leading from the NGF receptor to RhoA, however, will have to be addressed in future experiments. Based on the observations that activation of PKA is not necessary for NGF-induced differentiation of PC12 cells (173) we have to conclude that NGF might use other, not yet determined pathways to reach RhoA in order to induce the neuronal phenotype.

Differentiation and proliferation of clones expressing dominant negative N19RhoA

- The proliferation of N19RhoA expressing cells (PEN19 clones) in normal, serum-containing medium did not differ significantly from that of control cells.

This, together with the finding PEV14 cells also proliferate similar to wild type cells, reinforces the idea that the serum-induced proliferation pathways in PC12 cells do not involve RhoA.

- NGF-induced morphological differentiation was greatly enhanced in N19RhoA expressing cells. Upon NGF treatment, both PGN19 and PEN19 cells showed increased neurite initiation and neurite branching (Fig. 6, Table 1 and 2).
- LPA-induced neurite retraction was greatly diminished in N19RhoA expressing cells, indicating that in these clones expression of the dominant negative RhoA was sufficient to counteract activation of RhoA.

The opposite phenotypes of clones expressing the two different RhoA mutants support the inhibitory role of RhoA during the early events of NGF-induced neuronal morphogenesis.

The role of RhoA in neurite elongation

Clones with the steroid-inducible RhoA plasmid enabled us to up-regulate the expression of RhoA not only in naive cells, but also in later stages of neuronal differentiation, when cells are extending their already established neurites.

- Induced expression of V14RhoA after NGF priming did not cause the disappearance of neurites or the resumption of cell proliferation (Fig 7A, Table 3). Similarly, induction of N19RhoA did not increase neurite initiation or branching.
- However, induced expression of V14RhoA after NGF priming caused an ~100% increase in the mean neurite length per soma, whereas N19RhoA caused an ~50% reduction (Fig. 8).
- C3 treatment, which renders RhoA inactive, also decreased the mean neurite length per soma in NGF-primed, differentiating cells (Fig. 8), serving as an independent confirmation of the results with the N19 clones.

Our results indicate that in NGF-primed, neuronally committed PC12 cells RhoA controls the rate of neurite extension, and in general, the role of RhoA during this phase of differentiation is different from the role of RhoA during the early phase of differentiation.

To summarize these results:

We used stable transfected PC12 clones to study the role of RhoA in neuronal differentiation of PC12 cells. We decided on this model system because it makes possible the detailed analysis of signal trasduction pathways using biochemical/molecular biological methods.

After the initial, biochemical characterization of the clones that included PCR, Northern and Western blots (Fig 1.) to prove the expression of the mutated RhoA protein in the cells we designed further tests to prove that expression of these mutant proteins changed the balance of active and inactive RhoA in the cells, and this change was responsible for the effects observed. Although we were unable to use a direct method to measure the ratio of GTP and GDP bound RhoA (e.g.GTP-loading assay), we showed that the expression of dominant negative RhoA was sufficient to counteract activation of RhoA by LPA thus prevent LPA-induced neurite retraction; or the block of differentiation in activated RhoA-expressing cells could be partly neutralized by various Rho-inactivating treatments (Fig 4.).

In this study we focused on the role of RhoA in neuronal morphological differentiation. We established that RhoA has a role - although a different one - both in initiation and elongation of neurites. Using both the constitutively active (PE cells) and inducible (PG cells) expression systems enabled us to recognize the opposite role of RhoA in NGF-naive and NGF-treated, differentiating cells. This observation might help to resolve the contradiction of reports using different model systems thus investigating different stages of morphological differentiation.

Our data contributed to formation of the concept that a Rho-dependent but Ras-independent pathway is involved in the initiation and extension/retraction of neurites both during differentiation and regeneration.

For during the first phase of this project we focused on studying morphological differentiation, the observation that activated RhoA expressing cells failed to cease to proliferate on NGF treatment received relatively little attention, however, we found that the pattern of activation of ERKs is not affected. A RhoA-dependent but Ras-independent signaling pathway that renders neurons resistant to withdrawal from the cell cycle might be of fundamental importance given the probable relation to genesis of tumors. Our ongoing experiments aim to find the molecular targets of this pathway and to locate the site(s) of interaction between the Rho- and Ras-dependent signaling mechanisms.

The phenotype of dominant negative RhoA-expressing cells is characterized by enhanced neurite initiation and branching. As mush as we know this is the first attempt for a detailed morphometric analysis of neuronal differentiation in absence of RhoA activity. The interpretation of these data in a highly simplified cell culture system, however, is largely speculative. A possible explanation would be that in presence of the dominant negative RhoA the repellent signals that act through activation of RhoA and cause the disappearance of filopodia turning to unwanted directions are ineffective and this enables the stabilization of superfluous neurites and branches. Soluble even more extracellular matrix-associated molecules influencing neurite extension and pathfinding are widely studied, but we have not addressed this question. A collaborating research group, however, found that the inhibitory effect of myelin associated glycoprotein (MAG) on neurite extension might be due to

activation of RhoA, since contrary to wild type cells, dominant negative RhoA expressing PC12 (PEN19) cells can grow neurite on MAG matrix (172).

The observation that the induction of activated V14RhoA during later stages of neurite outgrowth increased the rate of neurite elongation is apparently contradictory to previous reports - including ours - stating that activation of RhoA by LPA cause retraction of neurites. The best explanation we are able to offer is that LPA elicited neurite retraction and NGF-elicited neurite extension might represent two different signaling modalities.

Based on these results, a simple model recognizing the different roles of RhoA in neuronal differentiation of PC12 cells was proposed: During the initial phase of differentiation, NGF inactivates RhoA, and inactivation of RhoA promotes neurite initiation. During later stages of morphogenesis, when cells are already committed to neuronal differentiation, activated RhoA promotes neurite extension without affecting neurite initiation.

Biological, physiological and medical significance of this project

Being a medical doctor by training who is fully engaged in basic research I feel compelled to relate my work to real-life problems, to seek the physiological relevance of my findings or rather to seek out problems with physiological/pathophysiological or direct medical pertinence. However fundamental the research is; and regardless of the methodology involving mainly *in vitro* experiments or the model system being an immortalized rat cell line, I firmly believe that this projects meets those criteria and is related to real medical problems and might help to answer biologically important questions.

To begin with the most general aspects this project deals with signal transduction mechanisms related to neuronal differentiation and proliferation of neuronal (precursor) cells. Understanding what is happening in the black box - the cell - when a signal - a hormone or growth factor - reaches it helped us lately to implement new diagnostic procedures or therapeutic methods, to have a clearer picture of the pathophysiological background of diseases. Just to name to examples, knowing that cholera toxin influences the heterotrimer G-protein subunit $G_{\alpha s}$ and increases cAMP levels in intestinal epithelial cells led to a better understanding of this disease and made cholera toxin a valuable research tool. Similarly, the finding that in certain leukemias RARα, a retinoic acid receptor gene is translocated and fused to an other gene, and this fusion protein appears to block cell differentiation thus promote proliferation, made us understand why and how is retinoic acid useful in treatment of this disease, moreover, this therapy can now be targeted to cases positive for this translocation.

The process of neuronal differentiation, the mechanism by which neuronal stem cells differentiate into neurons, and especially the mechanism by which neuronal cells create and regulate their complex morphology are poorly understood in spite of being subject to intense research. For it is a fundamental issue in developmental neurobiology to grasp how neurons elaborate axons and dendrites, their growth cones constantly interpreting environmental cues and steering the neurite in the right direction. The role of Rho family proteins in axonal pathfinding and in regulation of growth cone behavior is now supported by a plethora of data coming from biochemical studies, experiments with primary neuronal cells in cell culture or established clonal cell lines, and even from *in vivo* studies. Zipkin et al. (95), for example, found (in complete agreement with our data arising from a cell line) that after the expression of an activated Rho mutant in *C. elegans*, axons grew rapidly and in an unguided fashion, suggesting - based on *in vivo* evidence - that Rho proteins are involved in regulation of axon growth and pathfinding, but the detailed mechanism and signal transduction pathways involved in it remain to be elucidated. Our model system that is especially suitable for biochemical analysis might contribute to the clarification of these molecular mechanisms.

This research field is also of obvious practical - medical - importance because of its relationship with neuronal regeneration following neuronal injury or stroke. For example, a growth-factor like lipid molecule, lysophosphatidic acid (LPA) that is generated during blood coagulation from activated platelets is a strong and specific activator of RhoA, and causes rapid neurite retraction in PC12 cells and in other neuroblastoma cell lines as well. This suggests that activation of a Rho-mediated pathway might have a role in neuronal cell damage after intracranial hemorrhage and in inhibition regenerative sprouting. This projects stems partly from this observations, and our data presented here prove that activation of RhoA inhibits regeneration of neurites after a mechanical damage (8).

This thesis describes the generation of mutant RhoA-expressing clonal cell lines and

characterizes their morphological differentiation. Using this model system next we aim to study the role of this protein in regulation of proliferation and apoptosis of PC12 cells. RhoA being discovered as an oncogene and the data accumulating of it's role in regulation of proliferation justify this way of thinking; moreover, our finding that activated RhoA-expressing PC12 cells fail to cease to proliferate on NGF treatment (8) suggests that these cells can be used to investigate the problem. Regulation of proliferation is obviously related to oncogenesis, but some recent data indicate that understanding the role of Rho proteins in these processes might lead to new treatment options. Namely, farnesyl-transferase inhibitors, a family of drugs subject to extensive investigations lately are thought to exert their effect through inactivation of Rho proteins.

Future plans

Although first we focused on characterization of our mutant RhoA-expressing clones and on description of their neuronal morphological differentiation, these clones were originally generated with the intent to use them for biochemical studies. Both the literature and our own data support the concept that RhoA regulates the processes of proliferation-differentiation-apoptosis in PC12 cells. Thus, we would like next to concentrate on RhoA-mediated signal transduction pathways involved in these processes and attempt to identify the molecules involved in these pathways.The molecules we are most interested in are the cyclin dependent kinase inhibitors p21Cip1 and p27Kip1, and the transcription factor NFκB.

V. References

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VI. Appendix - Publications

1. Tigyi, G.; Fischer, D.J.; Sebok, A.; Yang, C.; Dyer, D.L.; Miledi, R., 1996a, Lysophosphatidic acid-induced neurite retraction in PC12 cells: control by phosphoinositide-Ca2+ signaling and Rho. J Neurochem. **66**(2): 537-48.
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3. Sebok, A., Nusser, N., Debreceni, B., Zhong, G., Santos, M.F., Szeberenyi, J., and Tigyi, G., 1999, Different Roles for RhoA in Neurite Initiation and Elongation During PC12 Differentiation. J. of Neurochemistry, **73**, 949-960.

VII. List of Abbreviations

I. Cell lines/cell types

Abbreviation	Explanation	Page #	Ref.
DRG-neurons	Dorsal Root Ganglion neurons	p15	102
IIC9 fibrolblasts	Chinese Hamster fibroblast cell line	p19	125
MDCK	Madin-Darby Canine Kidney epithelial cells	p10	75
M-M17-26	dominant negative Ras-expressing PC12 cells	p23	6
N1E-115	neuroblastoma cell line	p12	66
Neuro2A	neuroblastoma cell line	p16	111
NIH 3T3	mouse fibroblast cell line	p19	135
PC12	rat phaeochromocytoma cell line	p4	137
PE0	mock transfected PC12 cells, containing the same vector as PEV14/PEN19 cells	p24 - 25	8
PEN19	PC12 cell constitutively expressing dominant negative RhoA	p24 - 25	8
PEV14	PC12 cell constitutively expressing activated RhoA	p24 - 25	8
PG0	mock transfected PC12 cells, containing the same vector as PGV14/PGN19 cells	p24 - 25	8
PGN19	PC12 cell expressing dominant negative RhoA on steroid induction	p24 - 25	8
PGV14	PC12 cell expressing activated RhoA on steroid induction	p24 - 25	8
Swiss 3T3	mouse fibroblast cell line	p9	3

II. Enzymes

Abbreviation	Explanation	Page #	Ref.
ERK-1, 2	Extracellular Signal-Regulated Kinase	p19	137
FAK	Focal Adhesion Kinase	p10	36
MAPK	Mitogen Activated Protein Kinase	p12	6
MLCK	Myosin Light Chaine Kinase	p13	91
$p160^{ROCK}$	p160 Rho-actiovated Coiled Coil-containing Kinase	p9	5
p70S6K	p70 Ribosomal S6 Kinase	p21	145
PAK	p21 Activated Kinase	p14	104
PI3K	Phosphatidyl-Inositol-3 Kinase	p9	70
PI5K	Phosphatidyl-Inositol-4-Phosphate-5-Kinase	p9	55
PKA	Protein Kinase A, cAMP-activated Protein Kinase	p19	38
PKC	Protein Kinase C,	p8	57
PKN	Protein Kinase N	p8	57

PLD	Phospholipase D	p9	71
PRK-1	PKC-Related Kinase 1	p8	5
PRK-2		p9	5
ROK-II	Rho kinase II	p8	5
ROKα	Rho Kinase α	p8	5

III. Regulators of G-proteins

Abbreviation	Explanation	Page #	Ref.
BCR	Breakpoint Cluster Region	p17	28
Dbl	-	p6	28
FGD-1	Faciogenital Dysplasia Protein 1	p6	5
GAP	GTPase Activating Protein	p6	35
GDI	Guanine Nucleotide Dissotiation Inhibitor	p6	5
GDS	Guanine Nucleotide Dissotiation Stimulator (same as GEF)	p6	31
GEF	Guanine Nucleotide Exchange Factor (same as GDS)	p6	31
Graf	GAP for Rho Assotiated with FAK	p6	36
Lbc	-	p6	33
Myr5	5th Unconventianal Myosin from Rat	p6	37
Ost	-	p6	29
TIAM-1		p6	32

IV. Growth factors

Abbreviation	Explanation	Page #	Ref.
EGF	Epidermal Growth Factor	p7	25
Il-1, 2, 3, 6	Interelukin 1, 2, 3, 6	p21	25
NGF	Nerve Growth Factor	p7	25
PDGF	Platelet-Derived Growth Factor	p10	4
TNFα	Tumor Necrosis Factor α	p21	146

V. Heterotrimer G-protein subunits

Abbreviation	Page #	Ref.
$G\alpha_{12}$	p7	44
$G\alpha_{13}$	p7	45
$G\alpha_{i}$	p28	174
$G\alpha_{q}$	p28	174

VI. Monomer G-proteins

Abbreviation	Page #	Ref.
Rab	p5	9
Rac	p5	11
Ral	p5	9
Ran	p5	9
Rap	p5	9
Ras	p5	9

RhoA	p5	11
RhoB	p5	23
RhoC	p5	11
RhoD	p5	13
RhoE	p5	12
TTF	p17	114

VII. Protein domains

Abbreviation	Explanation	Page #	Ref.
CC-domain	Coiled Coil Domain	p8	5
CRIB-Motif	Cdc42/Rac Interactiv Binding Motif	p8	56
DH-Domain	Dbl Homology Domain	p6	28
MADs box		p20	141
PH-Domain	Pleckstrin Homology Domain	p8	28
REM-1	Rho Effector Binding Motif-1	p8	5
REM-2	Rho Effector Binding Motif-2	p9	5
SH3	Src Homology Domain 3, effector binding domain	p6	36

VIII. Second messengers and other small molecules

Abbreviation	Explanation	Page #	Ref.
ATP	adenosine-triphosphate	p7	-
Bt_2-cAMP	dibutyril cyclic AMP, a membrane permeable cAMP-analogue	p29	8
cAMP	cyclic adenosine-monophosphate	p19	-
DAG	Diacyl-Glycerol	p9	67
GDP	guanosine-diphosphate	p6	-
GTP	guanosine-trisphosphate	p9	-
LPA	Lysophosphatidic Acid	p18	25
PGE_2	Prostaglandyn E_2	p13	100
PIP2	Phosphatydil-inisitol-bisphosphate	p9	55

IX. Transcription factors

Abbreviation	Explanation	Page #	Ref.
c-fos	-	p20	143
DB1		p22	30
MEF-2		p20	141
MRF-4		p20	141
NFκB	Nuclear Factor kappa B	p21	146
Rel	-	p21	146
SRF	Serum Response Factor	p20	143
vezf-1		p22	165

X. Miscelleanous Proteins

Abbreviation	Explanation	Page #	Ref.
Abl	-	p18	5

BCR-Abl	-	p17	5
Crk	-	p7	47, 48
EDIN	Epithelial Cell Differentiation Inhibitor	p29	147
ERM-family	Ezrin-Radixin-Moesin Family of Proteins	p10	77
F-actin	Fibrillar Actin	p10	-
G-actin	Globular Actin	p10	-
IκB	Inhibitory kappa B protein	p21	156
I-CAM	Intercellular Adhesion Molecule	p21	147
MBS	Myelin-Binding Subunit	p9	28
MLC	Myosin light chain	p14	103
N-CAM	Neuronal Cell Adhesion Molecule	p21	153
NF	neurofilament	p11	59
p116Rip-1	Rho-interacting Protein	p9	66
p140mDia	Mammalian Homologue of Drosophyla diaphonus	p9	31
$p21^{WAF1}$	Member of the Cyclin-dependent Kinase Inhibitor Family	p18	124
$p27^{Kip1}$	Member of the Cyclin-dependent Kinase Inhibitor Family	p18	125
WASP	Wiskott-Aldrich Syndrome Protein	p10	9

XI. Miscelleanous

Abbreviation	Explanation	Page #	Ref.
C-terminus	Carboxy-terminus of proteins	p8	-
SRE	Serum Response Element	p20	143
C3 toxin	*C. botulinum* C3 toxin, an ADP-ribosylase exoenzyme	p4	7
PTX	Pertussis toxin	p	-
aa	Amino Acid	p8	-
N-terminus	Amino-terminus of proteins	p8	-
mRNA	messenger RNA	p19	-
PCR	Polymerase Chain Reaction	p19	-
Ph1	Philadelphia-Chromosome (BCR-Abl translocation) positive cells	p17	5
UV	Ultraviolet light	p5	19

XII. Ras and Rho Mutants

Abbreviation	Explanation	Page #	Ref.
V12 Ras	Constitutively Active Ras Mutant	p7	3,4
V12 Rac (1)	Constitutively Active Rac Mutant	p7	3,4
V12 Cdc42 (Hs)	Constitutively Active Cdc42 Mutant	p7	3,4
V14 RhoA	Constitutively Active RhoA Mutant	p7	3,4
N17Ras	Dominant Negative Ras Mutant	p7	3,4
N17Rac	Dominant Negative Rac Mutant	p7	3,4
N17Cdc42	Dominant Negative Cdc42Mutant	p7	3,4
N19RhoA	Dominant Negative RhoAMutant	p7	3,4