The Role of RhoA Protein in Neuronal Differentiation

Ph.D. Thesis Summary

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Introduction

The first Rho protein was found by (and named after) its homology to Ras proteins; 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. 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. 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 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 process of neuronal differentiation, the mechanism by wich neuronal stem cells differentiate into neurons, and especially the mechanism by wich neuronal cells create and regulate their complex morphology are poorly understood in spite of being subject to intense research. Understanding the regulation of neurite outgrowth is an essential prerequisite in designing successful interventions that promote neuronal regeneration after stroke or injury. The goal of our experiments was to elucidate the role of RhoA protein in development of the neuronal morphology by using the well established neuronal differentiation model of PC12 pheochromocytoma cell. These small, roundish cells, which proliferate in serum-containing medium, 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. Inhibition of Rho proteins by *Clostridium botulinum* C3 toxin also induces neuronal differentiation in PC12 cells. 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.

Specific Aims

1. To study the role of Ras and Rho GTPases in lysophosphatidic acid (LPA) -induced neurite retraction.

LPA is a normal constituent of serum, generated during blood clotting. LPA elicits various cellular responses in many different cell types, including neurons, which appear to effect two elementary cellular functions: cell proliferation and cell shape regulation. Among others LPA was shown to inhibit neurite outgrowth and and cause rapid retraction of neurites of NGF-differentiated PC12 cells.

Our goal was to test the role of Ras – which was shown to be necessary for NGF-induced differentiation – and Rho – which was known to be activated by LPA in fibroblast – in this process.

(Based on these results we decided to further analyse the role of Rho in neuronal differentiation.)

2. To test weather activation of RhoA prevents and inactivation of RhoA induces neuronal differentiation in PC12 cells, and to study the signal transduction pathways involved in these processes.

Beacuse C3-mediated inactivation of RhoA induces differentiation in PC12 cell, moreover, NGF-stimulation reduces the amount of free Rho available for ADP-rybosilation by C3 we hypothesized that the induction of neuronal differentiation changes the activity of RhoA by inactivating it. Based on this, we wanted to test whether inactivation of RhoA is a necessary for and is sufficient to induce differentiation. Second, we wanted to test the possible crosstalk between the well-established Ras-Erk and the putative Rho-mediated pathways.

3. To study the role of RhoA at later stages of neuronal morphological differentiation and during regeneration of neurites.

To test whether and how changes in Rho activity influence the neuronal morphology during later phases of neuronal differentiaiton, when cell are alredy committed to the neuronal lineage and after mechanichal or chemical demage of neurites, we generated PC12 clones expressing activated and dominant RhoA mutants from an inducible promoter.

Methods

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). For differentiation experiments cells were plated on plastic dishes coated with 100x diluted Matrigel and exposed to the differentiation agent in DMEM supplemented with N1 additives. 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.

3. Generation of Plasmid Constructs

List of plasmid constructs generated and used:

pGRE-N19RhoA; -V14RhoA , -wtRhoA

List of other plasmids used:

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

Human wild type and activated (V14) RhoA was provided subcloned in the mammalian expression vector pEXV-3. The wild type, dominant negative

(N19) and activated RhoA mutants were subcloned into the steroid-inducible pGRE-5 vector.

4. Stable transfection
Stable transfections done with the calcium-phosphate precipitation methode were used to generate PC12 cell lines expressing mutant RhoA proteins. 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.

5. PCR, asymmetric PCR
PCR was used for primary testing of Geneticin-resistant clones, asymmetric PCR (where the two primers were use in 1:100 ratio) was used for generating PCR-products for sequencing. The primers used were complementary to the last five codons of the myc-tag and codons 53-57 of RhoA.

6. Sequencing
Sequencing was used to verify the N19 mutation introduced into RhoA and to verify the presence of the correct RhoA mutation in the clonal cell lines.

7. Northern blot
Northern blot analysis was used to verify the expression and the streroid-inducibility of the introduced RhoA constructs. A 900 bp EcoRI fragment from the pEXV-RhoA vector containing full-length rhoA cDNA was used as a probe.

8. Western blots
Western blots were used to verify the expression of the introduced RhoA mutants and for membrane translocation assays. A mouse monoclonal antibody non cross-reactive with RhoB or RhoC (Santa Cruz, 26C4) was used for these experiments.

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) and to test the proliferation of mutant RhoA expressing cells. For these experiments riplicate or duplicate cultures were harvested resuspended in serum-containing normal medium by vigorous agitation to break up cell clums 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; Cell lines used were PC12, M-M17-26, A126-1B2, 123.7.); and 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. LPA and LPA-like phospholipids were applied in albumin-bound form. Neurite retraction was typically followed for 30 to 60 minutes. During this time, cells were kept at 37 °C and at 5% $C0_2$ levels.

Neurite lengths were measured using the NIH Image software . 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

11. Morphometric Analysis of Differentiated PC12 cells

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 24-well plates in 2.8-5.6 x 10^3 cells/cm^2. At these low densities, individual neurites could be traced.

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 was definied by dividing the number of neurites per soma.

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

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.)

5×10^6 cell were treated with NGF or Bt_2-cAMP. A crude membrane fraction was prepared by centrifugation at 50.000 g for 30 min. 40 μg samples of the membrane proteins were loaded per well and electrophoresed. Western blotting was performed as described above.

Results

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

- LPA-induced neurite retraction, was independent of pertussis toxin treatment, thus independent of G_i. In fibroblasts, however not in PC12 cells, LPA was proven to activate Ras through a G_i-mediated pathway.
- 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.

Taken together, we concluded that the LPA-induced neurite retraction in PC12 cells is independent of G_i and 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 similarly to what was reported on N1E-115 neuroblastoma cells, suggesting that Rho is involved in cell shape changes mediated by LPA.
- We 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. When analyzing the connection between the cAMP system and Rho, we found that C3 still induced neurite outgrowth in PKA-deficient 123.7 cells; and C3 differentiated 123.7 cells, just like C3-treated wild type PC12 cells, were resistant to LPA-induced neurite retraction. 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. 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. 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.

Regarding differentiation and proliferation of clones expressing activated V14RhoA we found that:

- 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.
- Inactivation of the mutant V14RhoA either by C3 toxin or by Bt_2-cAMP-mediated activation of PKA 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, 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.

- 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.

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.

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 we have to conclude that NGF might use other, not yet determined pathways to reach RhoA in order to induce the neuronal phenotype.

Regarding differentiation and proliferation of clones expressing dominant negative N19RhoA we found that:

- 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.
- 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.

Regarding the role of RhoA in neurite elongation we found that:

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. 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.
- C3 treatment, which renders RhoA inactive, also decreased the mean neurite length per soma in NGF-primed, differentiating cells, 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.

Conclusions

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 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. 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.

First 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. (See Specific aim #3.)

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. (See Specific aim #1.)

Our data support the hypothesis that inactivation of RhoA is necessary for NGF-induced differentiation of PC12 cells, however, we could not induce differentiation merely by expressing a dominant negative RhoA mutant. This might suggest the importance of other Rho variants (RhoB or RhoC). (See Specific aim #2.)

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.

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.

Publications

1. The thesis was based on these 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.
2. Tigyi, G.; Fischer, D. J.; **Sebok, A.**; Marshall, F.; Dyer, D. L.; Miledi, R., 1996b, Lysophosphatidic acid-induced neurite retraction in PC12 cells: neurite-protective effects of cyclic AMP signaling. J Neurochem **66**(2): 549-58.
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.

2. Other, related publications:

1. Lehmann, M.; Fournier, A.; Selles-Navarro, I.; Dergham, P.; **Sebok, A.,** Leclerc, N.; Tigyi, G., McKerracher, L. 1999, Inactivation of Rho signaling pathway promotes CNS axon regeneration. J Neurosci, **19**(17): 7537-47.

2. Abstracts, posters and oral presentations:

1. Tigyi, G., **Sebők, Á.,** Szeberényi, J., Identification of novel lipid mediators and lipid second messengers that cause neurite retraction in PC12 cells. Soc. Neurosci. Abstr. 607.1 p1474 1994.
2. **Á. Sebők**, J. Szeberényi, G. Tigyi. ADP-ribosylation of Rho by C3 toxin induces neuronal differentiation independent of Ras and PKA. 9th International Conference On Second Messengers and Phospholipids, Nashville, TN, 1996.
3. **Á. Sebők**, D. J. Fischer, N. Nusser, M. F. Santos, Y. Zheng and G. Tigyi. RhoA regulates neurite outgrowth in PC12 cells. Soc. Neurosci. Abstr. 297.6 p737, 1996.
4. **A. Sebok,** Santos M. F., Nusser N., Debreceni B., Tigyi G.: Dual role for RhoA in the NGF-induced differentiation of PC12 cells: opposing control over neuronal commitment versus neurite extension.(1997) Soc. Neurosci. Abstr. 23:241.20, p.600.

5. **Sebők Ágnes,** Nusser N., Debreceni B., M.F. Santos, Z.Guo ésTigyi G. A Rho GTP-áz kettős szerepe PC12 sejtek neuronális differenciációban: elősegíti a neuritok növekedését, de gátolja a differenciáció korai fázisát. VI. Sejt- és Fejlődésbiológiai Napok Szeged, 1998. január.
6. **Sebők Ágnes:** A Rho fehérjék szerepe a neuronális differenciációban. Jelátvitel Magyarországon. V. Kecskemét, 1998. október.
7. **Sebők Ágnes**: A Rho fehérje variánsainak szerpe a PC12 sejtek neuronális differneciációjában. VII. Sejt- és Fejlődésbiológiai Napok, Budapest, 1999. január.
8. **Sebők Ágnes,** Kiss Katalin és Szeberényi József:: A RhoA fehérje szerpe a PC12 sejtek proliferációjában. VIII. Sejt- és Fejlődésbiológiai Napok, Pécs, 2000. január.