The astrocytic cytoskeleton: Unravelling the role of GFAP

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Assembly compromised GFAPδ has a decreased subunit exchange rate in an intermediate filament network

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Abstract

Glial fibrillary acidic protein (GFAP) is the characteristic protein in the intermediate filament (IF) network of astrocytes. At least 10 different GFAP splice isoforms are known to be expressed, and the main isoforms are GFAPα and GFAPδ. The canonical GFAPα isoform is able to assemble into filaments by itself. GFAPδ, however, is assembly compromised, but low amounts of GFAPδ (~ 10% of total GFAP) are tolerated into an IF network composed of vimentin or GFAPα. An increasing amount of GFAPδ will lead to an imbalance with the other IFs present in the cells, and will cause a collapse of the IF network. An IF network is a dynamic structure and IF proteins continuously assemble and disassemble into the network. To study potential differences in the dynamic properties of GFAPα and GFAPδ, both in a physiological and a collapsed IF network, we performed a fluorescence recovery after photobleaching (FRAP) analysis on astrocytoma cells transfected with fluorescently-tagged GFAPs. Here we show for the first time that exchange of GFP-GFAPδ (t1/2 = 2.3 min) into the IF network of astrocytoma cells was significantly slower than the exchange of GFP-GFAPα (t1/2 = 1.1 min). Furthermore, we provide preliminary data that a collapsed IF network, induced by a high GFAPδ expression, led to an even more pronounced decrease in fluorescent recovery of both GFP-GFAPα (t1/2 = 4.5 min) and GFP-GFAPδ (t1/2 = 5.5 min). In the collapsed condition, however, the difference in dynamic properties between GFP- GFAPα and GFP-GFAPδ was not present anymore. Our study provides a first insight into biochemical properties of two GFAP isoforms that leads to differences in IF network dynamics. As the IF cytoskeleton has been implicated in cell signaling, a change in the dynamic properties of the network could play a role in regulating cellular processes. More importantly, a GFAP collapse mimicks GFAP aggregates present in Rosenthal fibers in Alexander Disease and some glioma subtypes, therefore our data might help to understand the functional consequences of these aggregates in astrocytes.
Introduction

Intermediate filaments (IF) are part of the cytoskeleton. Together with actin filaments and microtubules they form an integrated system that regulate many cellular processes, such as cell morphology, cell signaling, cell migration, and proliferation (Goldman et al., 2008; Ivaska et al., 2007; Pekny, 2001; Rutka et al., 1994). The main IF protein expressed in astrocytes is glial fibrillary acidic protein (GFAP). GFAP has 10 different isoforms formed by alternative splicing, of which GFAPα is the canonical isoform (Kamphuis et al., 2012; Middeldorp and Hol, 2011). The function of GFAP and its isoforms is still elusive, but there is emerging evidence that at least one isoform, GFAPδ, alters the properties of the IF network. GFAPδ differs from GFAPα only in its C-terminal tail, and is expressed in specific types of astrocytes; i.e. the adult neural stem cells in the human subventricular zone and subpial astrocytes (van den Berge et al., 2010; Roelofs et al., 2005). In pathological conditions GFAPδ can be expressed in certain types of reactive gliosis and glial tumours (Andreiuolo et al., 2009; Choi et al., 2009; Martinian et al., 2009; Heo et al., 2012; Kamphuis et al., 2014). The tail of GFAPδ disables the protein to form homodimers and this renders GFAPδ to be assembly compromised by itself (Nielsen and Jorgensen, 2004). Together with other type III IF proteins GFAPδ is able to form heterodimers and can be integrated in an IF network. Depending on the level of expression and the concentration of other IFs present, GFAPδ is either tolerated in the network or causes the whole IF network to collapse in a perinuclear fashion (Perng et al., 2008; Roelofs et al., 2005). Assembly experiments in a cell free environment showed that GFAP networks start to collapse when there is more then 10% of GFAPδ protein present in the network (Perng et al., 2008).

In the cell IF proteins are present in a soluble form in the cytoplasm and in filamentous structures that form an important part of the cell’s cytoskeleton. In vitro these IF networks are highly motile structures, that are constantly rearranged. The proteins within the filaments are also dynamic since there is an active exchange between the filamentous and non filamentous pool of IF proteins (Blikstad and Lazarides, 1983; Colakoglu and Brown, 2009; Lu, 1993; Soellner et al., 1985). Already formed IF networks can be actively disassembled by phosphorylation of IF proteins and a lack of dephosphorylation will hamper new IF assembly networks (Chou et al., 1989; Ku et al., 1996). It has been shown that phosphorylation of GFAP by kinases, such as Aurora B or CF kinase, at the N-terminal head domain are important for proper dissociation from the filaments during cytokinesis (Goto et al., 2000; Izawa and Inagaki, 2006; Ku et al., 1996; Omary et al., 2006).
In vivo it is known that physiological levels of GFAPδ are tolerated in a GFAPα network (Roelofs et al., 2005; Perng et al., 2008; van den Berge et al., 2010), although it has been shown in vitro that a high expression can lead to an IF network collapse (Nielsen and Jorgensen, 2004; Roelofs et al., 2005; Perng et al., 2008). These collapses resemble aggregates of GFAP proteins which occur when cells are transfected with mutant R416W GFAP (Perng et al., 2006). Mutations in GFAP are the cause of Alexander disease (AxD), a fatal neurodegenerative disease characterized by leukodystrophy, macrocephaly and psychomotor retardation (Brenner et al., 2001). A pathological hallmark of this disease is the presence of Rosenthal fibers (RF), which are astrocytic aggregates that are comprised of GFAP, ubiquitinated proteins, and stress proteins such as heat shock proteins like αB-crystallin (CRYAB) and heat shock protein 27 (HSP27) (Goldman and Corbin, 1988; Iwaki et al., 1989; Tomokane et al., 1991; Tang et al., 2006; Perng et al., 2006). IF associated proteins like plectin are also present in RFs (Tian et al., 2006). AxD mutations in GFAP and the subsequent collapse of the network influence cell viability, glutamate transport and astrocyte morphology (Hagemann et al., 2009; Tian et al., 2010; Chen et al., 2011; Sosunov et al., 2013). The research on mutant AxD GFAP shows that aggregates or accumulations of IF proteins can have a profound effect on the cell biology and physiology.

There is increasing evidence that GFAPδ changes IF properties. Here we study in more detail the differences in dynamic exchange of GFAPα and GFAPδ with the IF network in vitro. Dynamic differences between GFAP isoforms could elucidate functional consequences in GFAPδ expressing cells.

**Methods**

**Cell culturing and transfections**

U251MG (gift from Dr. B de Leeuw (Erasmus medical center, Rotterdam, The Netherlands)) and U343MG (gift from Prof Dr. R. Quinlan (Durham University, Durham, UK)) human astrocytoma cells were cultured in DMEM Glutamax (Gibco) mixed 1:1 with Ham’s F10 medium (Gibco) containing 10% Fetal bovine serum (FBS) (Gibco) and 10 U/ml Penicillin streptomycin (P/S) (Invitrogen). All cells were cultured in uncoated plastic 10 cm dishes (Corning) at 37°C in a humidified atmosphere, with 5% CO2.

Human GFAPα and GFAPδ cDNA sequences (Roelofs et al., 2005) were cloned in frame after the eGFP sequence in peGFP using BAMHI and HindIII as restriction sites (Clontech).
to create N-terminal eGFP tagged GFAPs. The N-terminal side was chosen for the eGFP tag since GFAPα and GFAPδ differ in their C-terminal tail. All plasmids were sequenced.

Cells were transiently transfected using polyethylenimine (PEI) (Polysciences) or Lipofectamine (Invitrogen) according to manufactures descriptions. 2.5 μg of plasmid DNA was used for PEI and 1.6 μg for Lipofectamine (Kamphuis et al., 2012) in sub-confluent 24-wells dishes.

### Live cell imaging

U343MG cells were imaged for 48 hours (h) using a Leica IR-BE (Leica Microsystems GmbH) inverted wide field microscope at 37ºC in an custom build incubator containing 5% CO2. Phase contrast and fluorescence images were acquired with a 40x objective at 10 and 30 minute (min) time intervals during 48 h. The single images were reconstructed and rendered into a time-lapse using Huygens software (Scientific Volume Imaging) and Image Pro Plus (Mediacybernetics).

### Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 10 min, washed in phosphate buffered saline (PBS), and incubated in SuMi buffer (50mM Tris, 150mM NaCl, 0.25 % gelatin (Difco) and 0.5% Triton X-100 (Sigma Aldrich), pH 7.4) for 10 min. Primary antibodies were diluted in SuMi and incubated at 4 °C on a shaker overnight. Cells were washed 3 times in PBS and, subsequently incubated with donkey secondary antibodies directed against mouse, rabbit or chicken (Jackson Immune research, 1:1400) and Hoechst 33258 (1:1000 dilution) (Invitrogen) diluted in SuMi at RT for 1 h. The primary antibodies used are listed in Table 1. Cells were washed again in PBS, before the coverslips with cells were mounted on slides with Mowiol (0.1 M Tris-HCl pH 8.5, 25% glycerol, 10% Mowiol (Calbiochem)).

### Table 1: Primary antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Cat #</th>
</tr>
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<td>panGFAP Dako</td>
<td>Dako</td>
<td>1:4000 (1:8000 WB)</td>
<td>Z0334</td>
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<tr>
<td>hGFAPδ</td>
<td>Manufactured in house (10-05-2001 Bleed)</td>
<td>1:1000 (1:1300 WB)</td>
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<td>Sc-6170</td>
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<td>Vimentin</td>
<td>Chemicon</td>
<td>1:3000</td>
<td>AB5733</td>
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<tr>
<td>GAPDH</td>
<td>Abcam</td>
<td>1:4000 (WB)</td>
<td>AB14247</td>
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Western blot

U251MG Cells were scraped with a cell scraper and lysed on ice for 15 min in 100 μl mild extraction buffer (20 mM Tris-HCl [pH 7.6], 140 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA)(Sigma Aldrich), 1 mM ethylene glycol tetraacetic acid (EGTA) (Sigma Aldrich), 0.5% [v/v] NP-40 (AppliChem) and two protease inhibitors; 1 mM phenylmethanesulfonylfluoride (PMSF;Roche Diagnostics) and 0.005 mM leupeptin; Roche Diagnostics). Cell lysates were centrifuged at 2,080 rcf for 2 min at 4°C. The supernatant was stored at -20°C. The pellet was dissolved as much as possible in 100 μl mild extraction buffer. Protein concentrations were measured using a Bicinchoninic Acid (BCA) kit (Pierce, Thermo Scientific) according to manufactures descriptions. Proteins were mixed with 2X loading buffer (2X: 100 mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.2M dithiothreitol and bromophenol blue) and heated for 5 min at 95°C before loading on a 7.5% SDS-PAGE reducing gel. After electrophoresis proteins were blotted on Whatman Protran membranes (GE Healthcare) using a semi-dry Trans-Blot system (Biorad). Blots were incubated with SuMi for 10 min to block aspecific binding before it was incubated with primary antibodies overnight at 4°C. Blots were subsequently washed in TBS-T (100 mM Tris-Hcl pH 7.4, 150 mM NaCl with 0.2% Tween-20) 3 times before secondary antibodies, diluted in SuMi, were incubated at RT for 1 h. Secondary antibodies used were donkey IRdye 800 (1:2000) (LI-COR, NE, USA) and donkey Dyelight Cy5 (1:4000) (Jackson Immune Research) directed at either mouse, rabbit, mouse or chicken, depending on the primary antibody. Blots were washed again 3 times in TBS-T before scanned with an Odysee scanner (LI-COR). The mean fluorescent intensity of the bands were analysed using ImageJ software (W.S. Rasband, NIH, Bethesda, MD, version 1.46f, http://imagej.nih.gov/ij/). The fluorescence was corrected for GAPDH fluorescence and the ratio soluble to insoluble was calculated by dividing the corrected mean fluorescent values of the supernatant to the pellet.

Fluorescent Recovery After Photobleaching (FRAP)

FRAP analysis was performed on transiently transfected U251MG cells. During imaging the temperature was maintained at 37°C in a humidified incubator chamber (OKO labs). Cells were analysed 24 hours after transfection. To monitor dynamics of GFAP in collapsed networks we transfected the cells with GFAPδ in combination with either GFP-GFAPα or GFP-GFAPδ. FRAP experiments were carried out on a SP5 Leica Confocal Microscope (Leica, Germany) with a 63x objective. The pinhole was set on 209.99 μm and the scanning
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...speed at 400Hz with a resolution of 512x512 pixels. Bar-shaped regions of interest (ROI) were bleached with a 488nm Argon laser (100% power) until at least 50% of the fluorescence was bleached. ROI sizes were kept constant for all measurements (1.5 µm x 10 µm). After bleaching, a time-series of capturing 10 frames with a 30 second interval was made. Then, z-stacks were taken manually every 5 min up to 30 min after bleaching. To ensure that the bleached ROI did not drift out of focus z-stacks were made throughout the whole cell. 3 ROIs were bleached per cell at different locations within the IF network. FRAP experiments were performed at different days in at least 3 separate experiments. This resulted in the following amount of ROIs measured; 33 x GFAPα in a network, 30 x GFAPδ in a network, 8 x GFAPα in a collapse and 8 x GFAPδ in a collapse.

**FRAP analysis**

ROIs were positioned in post bleach pictures manually. Per time point the position of the ROI was corrected for cell movement and average fluorescence was measured using Image J. The average fluorescence was plotted in time to obtain fluorescence recovery curves for every ROI. The half time was calculated by interpolating the time at 50% of the fluorescence of the maximum fluorescence at 30 min. The immobile fraction was calculated by comparing the fluorescence in the bleached area after recovery ($F_\infty$) with the fluorescence before bleaching ($F_i$) and just after bleaching ($F_0$). The immobile fraction is defined as $1 - (F_\infty - F_0)/(F_i - F_0)*100$. For $F_\infty$ the average of the last three timepoints was used for analysis.

**Statistics**

Data was tested for normality with a Kolmogorov-Smirnoff test. Mann-Whitney U tests or Kruskal-Wallis tests with a Dunns post hoc were performed using Graphpad Prism (version 6.02 Graphpad software inc.). $p$-Values were considered significant when < 0.05.

**Results**

**GFP tagged GFAP incorporates into endogenous IF network but affects assembly**

To perform live cell imaging experiments, a fluorescent tag is needed to visualize GFAP. Since we were investigating differences between GFAPα and GFAPδ, which differ in their C-terminal tail, we used an N-terminal tag for GFAP imaging. U251MG cells endogenously express vimentin, nestin and synemin. GFAP levels are low in U251MG cells compared
to U343MG cells (Chapter 2 and unpublished observations). Both GFP-GFAPα and GFP-GFAPδ did incorporate into the endogenous IF network of U251 cells (Fig 1A, B). The endogenous IF network is visualized by the vimentin staining. 24 hours after transfection, the GFP-GFAPα transfected cells showed a spread out network (Fig 1A). GFAPδ is known to cause a concentration dependent collapse of the network (Nielsen and Jorgensen, 2004; Perng et al., 2008). Therefore, 24 hours after transfection GFP-GFAPδ expressing cells showed a mixture of cells with either a spread out network (Fig 1B) or a collapsed IF network (Fig 1C). About 30% of the GFP-GFAPδ expression cells in our cell culture condition showed a collapse at this timepoint, although the exact percentage varied between experiments and was most likely dependent on the transfection efficiency.
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Figure 2: Collapse of the IF network due to high GFP-GFAPδ expression. A) Stills from a representative live cell imaging experiment. U343MG cells were transfected with GFP-GFAPδ and imaged for 48 hours. The GFP-GFAPδ was initially incorporated into the IF network (arrows at t=12h), but as the amount of GFP-GFAPδ increased over time, it eventually caused a collapse of the network (t=18h). During the process of collapsing, thicker and shorter filamentous structures are visible in the cell, which are moving into the direction of the collapsed network (arrowheads in t=30h). B) These small filaments sometimes co-localized with vimentin in U251MG cells as well (arrows). Scale bars represent 20 μm.
**Figure 3:** Different dynamics between GFAPα and GFAPδ. A) FRAP experiments consisted of bleaching ROI and measuring the fluorescence recovery up to 30 min after the bleach in U251MG cells. Within 150 seconds most of the fluorescence was recovered, but recovery was never complete. B) FRAP experiments were performed for GFAPα, GFAPδ and GFAPδ in a collapse and half times were calculated. There is a significant difference in half time between GFAPα (median = 1.1 min) and GFAPδ (median = 2.3 min) (p<0.05 n=33, n=30), and between GFAPα and GFAPδ in a collapse (median= 3.8 min) (p=0.0001 n=32). There was no significant difference in the half time of GFAPδ in spread out or a collapsed network, although there was a trend that a collapse decreased the half time of GFAPδ. C) The immobile fractions were not significantly different between GFAPα (40.2 %), GFAPδ (49.1%) (p=0.7 n=29 and n=30). GFAPδ in a collapse did have a significantly different immobile fraction (56.3%; n=32) compared to GFAPα (p=0.03) and GFAPδ (p=0.04) in a network. Graphs B and C show median values with interquartile range. D) Medians of FRAP curves for GFAPα, GFAPδ and GFAPδ in a collapse show the recovery after bleaching. Non parametric tests were performed on the data extracted from the FRAP measurements so non overlapping curves do not equal significant differences in this graph.
Live cell imaging of the GFAPδ network collapse

To visualize the dynamics of the collapsing network over time, astrocytoma cells transfected with GFAP-GFAPδ were imaged for 48h, starting 4 h after transfection. U343MG cells were used here since they are less motile than U251MG cells which was preferable for longer imaging (unpublished results). As the fluorescence became visible, it was clear that GFP-GFAPδ was located throughout the IF network in the whole cell (arrow in Fig 2A t=12h). As the expression of GFP-GFAPδ increased the fluorescence started to condensate around the nucleus (Fig 2A). Sometimes small aggregates or condensations were seen which were also accumulating in the collapse (arrowheads Fig 2A t=30). During and after the process of the accumulation, cells were still migrating and the collapsed IF network was a motile structure in both stationary and moving cells. These experiments showed that also GFP-GFAPδ, in small amounts, is incorporated into the endogenous IF network before it caused a collapse of the IF network. Images from fixed cells stained for GFP, GFAP and vimentin showed that the squiggles of GFP-GFAPδ sometimes co-localized with vimentin and were not part of larger filaments (Fig 2B, arrow). This shows that the assembly of the IF network is disturbed.

Dynamic properties of GFP-GFAPα and GFP-GFAPδ isoforms

To assess the dynamic properties of GFAPα and GFAPδ, FRAP experiments were performed on U251 astrocytoma cells. Cells were transfected with the GFP-GFAP isoforms and the FRAP analysis was started 24 h later. Cells with non-collapsed and collapsed networks were measured and analysed separately. Fluorescent GFAP networks were bleached and the fluorescence recovery was imaged up to 30 min after bleaching. A typical example is shown in Figure 3A. The median of all FRAP curves for GFAPα and GFAPδ and GFAPδ in a collapsed network are shown in Figure 3D. The time needed to recover to 50% of the final fluorescence (half time: t½) and the immobile fraction were calculated. The median t½ value of GFAPδ was 2.3 min and this was significantly (p<0.05) longer than the median t½ of GFAPα, which was 1.1 min (Fig. 3B). Since high GFAPδ expression leads to a collapse of the IF network, the dynamic properties of GFAPδ were investigated in a collapsed network as well. Although not significant, there was a clear trend visible that GFAPδ had a longer t½ (median = 3.8 min) when in a collapsed network compared to a spread out network (Fig 3B), indicating a slower on/off rate from the IF network. We also calculated the percentage of fluorescence that did not recover from the FRAP curves, which represent the immobile fraction of the GFP-GFAPs. We observed that the immobile fraction did not significantly differ between GFAPα (median =
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40.3%) or GFAPδ (median= 49.1%). There was a significant difference between the immobile fraction of GFAPδ in a collapse (median = 56.4%) and GFAPα or GFAPδ in a network (Fig 3C).

To assess whether the change in dynamic properties between GFAPδ in a network and in a collapse was due to the collapse, we performed a pilot experiment in which we also measured the dynamics of GFAPα in a collapsed network. In order to study this, we transfected cells with GFAPδ to induce the collapse, and either GFP-GFAPα or GFP-GFAPδ. First we confirmed that GFP-GFAPα is incorporated in the collapsed network. We indeed observed that both GFP-GFAPα (Fig. 4A) and GFP-GFAPδ (Fig. 4B) were incorporated into the collapse as can be seen by vimentin stainings. Vimentin is highly expressed in U251 cells and shows the endogenous IF network. A typical example of a bleached collapsed network and the subsequent recovery is shown in Figure 5A. Median t½ for GFAPα (4.4 min) and GFAPδ (5.5 min) did not differ significantly between eachother (p=0.8) (Fig. 5B). The t½ measured for GFAPα was remarkably higher then when not in a collapsed network (Fig. 5B). The fluorescence after 30 min of recovery was still low and did not always show a clear plateau. Therefore the immobile fractions were not calculated for GFAPα in a collapse. Taken together we show that GFAPα

Figure 4: Incorporation of GFP-GFAP isoforms in a collapsed IF network. U251MG cells expressing GFAPδ showed a collapse of the IF network, as seen in A and B by analyzing GFAP and vimentin fluorescence. When co-expressed with GFAPδ, both GFP-GFAPα and GFP-GFAPδ were incorporated into the collapse (arrows). GFP fluorescence co-localized with GFAP and vimentin immunostaining showing that the dynamics measured in these experiments reflect GFAP in a collapsed network. Cells transfected with GFP-GFAPδ showed a similar collapsed structure as the GFP-GFAPα cells. Scale bar represents 20 µm.
and GFAPδ have different exchange dynamics (as measured by $t_{1/2}$) and the dynamics show a trend for higher $t_{1/2}$ when the IF network is collapsed.

**Lower dynamics of GFAPδ are not due to an absence of a soluble pool**

There is a constant exchange of IF proteins from the filamentous forms to non-filamentous forms and back. The non-filamentous IF proteins are part of a soluble pool of IF proteins. Since we observed differences in dynamics between GFP-GFAPα and GFP-GFAPδ we investigated whether a change in the equilibrium between the soluble and insoluble pool of GFAP proteins could be detected. The soluble and insoluble fractions were separated by centrifugation and run on a gel. The blotted proteins were immunostained for pan-GFAP, recognizing both GFAPα and GFAPδ. To quantify differences between the GFAPα and GFAPδ soluble pools the α/δ ratio was determined. In figure 6A it is shown that the level of GFAPα and GFAPδ present in the transfected cells was similar. The endogenous level of GFAP in control cells...
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is visible in the last lane and is lower in comparison with the transfected cells. It is clear that more GFAPδ ends up in the insoluble fraction. Our data confirm earlier data by Perng and colleagues in U343MG cells (Perng et al., 2008). GAPDH was used as a loading control. In figure 6B the ratios soluble/insoluble are shown. Although no statistically significant differences were found in soluble/insoluble ratios between GFAPα and GFAPδ transfected cells from 3 independent experiments. All data points are shown and in all 3 experiments the ratio was lower for GFAPδ indicating that there is a trend (p=0.1 n=3) for more protein in the pellet for GFAPδ then for GFAPα.

Discussion

In this study we show that GFAPα, with a median half time of 1.1 min, exchanges faster then GFAPδ which has a median half time of 2.3 min. When the IF network collapses it slows down the exchange of GFAPδ to a half time median of 3.8 min. The immobile fraction increased significantly for GFAPδ when the IF network collapsed. In a pilot experiment, where
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The dynamics of GFAPα and GFAPδ were measured in a collapsed network, both GFAPα and GFAPδ showed a trend for a slower exchange compared to in a network. This difference in exchange probably leads to an increase in the pool of filamentous proteins relative to the soluble pool in cells expressing GFAPδ.

**Dynamics of GFAP compared to other IFs**

Dynamic properties of IFs have been studied before using FRAP. Half times of fluorescent recovery of IFs into the IF network vary between different IFs, as has been shown by studies from the Goldman lab (Yoon et al., 2001, 1998). The half time we have measured for GFAPα of 1.1 min is faster than vimentin (~5 min in BHK-21 cells with GFP-vimentin) and keratins (~106 min in Ptk2 cells with GFP-Keratin8). In these experiments GFAP was tagged to the the N-terminus of both vimentin and keratin. A previous study on GFAP dynamics (using GFAPalpha) showed a half time of 217 ±30 sec (3.6 min) for a GFP- GFAP fusion protein (N-terminal tag). This is longer compared to the half time we have observed. Li and colleagues however investigated mouse GFAP and used C6 cell lines, derived from rat glioma (Li et al., 2006), where we used human GFAP and human astrocytoma cells. The differences in half time of GFAP could be due to species differences or temperature differences during the imaging. Li and colleagues did not state at what temperature the experiments were done. If these were at room temperature it could explain the lower exchange speed. It is unknown why different IF proteins have such different exchange rates. The differences are likely due to phosphorylation or structural properties that affect the assembly. The latter is reflected in higher half time values for lamins (~140 min)(Gilchrist et al., 2004; Moir et al., 2000) which forms IgG like folds during assembly (Kreplak et al., 2004). The higher half time for lamins could also be due to the localization of the lamin network in the nuclear lamina compared to GFAP and vimentin which are located in the cytoplasm.

**The C-terminus and GFAP exchange rate**

In this study we showed that GFAPδ incorporates and dissociates slower from an IF network than GFAPα. Since the C-terminal tail is the only difference between GFAPα and GFAPδ the differences in dynamics must be due to this specific sequence. Phosphorylation is the main regulator of IF protein exchange (Ku et al., 1996; Nakamura et al., 1992; Skalli et al., 1992). In the part of the C-terminus where GFAPα and GFAPδ differ, in both isoforms 7 residues are present which could be potentially phosphorylated. The position of these residues is however different, as the aminoacid sequence differs between the two C-terminal
tails (see chapter 6). Taking 3D folding into account this difference in localization could lead to a different availability of phosphorylatable residues. Mutation experiments altering the 9 phosphorylation sites in the C-terminal end of GFAPα and GFAPδ could help elucidate whether phosphorylation of the C-terminus indeed influences IF dynamics. Earlier experiments have shown that ablation of the whole C-terminal tail of IF type III proteins inhibits IF assembly (Chen and Liem, 1994; Chen et al., 2011), but phosphorylation of vimentin at the C-terminal side did not affect its assembly (Chou et al., 1996). These latter experiments have not been done yet for GFAP. Thus further research is needed to find out whether phosphorylation of residues in the tail of GFAP plays a role in GFAP exchange.

What could be the functional consequence of a GFAP protein with a slower exchange rate?

The slower exchange rate of GFAPδ suggests that the GFAPδ proteins have a larger affinity to the filamentous pool than the soluble pool, which is in contrast to GFAPα. This would lead to an increase in the amount of protein in the filaments compared to the soluble pool, as we could confirm with our western blot experiments. And it would lead to an increase in immobile fraction of GFAPδ in a collapse which we also observe in our data. This suggests that GFAPδ cannot get phosphorylated as efficiently as GFAPα.

It has been proposed by others that IF networks can function as a kinase sink and thereby regulate several signaling pathways (Ku and Omary, 2006; Pallari and Eriksson, 2006). The presence of an IF network, full of proteins that can be phosphorylated, would provide a substrate for different kinases which consequently cannot phosphorylate other substrates at that moment. IF networks with GFAPδ could have less potential for phosphorylation then networks with only GFAPα. The regulatory role of different GFAP isoforms would then be in the availability of phosphorylatable GFAP proteins in the network. Such a role has been proposed for keratins in regulating mitosis by inhibiting 14-3-3 to bind to Cdc25 (Margolis et al., 2006). 14-3-3 is a protein which binds to several signaling proteins like receptors, kinases and phosphatases (Dougherty and Morrison, 2004). 14-3-3 also binds to phosphorylated GFAP (Li et al., 2006). The lower protein exchange of GFAPδ could alter the amount of phosphorylated GFAP, thereby influencing the availability of GFAP to bind to 14-3-3, and thus altering several signal transduction pathways.

Further studies are needed and it would be crucial to have the right amount of GFAPδ incorporated in an IF network which is studied. Too high GFAPδ levels will cause a collapse of the network and we show here that the collapse per se also affects the protein dynamics and immobile fraction of the GFAP network.
Collapsed networks alter GFAP dynamics

A collapse of the IF network is a dynamic process and exchange of IF proteins is possible before and after the collapse. When GFAPδ is expressed, the solubility of GFAP networks decreases, a collapse occurs, and less GFAPδ is present in the soluble pool compared to GFAPα (Perng et al., 2008). It was not studied before whether this affects the dynamic properties of GFAP. To address this question we expanded our experiments with a pilot study of GFAP isoform dynamics in collapsed networks. We demonstrate here that in a collapsed network the dynamics of both GFAPα and GFAPδ are reduced. This suggests that IF protein dynamics are not only dependent on protein sequence intrinsic properties but also on the structure of the IF network (collapsed or not collapsed). Since phosphorylation is essential for IF assembly, it could be that phosphorylation sites are not accessible anymore when IF proteins are in a collapsed network. This would explain the decrease in GFAP half time and the increase in immobile fraction we observed in the current study (Fig. 3B and 5B).

Many different proteins are associated with collapses of GFAP in astrocytes. Alpha B-crystallin (CRYAB) associates with IF filaments and co-localizes with GFAPδ proteins in collapsed IF networks (Perng et al., 2008). CRYAB can bind to human GFAP in both folded and unfolded states and mediates GFAP assembly and solubility (Nicholl and Quinlan, 1994). CRYAB can inhibit some of the toxic effects of GFAP collapses (Hagemann et al., 2009) and it reduces GFAP filament formation when purified GFAP is assembled in a cell free system (Nicholl and Quinlan, 1994). The presence of CRYAB in GFAP accumulations shows that at some point CRYAB is not able to maintain normally distributed filaments anymore in the presence of GFAPδ. Expression of low amounts of GFAPδ in U343MG cells already shows an increase in association with CRYAB, but this increase is also seen in collapses caused by mutant or GFP tagged GFAP. This illustrates that not only GFAPδ, but also the reorganization of GFAP can cause differences in protein binding to IF filaments (Perng et al., 2008).

Another protein which binds to IF is plectin. Plectin is a linker protein able to interact with actin, microtubules, integrins and IFs (Wiche and Winter, 2011). Like CRYAB, plectin is localized to GFAP aggregates in cells expressing mutant GFAP but also affects GFAP network distribution (Tian et al., 2006; Wiche and Winter, 2011). Expression of mutated GFAP, resulted in a decrease in plectin expression suggesting that the levels of plectin could be partially responsible for the collapse of the network (Tian et al., 2006). It is not known whether GFAPδ has lower affinity to bind plectin and could thereby mediate the collapse. Whether plectin plays a role in the collapse in GFAPδ expressing cells needs to be investigated. The effect of
GFAP binding proteins on GFAP exchange rates could help explain why the exchange rates are lower in a collapsed network.

**Conformational changes and GFAP dynamics**

The solubility of GFAP collapses is lower compared to non collapsed networks (Hagemann et al., 2009; Perng et al., 2006, 2008). Electron microscopy on GFAP collapses caused by GFAPα overexpression shows that 10 nm filaments are still present but are disorganized (Koyama and Goldman, 1999). It is not known whether there are conformational changes in the IF proteins when there is a collapsed network. Increased filament-filament associations could be the cause for different GFAP dynamics and increased immobile fractions, since both the solubility of the collapses and the dynamics of the protein are different. If conformational changes occur when IF proteins collapse, protein binding, and therefore IF functions dependent on protein binding, could be substantially altered.

**FRAP to measure IF protein dynamics**

FRAP measures the recovery of fluorescent signal at the bleached ROI. Fluorescence can only recover when the bleached protein moves away from this position and is replaced by another protein. With our experiments we were not able to directly link the recovery to exchange from a soluble pool. Experiments where the soluble pool is depleted (by permeabilizing cells for instance) and the recovery is measured would proof if all of the exchange we see comes from the soluble pool. Another point to mention is that we are aware of the detrimental effect of the GFP tag on the assembly properties of GFAP. In order to perform FRAP experiments a fluorescent tag is needed and therefore we spike in a small amount of GFP-tagged GFAP which is still able to form a network with the endogenous network. The exact half time measured, could however be different compared to untagged GFAP, but here we focused on differences between GFAP isoforms and not absolute half times of GFAP.

Taken together we show that the dynamic properties between GFAPα and GFAPδ are different and that a collapse of the IF network likely changes the dynamics of GFAP. It is not known yet what mediates this difference in dynamic exchange and what the functional consequences are. Studies on phosphorylation of the C-terminal tail of GFAP isoforms will help elucidate the potential role of phosphorylation in the difference in exchange rates.
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