Dynamics of ataxin-1 in spinocerebellar ataxia type 1
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Citation for published version (APA):
Krol, H. A. G. (2009). Dynamics of ataxin-1 in spinocerebellar ataxia type 1
CHAPTER 4

Interaction of ataxin-1 and protein kinase Cγ mediates their phosphorylation and cellular localization status
Interaction of ataxin-1 and protein kinase Cγ mediates their phosphorylation and cellular localization status

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Abstract

Spinocerebellar ataxias (SCAs) comprise a group of 30 neurodegenerative disorders caused by different types of mutations in various genes. Despite the genetic heterogeneity, the disease phenotypes show large overlap, as all subtypes are characterized by prominent loss of Purkinje cells. It is speculated that multiple SCA genes participate in common pathogenic pathways leading to neurodegeneration. Here, we studied whether the SCA1-related ataxin-1 and SCA14-related PKCγ proteins are functionally linked. Immunoprecipitation experiments showed that ataxin-1 and PKCγ form complexes in vitro and in vivo. Furthermore, co-expression of ataxin-1 and PKCγ increased ataxin-1 phosphorylation levels, induced the formation of ataxin-1 nuclear bodies and increased their dynamics. Polyglutamine (polyQ)-expanded ataxin-1 also affected the localization and autophosphorylation levels of wildtype PKCγ in vitro and in vivo. We conclude that ataxin-1 and PKCγ indeed are functionally linked and our data point at a common biological pathway that underlies SCA1 and SCA14.

Keywords: Ataxin-1, PKCγ, spinocerebellar ataxia, Purkinje cell, phosphorylation, nuclear bodies

Introduction

Spinocerebellar ataxias (SCAs) comprise a group of 30 neurodegenerative disorders that share a majority of their clinical symptoms and neuropathological characteristics. The major feature is atrophy of the cerebellum with a prominent loss of Purkinje cells. In addition, other brain regions are affected such as the brainstem and spinal cord. This neurodegeneration leads to clinical symptoms including ataxia, dysarthria, and
oculomotor problems (Schols et al., 2004). The diseases manifest around midlife but the severity of the disease may vary between patients with identical SCA types or even between family members. SCA is a genetically heterogeneous disorder. Up to date, 15 different SCA genes have been identified, and an additionally 15 SCA loci have been mapped (Duenas et al., 2006). Intriguingly, the different SCAs can be caused by various mutational mechanisms such as coding polyQ (CAG) repeat expansions, non-coding (CTG, CAG) repeat expansions and missense or deletion mutations in the coding region of a SCA gene (Duenas et al., 2006). The fact that different SCA genes with diverse types of mutations show similar disease phenotypes and neurodegenerative features suggests that common underlying biological mechanisms or pathways are involved in the neuropathogenesis of these diseases.

SCA1 is caused by an expansion of the polyQ rich region in the protein ataxin-1. In the mutant ataxin-1 protein, the polyQ repeat numbers exceed the disease-inducing threshold of 36-40 glutamine residues. The resulting toxic gain of function leads to dysfunction and degeneration of Purkinje cells in the cerebellum, specific brain stem neurons, and loss of motoneurons in the spinal cord (Matilla-Duenas et al., 2008; Robitaille et al., 1998). Little is known about the precise function of ataxin-1 which complicates the understanding of the biology of the disease. Ataxin-1 can shuttle between the nucleus and cytoplasm of cells and interacts with RNA and proteins that are implicated in regulation of transcription and RNA binding and stability including capicua, RORα, RBM17 and pumilio-1 (Irwin et al., 2005; Krol et al., 2008; Lim et al., 2008; Lim et al., 2006; Serra et al., 2006; Yue et al., 2001). These data suggest that ataxin-1 may be involved in particular stages of transcription and/or translation, such as transcription regulation, mRNA nuclear export, mRNA stability and splicing. Ataxin-1 is predominantly localized in the nucleus of most neurons, but it is present in both nucleus and cytoplasm of Purkinje cells (Klement et al., 1999; Koshy et al., 1998). The sensitivity of Purkinje cells for mutated ataxin-1 may be caused by this difference in localization. On the other hand, nuclear localization of ataxin-1 seems to be critical for the pathogenesis of SCA1 as transgenic mice expressing ataxin-1 with a mutated nuclear localization signal (NLS) do not develop the disease (Klement et al., 1998). Overexpression of wildtype or mutant ataxin-1 protein leads to the formation of nuclear bodies in vitro (Krol et al., 2008; Stenoien et al., 2002), which is in accordance with transgenic mice studies that show similar nuclear structures upon overexpressing of wildtype or mutant human ataxin-1 (Koshy et al., 1998). These nuclear bodies may represent functional protein complexes containing ataxin-1, as these structures are dynamic and are not insoluble polyQ aggregates (Krol et al., 2008). The formation of nuclear bodies is also regulated by the phosphorylation status of ataxin-1, as ataxin-1 lacking serine 776 phosphorylation does not form nuclear bodies and exhibit a reduction of mutant ataxin-1 pathogenesis (Emamian et al., 2003). Moreover, this mutation disrupts the interaction with 14-3-3 in vitro and in vivo and impairs the formation of ataxin-1-capicua complexes (Chen et al., 2003a; Lam et al., 2006). These findings suggest that not only polyQ expansion, but also altered nuclear localization and phosphorylation levels can affect ataxin-1 function.

The role of altered phosphorylation in SCAs is underscored by SCA12 and SCA14 variants of the disease (Holmes et al., 1999; Yabe et al., 2003). SCA12 is caused by a muta-
Interaction of ataxin-1 with protein kinase Cγ in the brain-specific regulatory subunit of the protein phosphatase PP2A. SCA14 is caused by missense mutations in the protein kinase C gamma (PKCγ), which is the brain-specific isoform of the large serine/threonine kinase PKC family and is highly expressed in Purkinje cells (Chen et al., 2003a; Daniel et al., 1998; Schrenk et al., 2002). Throughout the coding region of PKCγ, 23 mutations have been identified, but particularly the C1B subdomain is a mutational hotspot since 75% of all SCA14 mutations are located in this particular subdomain (Chen et al., 2005). These mutations affect C1B accessibility and PKCγ kinase activity, leading to an aberrant MAPK signaling in SCA14 (Verbeek et al., 2008). PKCγ is known to be an important regulator of long term depression by controlling synapse plasticity at the parallel fiber of the Purkinje cell synapse (Daniel et al., 1998). However, the molecular mechanisms by which PKCγ controls these processes are still unknown. Furthermore, active PKCγ has an inhibitory effect on the growth and size of the dendritic tree of Purkinje cells (Abeliovich et al., 1993; Chen et al., 1995; Metzger and Kaffhammer, 2000; Newton, 2001; Schrenk et al., 2002). PKCγ is mostly present in neurites of Purkinje cells, including the dendritic shafts and dendrites. In other neurons such as pyramidal cells, the protein is predominantly located in the cytoplasm of the cell body (Sakai et al., 2004; Tanaka and Nishizuka, 1994).

Similarly to the altered distribution of ataxin-1 in Purkinje cells, the different location of PKCγ may contribute to the prominent Purkinje cell loss observed in SCA. There are indications that SCA1 and SCA14 have common underlying mechanisms that cause Purkinje cell degeneration, as ataxin-1 and PKCγ may be functionally linked. In a SCA1 mutant mouse model, transcriptionally downregulated genes in glutamate signaling were observed in Purkinje cells (Serra et al., 2004). This downregulation may be related to PKCγ as activated glutamate signaling activates PKC and also affects the intracellular levels of Ca2+, a second messenger of PKC (Conn and Pin, 1997; Serra et al., 2004). In addition, loss of PKCγ at the dendritic cell membrane and a decrease in total protein levels was observed in the SCA1 mouse model, whereas the mRNA levels were not changed. Moreover, PKCγ appeared to be mislocalized in cytoplasmic vacuoles in Purkinje cells (Skinner et al., 2001). In addition, the finding that the cerebellum of a SCA14 patient also showed reduced ataxin-1 immunostaining (Chen et al., 2003a) strengthens the hypothesis that ataxin-1 and PKCγ affect each other and are functionally linked.

To test whether SCA1 and SCA14 are functionally related, we investigated whether ataxin-1 and PKCγ are interaction partners and localize in complexes. Here, we show that PKCγ and ataxin-1 interact both in vitro and in vivo in mouse brain. Furthermore, we show that PKCγ can induce the phosphorylation of ataxin-1 and increases the formation and dynamics of ataxin-1 nuclear bodies. On the other hand, PKCγ phosphorylation is increased, and PKCγ is translocated to the plasma membrane of Purkinje cells in a SCA1 mutant mouse model.

Materials and Methods

DNA constructs. The generation of the human wildtype PKCγ-GFP and SCA14-mutant PKCγ-GFP and RFP constructs (G118D and V138E) have been described previously (Verbeek et al., 2008; Verbeek et al., 2005). The expression constructs of GST-tagged PKCγ were generated by amplifying the PKCγ cDNA using primers: forward 5’-GCCGGATCCACCATGGTCTGGGGCCC-3’ and reverse 3’- CGGGCGGCGGC- CATGACGGCACAGGCACGTG-5’ generating
a BamHI site at the 5’-end and a NotI site at the 3’-end. This facilitated cloning into the pEBB-GST plasmid. The GFP-Atx1[2Q], GFP-Atx1[85Q], FLAG-Atx1[30Q] and FLAG-Atx1[82Q] constructs were kindly provided by Dr. Huda Zoghbi (Houston, TX) and Dr Ronald Evans (San Diego, CA), respectively. The Ser694Ala mutation was introduced into the ataxin-1 cDNA with the Quickchange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) using the following primers: forward 5’-GAACCTGAAGAACGGCGCTGTTAAAAAGGGCCAG-3’ and reverse 5’-CTGGCCCTTTTTAACAGCGCCGTTCTTCAGGTTC-3’. All constructs were verified by sequencing.

**Cell culture and transfection.** COS-7 and mouse neuroblastoma N2A cells were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 5% penicillin (100 U/ml), streptomycin (100 mg/ml) and glutamine (100 mg/ml). Cells were maintained at 37°C in an atmosphere of 5% CO₂. For live cell microscopy, 2 x10⁵ cells were plated on glass coverslips (24 mm; Fischer Scientific, Braunschweig, Germany). The cells were subsequently transfected with the different constructs after 24 h using Lipofectamine 2000 transfection reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). HEK 293T cells were cultured in Iscove’s Modified Dulbecco’s Medium supplemented with high glucose, 10% fetal bovine serum, 5% penicillin (100 U/ml), streptomycin (100 mg/ml) and glutamine (100 mg/ml) in an atmosphere of 5% CO₂. For live cell microscopy, 2 x10⁵ cells were plated on glass coverslips (24 mm; Fischer Scientific, Braunschweig, Germany). The cells were subsequently transfected with the different constructs after 24 h using Lipofectamine 2000 transfection reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). HEK 293T cells were cultured in Iscove’s Modified Dulbecco’s Medium supplemented with high glucose, 10% fetal bovine serum, 5% penicillin (100 U/ml), streptomycin (100 mg/ml) and glutamine (100 mg/ml) in an atmosphere of 5% CO₂. One day before transfection, 2.2 x 10⁶ cells were seeded into 10 cm dishes and transiently transfected with 4 μg DNA using polyethyleneimine (PEI; Polysciences, Warrington, PA). After transfection, cells were cultured at 37°C for 48 h before analysis.

**Confocal laser scanning microscopy (CLSM) and fluorescence recovery after photobleaching (FRAP) analysis.** Transiently transfected N2A cells were categorized based on co-expression of GFP-Atx1[2Q/85Q] and PKCy-138-RFP by selecting GFP- and RFP-positive cells. Based on our previous data, cells were selected that contained ataxin-1 nuclear bodies that were approximately 1.5 μm in size (Krol et al., 2008). FRAP analysis was performed using an SP2 CLSM adapted for living cell analysis using a 63x oil immersion objective (Leica, Mannheim, Germany). A selected ataxin-1 nuclear body was repeatedly bleached in 10 frames at maximum laser power, resulting in a reduction of fluorescence to less than 10% of the initial value. Fluorescence recovery was measured as previously described (Krol et al., 2008).

**GST-fusion protein pull-down assay and immunoprecipitation.** To study interactions between ataxin-1 and PKCy, HEK 293T cells were transiently transfected with full length PKCy-138-GST and GFP-Atx1[2Q] and GFP-Atx1[85Q]. The transfected cells were lysed in buffer A (50 mM NaHPO₄, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA and 1% Triton) supplemented with complete mini protease inhibitor cocktail (Roche, Palo Alto, CA) and phosphatase inhibitor cocktail (Sigma, St. Louis, MO), at 48 h after transfection. The protein quantity was determined using the Bradford protein analysis. The detergent-soluble fraction was incubated with glutathione–Sepharose at 4°C for 2 h. The glass beads were washed twice in buffer A, and subsequently three times in buffer B (buffer A supplemented with 300 mM NaCl). The GST-bead bound proteins were analysed by SDS-PAGE and immunoblotting. Antibodies were used following the manufacturer’s description (anti-GST; TebuBio, Heerhugowaard, NL and anti-GFP; Clontech, Mountain View, CA). The proteins were visualized using enhanced chemiluminescence on radiograph films.

Cerebellum of control mice and the brains of A05 and B05 mice, kindly provided by Dr. Harry Orr (University of Minnesota, Minneapolis, US (Burright et al., 1995) were homogenized in Tris pH 8.0 supplemented with complete mini protease inhibitor cocktail and phosphatase inhibitor cocktail using glass beads and subsequently subjected to three freezing and thawing cycles. The protein quantity was determined using the Bradford protein analysis. Equal amounts of the soluble fractions were pre-cleared with washed protein A/G beads at 4°C for 4 h. Next, the pre-cleared soluble
fractions were incubated with 5 µg of the primary antibody (anti-Atx1; kindly provided by Dr. H. Orr or anti-PKCγ; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The following day, 50 µl washed protein A/G beads were added to the fractions and incubated at 4°C for 3 h. The beads were pelleted, and washed twice in buffer A, and subsequently three times in buffer B. The bound proteins were analysed as described above.

**PKCy and phorbol ester induced phosphorylation of ataxin-1.** To induce phosphorylation of ataxin-1, HEK 293T cells were transiently transfected with FLAG-Atx1[30Q] and FLAG-Atx1[85Q] and treated with 400 nM phorbol myristate acetate (PMA) for 2 h. In addition, HEK293T cells were co-transfected with FLAG-Atx1[30Q/85Q] and PKCy-138-GST and subsequently stimulated with 400 nM PMA. After 2 h, the cells were lysed in buffer A and the detergent-soluble fraction was incubated with agarose-A/G beads (Santa Cruz), which were pre-bound with anti-FLAG antibody (Sigma) for 1 h at 4°C, overnight at 4°C. The beads were washed twice with buffer A and three times with buffer B. Bound ataxin-1 was analysed on 10% SDS-page gels by immunoblotting using a phospho-(Ser)-PKC-substrate antibody following the manufacturer’s instruction (Cell Signalling, Danvers, MA). The proteins were visualized using ECL on films.

**Immunoblotting.** The HEK293T cells were harvested at 48 h after transfection and lysed immediately in 1x SDS sample buffer. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane filter (Schleicher & Schuell, Dassel, Germany) and blocked in 5% milk to prepare for western blotting. The western blots were analyzed with anti-Atx1 (kindly provided by Dr. H. Orr), anti-PKC (Santa Cruz), anti-pT514, anti-pT655, anti-pT674 (all Cell Signalling), anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-p44/42 MAP kinase (both from Cell Signalling), and anti-actin (MP Biochemicals) antibodies.

**Ataxin-1 aggregate scoring assay.** To determine whether PKCy-induced phosphorylation altered the number and distribution of ataxin-1 nuclear bodies, COS-7 and N2A cells were transiently transfected with either GFP-Atx1[Q2] or GFP-Atx1[85Q] and GFP-Atx1[Q2]/[85Q]-S694A and co-transfected with PKCy-138-RFP. Next, the percentage of cells with nuclear ataxin-1 bodies versus cells with diffuse nuclear staining were scored using a IRB inverted fluorescent microscope (Leica). The scoring assays were performed in triplicate by an independent scorer.

**Immunohistochemical staining of mouse SCA1 cerebella.** Brains of SCA1 transgenenic mice A02 and B05, expressing Atx1[Q30] and Atx1[Q82], respectively, under a Purkinje cell-specific promoter were kindly provided by Dr. Harry Orr (University of Minnesota, Minneapolis, US) (Burright et al., 1995). The brains of the sacrificed mice were frozen in liquid nitrogen, stored at -80°C and cut using a cryostat (section thickness 10 µm). Sections were post-fixed in 4% buffered paraformaldehyde for 10 min and blocked in 10% normal goat serum with 0.4% Triton-X100 in 0.05 M phosphate buffer (pH 7.4) for 1 h at room temp. For immunostaining, we used the dilutions 1:2500 and 1:8000 for the PKCy-antibody (Santa Cruz) and 11750V-antibody (provided by Huda Zoghbi), respectively. Immunostaining of phosphorylated PKCy was performed using the antibodies pT655 and pT674 (both Invitrogen) in a dilution of 1:500. Incubations with primary antibodies were performed overnight at 4°C in PBS containing 0.1% Triton and 1% FCS (PH 7.4) followed by an incubation with secondary goat anti-rabbit Cy3 (Jackson) in a dilution of 1:100 in PBS containing 1% FCS for 60 min at room temp. The sections were washed in PBS and cover slipped using vectashield (Vector Laboratories, Burlingame, CA).

**Results**

**PKCy interacts with ataxin-1.** To test whether ataxin-1 and PKCy interact, GST-pull down experiments were performed in HEK293T cells expressing both GST-tagged PKCy and FLAG-tagged ataxin-1 (FLAG-Atx1[30Q]/[85Q]). As a negative control, FLAG-Atx1[30Q] was co-expressed with a
non-fused GST. When PKCγ-wt was immuno-precipitated from lysates of cells transfect- ed with PKCγ and ataxin-1, both Atx1[30Q] and Atx1[82Q] were co-immunoprecipitated (Fig. 1A). Both Atx1[30Q] and Atx1[82Q] were also detected in the immunoprecipitates of SCA14-mutant PKCγ (V138E). The interaction between SCA14-mutant PKCγ and wildtype ataxin-1 appeared to be stronger.

When the interactions were examined by pulling down ataxin-1, only Atx1[85Q] was co-immunoprecipitated with PKCγ (compare lane 2 and 3, Fig. 1B), as Atx1[30Q] failed to pull down any of the PKCγ. To test whether ataxin-1 and PKCγ interact in vivo, we investigated the interaction in the brain of transgenic ataxin-1 mice overexpressing either human Atx1[30Q] or Atx1[82Q]. Here,
both ataxin-1[30Q] and [82Q] were present in the immunoprecipitates of PKCγ (Fig. 1C left panel, lane 1 and 2). Similar to the in vitro data, PKCγ failed to be pulled down with ataxin-1 in 5 week old mice (Fig. 1C; right panel).

Taken together, these findings suggest that PKCγ and ataxin-1 interact, but that this interaction can only be visualised via pull-down of PKCγ. This may be explained by exclusive interaction in the cytoplasm, as most ataxin-1 is located in the nucleus.

**PKCγ can phosphorylate ataxin-1.**
Because ataxin-1 and PKCγ interact, we examined whether PKCγ can phosphorylate ataxin-1 in vitro. To test the effect of PKCγ on phosphorylation of ataxin-1, we used an antibody that can detect all phosphorylated serines in PKC substrates. HEK293T cells were co-transfected with FLAG-Atx1[30Q] or [82Q] together with PKCγ-GST. In addition, cells expressing FLAG-Atx1[30Q] or FLAG-Atx1[82Q] were treated with 400 mM PMA for 30 min to activate endogenous PKCα and β. PMA induced increased phosphorylation of both wildtype and polyQ-expanded ataxin-1, as shown by the anti-phospho-(Ser)-PKC substrate antibody upon immunoprecipitation of ataxin-1 (Fig. 2; upper panel, lanes 3 and 4). Co-expression of PKCγ induced a similar phosphorylation level of Atx1[30Q/82Q] in the absence of PMA (Fig 2; compare lane 3, 4 with lane 5 and 6), suggesting that PKCγ can phosphorylate ataxin-1. Additional PMA stimulation did not significantly further increase the amount of ataxin-1 phosphorylation (Fig. 2; lane 7 and 8). These results suggest that ataxin-1 not only interacts with PKCγ but is also a substrate of PKCγ at a yet unknown amino acid position.

**PKCγ enhances ataxin-1 nuclear body formation.** Since the phosphorylation status of ataxin-1 can affect nuclear body formation and toxicity (Chen et al., 2003b; Emamian et al., 2003), we examined whether PKCγ protein levels affect the formation of ataxin-1 nuclear bodies or alter the kinetics of these structures. We overexpressed either GFP-Atx1[2Q] and GFP-Atx1[85Q] alone or together with PKCγ-RFP in COS-7 cells. Cells expressing GFP-Atx1 showed that endogenous PKCγ mostly resided in the cytoplasm, whereas ataxin-1 was predominantly present in the nucleus. Cells co-expressing both GFP-Atx1 and PKCγ-RFP showed no recruitment of PKCγ into nuclear ataxin-1 bodies (data not shown), suggesting that the interaction between PKCγ and ataxin-1 occurs elsewhere, for example after shuttling of ataxin-1 into the cytoplasm. Whereas a minority of COS-7 cells (25% and 32%) expressing GFP-Atx1[2Q] or GFP-Atx1[85Q] develop nuclear bodies (Fig. 3A; white bars), co-expression of PKCγ showed an increase in

![Figure 2](https://example.com/fig2.png)

**Figure 2.** PKCγ phosphorylates ataxin-1. Lysates of HEK293T cells co-transfected with FLAG-ataxin-1[30Q]/[85Q] and PKCγ-wt-GST and stimulated with or without PMA (400 nM) were immunoprecipitated using the anti-FLAG antibody. The lysates (input) and immunoprecipitates were analyzed by immunoblotting with either anti-FLAG, anti-GST anti-p(Ser)-PKC-substate antibodies. The blot is a representative of 3 independent experiments.
the number of nuclear bodies (42% and 53%, respectively; Fig. 3A). Co-expression of the SCA14-mutant PKCγ (V138E) showed less ataxin-1 nuclear bodies when compared to PKCγ-wt (38% and 46%; Fig. 3A). However, this decrease was not significant. These results imply that PKCγ stimulates the formation of nuclear bodies of both Atx1[2Q] and Atx1[85Q], but these effects are limited when PKCγ is mutated. As we showed earlier that SCA14 mutations lead to reduced PKCγ activity (Verbeek et al., 2008), this suggests that SCA14-mutant PKCγ induces less ataxin-1 phosphorylation and subsequently less nuclear body formation.

We used a motif scanner (http://scan-site.mit.edu/motifscan_seq.phtml) to detect possible PKC binding motifs present in the ataxin-1 protein sequence to identify the PKC phosphorylation motif in ataxin-1. A putative PKCa, β, γ site was detected at S694 using medium stringency (Supplementary Fig. 1A). In order to determine the role of this putative PKC-specific-phosphorylation site S694 on the formation of ataxin-1 nuclear bodies, we mutated the serine 694 to alanine mimicking a non-phosphorylated status. This mutation led to a small, but not significant reduction of the formation of nuclear bodies in cells expressing GFP-Atx1-S694A (Supplementary Fig. 1B). To examine whether this reduction of nuclear bodies was caused by the inability of PKCγ to phosphorylate S694, we co-expressed Atx1[2Q/85Q]-S694A with PKCγ. Unexpectedly, co-expression of PKCγ induced ataxin-1 nuclear bodies after Atx1-S694A transfection similar as for Atx1[2Q] transfection. However, co-expression of PKCγ with Atx1[85Q]-S694A also caused increased numbers of nuclear bodies when compared to Atx1[85Q] alone, but remained significantly different (p<0.05) when
compared to Atx1[85Q] co-expressed with PKCγ. These results suggest that phosphorylation of S694 plays a role in the formation of ataxin-1 nuclear bodies, but this site is not the primary PKCγ phosphorylation motif. Alternatively, the observed effect is phosphorylation-independent and may already be induced via the PKCγ-Atx1 interaction.

**PKCγ increases nuclear dynamics of ataxin-1.** Since cytoplasmic PKCγ can increase nuclear body formation of ataxin-1, we next examined whether PKCγ affected the dynamics of these structures that normally show a constant exchange of ataxin-1 between bodies and the nuclear environment. The on/off rate of ataxin-1 in nuclear bodies is affected by the length of the polyQ expansion in ataxin-1, with a higher on/off rate for ataxin-1 with an expanded polyQ tract (Krol et al., 2008). The on/off rate of ataxin-1 can be determined using FRAP, which was performed by measuring the fluorescent recovery of a single photobleached ataxin-1 nuclear body. The resulting $t_{1/2}$ (which is the time point where the fluorescence has recovered to 50% of its original fluorescence intensity level) was determined. FRAP was performed in N2A cells expressing only ataxin-1 (GFP-Atx1[2Q/85Q] + free mRFP) or GFP-Atx1[2Q] together with RFP-tagged PKCγ-wt or SCA14-mutant PKC (V138E). Interestingly, co-expression of PKCγ-wt significantly reduced the $t_{1/2}$ of Atx1[2Q] by 20 seconds (Fig. 3B). This is similar to the on/off rate of polyQ-expanded ataxin-1, with the $t_{1/2}$ of Atx1[85Q] (Fig. 3B), which is in accordance with our earlier data (Krol et al., 2008). Expression of SCA14-mutant PKCγ did not significantly decrease the $t_{1/2}$ of Atx1[2Q]. These data indicate that increased levels of PKCγ can accelerate the on/off rate of ataxin-1 nuclear bodies, which is similar to the effects of ataxin-1 polyQ-expansion (Krol et al., 2008).

**Ataxin-1 alters PKCγ localization and autophosphorylation in a SCA1 mouse model.** Since PKCγ and ataxin-1 can interact, and PKCγ can alter ataxin-1 kinetics, we investigated whether ataxin-1 also affected the intracellular location of PKCγ in vivo. We used a SCA1 mouse model that overexpresses either human Atx1[Q30] or Atx1[Q82] driven by a Purkinje cell specific promoter (Burrig et al., 1995). The endogenous PKCγ protein levels and localization in cerebellar slices of Atx1[Q30] and Atx1[Q82] mice were determined in mice that were 5 or 12 weeks old. Atx1[Q30] mice displayed a diffuse intracellular PKCγ staining in Purkinje cells (Fig. 4A, top panels) similar to wildtype mice Purkinje cells, whereas PKCγ was predominantly present at the plasma membrane in Atx1[Q82] mice (Fig. 4A, lower panels) irrespective the age of the mice. This was accompanied by a slight loss of PKCγ protein expression, as was noticed previously by others (Skinner et al., 2001). Little staining of the dendrites was observed in Atx1[Q30] which may be due to overexpression of Atx1[30Q], that has been described to induce slight clinical symptoms (Fernandez-Funez et al., 2000).

To investigate whether the change in PKCγ protein localization correlated with altered PKC phosphorylation, we analyzed brain lysates of the Atx1[30Q] and Atx1[82Q] mice by immunoblotting using PKCγ-phospho-specific antibodies (pT655 and pT764, respectively). We observed increased phosphorylation levels of PKCγ in Atx1[82Q] mice that were 5 weeks old when compared to Atx1[30Q] mice with both antibodies. This suggests that mutant ataxin-1 affects endogenous PKCγ activation as well as its cellular localization. A similar increase in PKCγ phosphorylation was also observed in 12 week-old mutant Atx1 mice compared...
Figure 4. Mutant ataxin-1 affects localization and autophosphorylation of PKCγ in Purkinje cells. (A) Confocal images of cerebella of 5 and 12 weeks old A02 and B05 mice and of a 12 week old wild-type mouse. Sections were stained with anti-PKCγ antibody and visualized at 63x magnification. (B) Lysates of brains of A02 and B05 mice that were 5 and 12 weeks old were analyzed using immunoblotting with anti-PKCγ, anti-ataxin-1, anti-PKCγ-pT514, anti- PKCγ-pT655, and anti-PKCγ-pT674 antibodies. (C) Cerebellar slices of 5 and 12 weeks old A02 and B05 mice and of 12 weeks old wild-type mouse were investigated immunohistochemically using anti-PKCγ-pT655, and anti-PKCγ-pT674 antibodies.
to a Atx1[30Q] mouse. However, no mutant ataxin-1 allele in the [82Q] mice was observed, but increased levels of ataxin-1 were observed when compared to Atx1[30Q] mice (Fig. 4B; top panel, lanes 1 and 3 versus lanes 2 and 4). As the mutant Atx1 is driven under a Purkinje cell-specific promoter only this type of cell expresses mutant Atx1. Therefore, protein levels are too low to be detected when analyzing complete mouse brain. However, both findings are in concurrence with previous reports (Klement et al., 1998). In addition, when Atx1[30Q/85Q] and PKCγ-wt/V138E were cotransfected in HEK293T cells, an increase in PKCγ pT655 and pT674 was also observed when compared to cells only overexpressing PKCγ using immunoblotting (Supplementary Fig. 2). Furthermore, phosphorylation levels of PKCγ-wt and mutant upon overexpression of Atx1 were not observed.

To confirm the increased PKCγ autophosphorylation levels in Purkinje cells of Atx1[82Q] mice, we performed immunohistochemistry to detect phosphorylated PKCγ. A significant amount of phosphorylated PKCγ (T655 and T674) was present at the plasma membrane of Purkinje cells of Atx1[30Q] and Atx1[82Q] mice that were 5 and 12 weeks old (Figure 4C). However, increased phosphorylation levels (T655 and T674) were found in Atx1[82Q] Purkinje cells when compared to Atx1[30Q] and wild-type mice (Fig. 4C; compare top panels versus middle panels).

Taken together, these results underscore our hypothesis that ataxin-1 and PKCγ are functionally related by regulating each others functionality by controlling nuclear ataxin-1 complex formation and local PKCγ activity in Purkinje cells.

Discussion

In SCAs, mainly the cerebellum is affected with a massive degeneration of Purkinje cells. The biological pathways leading to neurodegeneration are not understood yet and depend on both the function of the wildtype SCA protein as well as the context of the mutation. As most of the SCA genes do not show any homology in their mutational mechanism, protein function, and structure, this may be complex. However, recent studies led to the identification of some shared pathways leading to ataxia, consisting of dysfunction in gene expression, transcription, synaptic transmission or other intracellular signaling pathways such as calcium or glutamate signaling (Carlson et al., 2009; Lim et al., 2006; Seeley et al., 2009). Whether these biological pathways function independently or are interconnected remains to be determined. The fact that some of the ataxia proteins may interact directly or indirectly via protein-protein interactions (Lim et al., 2006), strengthens the hypothesis that shared pathways are indeed associated with ataxia.

Our study provides biochemical and cell biological evidence for a shared underlying mechanism involving two SCA-causing proteins, ataxin-1 (SCA1) and PKCγ (SCA14). By co-immunoprecipitation we showed that ataxin-1 and PKCγ are in a complex in vitro and in vivo (Fig. 1). It is yet not clear whether this is a direct or indirect interaction or that PKCγ and ataxin-1 both are present in a larger protein complex. Since their interaction could only be visualized via pull-down of PKCγ, it suggests that the interaction between the two proteins may occur only in the cytoplasm of Purkinje cells. Since ataxin-1 is solely restricted to the nucleus in other neuronal cell types it may be that their interaction occurs exclusively in Purkinje cells (1998; Klement et al., 1999; Koshy et al.,
1998; Orr, 2001). If this hypothesis is true, it may explain the increased sensitivity of Purkinje cells for mutations in SCA proteins when compared to other neuronal cells which also express the disease-related proteins. Intriguingly, SCA14-mutated PKCγ seemed to be stronger in complex with ataxin-1 when compared to wildtype PKCγ, which suggests that mutated PKCγ leads to alterations in ataxin-1 wildtype function that eventually also contributes to pathology.

In addition, overexpression of PKCγ increased phosphorylation levels of ataxin-1 and the formation and mobility of ataxin-1 nuclear bodies (see Figs. 2 and 3). Since activation of endogenous PKCa and β using a phorbol ester had a similar effect on ataxin-1 phosphorylation, this functional link is not exclusive for PKCγ. However, endogenous PKCa/β needed to be activated to induce ataxin-1 phosphorylation whereas PKCγ overexpression alone without PMA activation was sufficient to phosphorylate ataxin-1. This may be due to higher specificity of PKCγ for ataxin-1 than that of PKCa and β.

Overexpression of both wildtype and SCA14-mutant PKCγ significantly increased the number of ataxin-1-[2Q] and [85Q] nuclear bodies in vitro, suggesting that 1) the PKCγ-ataxin-1 interaction or 2) the phosphorylation of ataxin-1 by PKCγ is involved in the generation of additional functional ataxin-1 complexes. However, slightly less ataxin-1 nuclear bodies were observed in the SCA14-mutant PKCγ-expressing cells compared to wildtype PKCγ expressing cells. Given the fact that 1) SCA14-mutant PKCγ binds stronger to ataxin-1 than wildtype PKCγ, and 2) SCA14-mutant PKCγ is less active than wildtype but that 3) both PKCγ proteins induce the formation of nuclear bodies, suggest that alterations in ataxin-1 phosphorylation levels modify the formation of functional ataxin-1 complexes. This is in accordance with a previous study by Emamian et al. (Emamian et al., 2003) that showed phosphorylation by Akt at ataxin-1-S776 to be crucial for nuclear body formation. Here, mutation of this amino acid to alanine (A776) impaired ataxin-1 phosphorylation at this site and resulted in a decrease of nuclear bodies (Chen et al., 2003b). In addition, phosphorylation plays an important role in the translocation of ataxin-1 from cytoplasm to the nucleus, as 80% of all phosphorylated ataxin-1 at serine 776 is present in the nucleus of cerebellar neurons (Dr. H. Orr, personal communication). Atx-S694 was predicted to have a putative PKCγ phosphorylation site. Therefore, we investigated whether mutation of this site to alanine had effect on ataxin-1 nuclear body formation. As Atx1[2Q]-A694 showed slightly reduced numbers of nuclear bodies, we suggest that also this site may play a role in ataxin-1 body formation. However, S694 is not the main PKCγ phosphorylation site in ataxin-1 as co-expression of PKCγ with Atx1[2Q]-A694 also resulted in significant increased numbers of nuclear bodies (Supplementary Fig. 1B). Unfortunately, we were unable to identify which phosphorylation site of ataxin-1 was targeted by PKCγ.

Overexpression of PKCγ also increased the mobility of ataxin-1 nuclear bodies in COS-7 cells (Fig. 3). We hypothesize that ataxin-1 oligomerization is modified because of additional PKCγ phosphorylation, leading to an altered conformation of the ataxin-1 protein. This matches with our previous finding that altered ataxin-1 protein conformations due to the expanded polyQ tract also led to increased ataxin-1 mobility in nuclear bodies (Krol et al., 2008).

Interestingly, cytoplasmic PKCγ is translocated to the plasma membrane in Purkinje cells of mutant-ataxin-1 mice (Fig. 4).
This change in cellular PKCγ location suggests that mutant ataxin-1 induces changes in PKCγ activity and subsequently its function. To investigate whether translocation of PKCγ to the plasma membrane is associated with increased PKCγ activity, we determined PKCγ autophosphorylation levels (Fig. 4 and Supplementary Fig. 2). We observed a significant increase in PKCγ autophosphorylation at T655 and T674 in brain lysate of mutant SCA1 mice, and in cells overexpressing both ataxin-1 and PKCγ proteins, using immunoblotting. This increase in autophosphorylation was confirmed by immunohistochemistry in Purkinje cells of mutant SCA1 mice. Moreover, we also noticed the loss of total PKCγ protein as has been described before by Skinner et al. (2001). Therefore, our results suggest that loss of intracellular PKCγ protein is compensated by increased PKCγ autophosphorylation and subsequently leads to increased kinase activity as was shown in vitro and in vivo.

In summary, we conclude that the two SCA proteins ataxin-1 and PKCγ are present in the same protein complex and may function in a common biological pathway that is specifically vulnerable to alterations in Purkinje cells. As mutant ataxin-1 has been shown to cause downregulation of proteins involved in glutamate signaling in neurons (Lin et al., 2000), we suggest that PKCγ is affected as it is implicated in regulating glutamate signaling (Zheng and Keifer, 2008). The change in PKCγ localization and autophosphorylation may thus be caused by 1) disturbed glutamate signaling or 2) loss of total PKC protein. As a consequence, additional important downstream signaling pathways such as calcium signaling and MAPK signaling are affected resulting in dysfunction of Purkinje cells and subsequent neurodegeneration.

**Acknowledgements**

We would like to thank Dr. Harry Orr and Dr. Huda Zoghbi for providing the ataxin-1 constructs, mouse material, and the ataxin-1 antibody.
**Supplementary Figures**

**Supplementary Figure 1.** PKC motif at ataxin-1-S674 is not the unique action site for PKCγ. (A) Ataxin-1 contains a predicted PKCα, β, γ phosphorylation motif at S674. (B) COS7 cells were transfected with either ataxin-1[2Q]/[85] (S697 and A697) or co-transfected with PKC-wt-RFP. Ataxin-1 nuclear body formation was scored manually and analyzed with an unpaired t-test (*, p-value<0.05).

**Supplementary Figure 2.** Ataxin-1 enhances PKCγ autophosphorylation levels. Lysates of HEK293T cells transfected with either FLAG-ataxin-1[30Q]/[85Q] or cotransfected with PKCγ-GFP (wt or SCA14-mutant) were analyzed with immunoblotting using anti-GFP, anti-ataxin-1, anti-PKCγ-pT514, anti- PKCγ-pT655, and anti-PKCγ-pT674 antibodies.
References


