Rab6 and unfolded protein response-mediated proteostasis in Alzheimer's disease
Elfrink, H.L.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Rab6 accumulation is associated with dysfunction of the autophagy/lysosomal pathway in Alzheimer’s disease

Hyung L. Elfrink, Jeroen J.M. Hoozemans, Rob Zwart, Frank Baas and Wiep Scheper
Abstract

Our previous work showed that Rab6 accumulates in Alzheimer’s disease (AD) brain and that this is in strong correlation with the increase of the unfolded protein response (UPR) of the endoplasmic reticulum (ER). In cell models, we showed that Rab6 actually reduces the UPR and associated ER stress toxicity. This effect is not mediated via UPR signaling, but via an unknown feedback mechanism that may involve lysosomes. Because the autophagy/lysosomal pathway (ALP) is compromised in neurons of AD patients, we further investigated in this study the connection between high Rab6 levels and dysfunction of the ALP. We showed that membrane bound Rab6 accumulates in cells deficient for autophagy and therefore this pool of Rab6 is a substrate for the ALP. In line with this we find increased levels of Rab6 in AD hippocampal neurons with increased LC3 and Cathepsin D levels, indicative of ALP dysfunction. Double immunohistochemistry shows that Rab6 accumulates both in- and outside of structures positive for ALP markers. We propose that impaired ALP function contributes to Rab6 accumulation in AD neurons as well to rendering Rab6 inactive or ineffective to perform its protective role during ER stress.
Introduction

Alzheimer’s disease (AD) is an age-related multifactorial disorder of which we are only beginning to understand molecular events that are early contributors to the pathogenesis. Activation of the unfolded protein response (UPR) of the endoplasmic reticulum (ER)\(^1\) is such an early event. The UPR is activated in response to disturbances in the ER that result in protein misfolding. It involves a transcriptional and translation- al program aimed to restore ER homeostasis after which tight feedback mechanisms switch off the UPR\(^88-100\). Prolonged UPR activation in the brain, however, may lead to neurodegeneration via depletion of synaptic proteins\(^285,286\) and initiation of an apoptotic program\(^235,236\).

We have previously shown that the small GTPase Rab6 is increased in AD brain and that this occurs in strong correlation with the increase of UPR\(^226\). We have shown that Rab6 has an ameliorating effect on the UPR; elevated Rab6 levels reduce UPR activity and inhibit ER stress toxicity\(^101\). Our previous work shows that Rab6 does not directly interfere in the upstream sensing and signaling of the UPR, but rather works via a feedback mechanism\(^101\). ER stress induces activity of the autophagy/lysosomal pathway (ALP)\(^233\). Indeed, during UPR activation, the ALP is the major pathway for protein degradation\(^169\). Knockdown of Rab6 induces a strong relocalization of lysosomes, accompanied with disturbed lysosomal activity. In addition, it impairs the dynamic repositioning of lysosomes during nutrient and ER stress (Chapter 5), suggesting a role for lysosomes in the Rab6 mediated downregulation of the UPR.

In AD brain UPR activation in neurons is high despite high levels of Rab6\(^226\). Therefore, the downregulating effect of Rab6 on the UPR is apparently compromised in AD brain. An imbalance between formation and degradation of lysosomal/autophagic vesicles causes accumulation in AD brain\(^247,270\). The accumulation of these vesicles may ultimately contribute to the formation of end-stage granulovacuoles\(^287,288\), granules associated with AD that represent disturbed ALP processes\(^288\). In AD hippocampus, neurons that exhibit this granulovacuolar degeneration (GVD) are strongly positive for UPR activation markers\(^64\). In addition, LC3, a substrate of the ALP, accumulates in neurons with an active UPR\(^169\). These observations suggest that prolonged UPR activation is associated with dysfunction of the ALP.

The cause of the Rab6 accumulation in AD brain is not known. Rab6 levels are not increased by ER stress; in fact UPR activation reduces the Rab6 levels and also inhibition of the proteasome does not lead to higher Rab6 levels\(^226\). Rab6 is therefore not a direct target of the UPR or the ubiquitin proteasome system. It is not known whether Rab6 is a substrate for the ALP, which could be a possible explanation for its accumulation in AD neurons with a defective ALP.

Here we further investigate the connection between Rab6 and the ALP in AD hippocampus.
Materials and methods

Materials

Cell culture media and reagents were obtained from Gibco/Invitrogen (Carlsbad, CA, USA) and other chemicals were from Sigma (St. Louis, MO, USA), unless indicated otherwise.

Post-mortem hippocampal material

Post-mortem hippocampal material was obtained from the Netherlands Brain Bank (Amsterdam, the Netherlands). Patients or their next of kin gave informed consent for autopsy and use of tissue and medical records for research purposes. Clinical diagnosis, age, gender and Braak staging of cases used in this study are listed in table 1, if available.

Table 1. Cases in this study

<table>
<thead>
<tr>
<th>Case</th>
<th>Clin. Diag.</th>
<th>Age (y)</th>
<th>Gender</th>
<th>Braak stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>S10-198</td>
<td>Con</td>
<td>83</td>
<td>f</td>
<td>1</td>
</tr>
<tr>
<td>S11-27</td>
<td>Con</td>
<td>80</td>
<td>m</td>
<td>1</td>
</tr>
<tr>
<td>S06-183</td>
<td>AD</td>
<td>62</td>
<td>m</td>
<td>6</td>
</tr>
<tr>
<td>S08-139</td>
<td>AD</td>
<td>U</td>
<td>U</td>
<td>6</td>
</tr>
</tbody>
</table>


Cell culture and treatment

Inducible mouse embryonic fibroblast (MEF) Atg5<sup>−/−</sup> cells were a kind gift of Dr. N. Mizushima<sup>256</sup>. MEF Atg5<sup>−/−</sup> cells were cultured in Dulbecco’s modified Eagle medium with GlutaMAX supplemented with 10% (v/v) fetal calf serum (Lonza, Basel, Switzerland), 100 U/mL penicillin and 100 µg/mL streptomycin. MEF Atg5<sup>−/−</sup> cells were maintained in the presence of doxycyclin (20 ng/mL) and cultured in the absence or presence of doxycycline for the Atg5<sup>+/+</sup> or Atg5<sup>−/−</sup> genotype, respectively. Omission of doxycycline was at least 72 hours to fully obtain the wildtype Atg5 expression. Cells were incubated at 37°C, 5% CO<sub>2</sub> and 95% humidity. Cells were plated in a desired wells format at a density of ~8.10<sup>3</sup> cells/cm<sup>2</sup> in complete culture medium.

Subcellular fractionation, SDS-PAGE and western blotting

Cells were cultured in Ø10 cm Petri dishes. After treatment, cells were harvested by scraping with a rubber policeman in a hypotonic lysis buffer (5 mM Tris.HCl pH 7.4, 250 mM sucrose [Merck, Darmstadt, Germany], 1 mM EDTA, Complete protease inhibitors and PhosSTOP phosphatase inhibitor cocktail [Roche, Penzberg, Germany]) and passed 10 times through a 27 gauge needle, all on ice. Post nuclear supernatant (800xg,
10 minutes, 4°C) protein content was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Post nuclear supernatants were transferred in detergent-free ultracentrifuge tubes and ultracentrifuged (120,000xg, 1 hour, 4°C). Membrane pellets were dissolved in 1% (v/v) Triton X-100 in PBS. Samples were analyzed for Rab6 on a 12% polyacrylamide gel. Membrane fraction protein loading was proportional to the protein content of the post nuclear supernatant and was checked using calnexin (see table 2) as a loading control. A TE77XP Semi-Dry Transfer Unit (Hoefer, San Francisco, CA, USA) was used for protein transfer to PVDF membrane (Millipore, Billerica, MA, USA). Antibodies and incubation conditions are specified in table 2. Visualization was performed with Lumi-Light Western Blotting Substrate (Roche) on a LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed as described previously by Nölle et al. Briefly, formalin-fixed, paraffin-embedded tissue was selected from age-matched non-neurological controls and Alzheimer’s disease cases (table 1). Sections (5 µm thick) were mounted on Superfrost Plus tissue slides (Menzel-Gläser, Braunschweig, Germany) and dried overnight (37°C). After deparaffinization, endogenous peroxidase activity was quenched (0.3% H2O2 in phosphate-buffered saline [PBS] for 30 min). All washes were performed with PBS. Sections were treated in sodium citrate buffer (10 mM, pH 6.0) and heated by autoclave (10 min) for antigen retrieval. Sections were incubated with Rab6 primary antibody in Dako antibody diluent (Dako, Glostrup, Denmark; see table 2 for more information). Sections were incubated with horseradish peroxidase (HRP) labeled secondary antibody complex (REAL EnVision/HRP, Dako, 30 min at room temperature). Color development was performed using 3,3’-diaminobenzidine (DAB, Sigma) as chromogen (10 min). Sections were treated with sodium citrate (10 mM, pH 6.0) and heated by autoclave (10 min) to denature bound antibodies. Sections were blocked in 10% normal goat serum (10 min at RT, Dako). Incubation with secondary primary antibody (LC3 or Cathepsin D, see table 2) was performed overnight (4°C). Sections were incubated with HRP labeled secondary antibody for 1 h. Color development was performed using Liquid Permanent Red (LPR, Dako) as chromogen. Sections were table 2.

### Table 2. Antibodies used for western blot and immunofluorescence

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Clonality</th>
<th>Manufacturer</th>
<th>Cat. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab6</td>
<td>Rabbit</td>
<td>Polyclonal (C-19)</td>
<td>Santa Cruz</td>
<td>sc-310</td>
</tr>
<tr>
<td>eEF2</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Cell Signaling</td>
<td>#2332</td>
</tr>
<tr>
<td>Calnexin</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Calbiochem</td>
<td>208880</td>
</tr>
</tbody>
</table>

**Western blotting**

- Incubations for western blot were performed 1:1000 in 5% (w/v) fat-free milk in PBS-T (0.05%, v/v), secondary HRP labeled antibodies were from Dako. Incubations for immunohistochemistry were performed 1:100, secondary dilutions were 1:300. Also used for immunohistochemistry.
counterstained with haematoxylin and mounted using DePeX (BDH Prolabo/VWR, Radnor, PA, USA). Negative controls were generated by omission of primary antibodies. Microscopy was performed on a Leica microscope with a 20x objective. At least three fields of view were photographed per patient. Images were acquired and spectrally separated using Nuance Multispectral Tissue Imaging System and software (Nuance software, CRi/PerkinElmer, Waltham, MA, USA). Spectral grey-scale images were converted to indexed pseudo-fluorescence images using Photoshop CS6 (Version 13.0.1 x32, Adobe Systems Incorporated, San Jose, CA, USA).

**Figure 1.** Membrane bound Rab6 increases if autophagy is impaired.

Total and membrane associated Rab6 was determined by western blot in MEF Atg5-/- cells. Doxycycline (20 ng/mL) was used to induce the Atg5-/- genotype and doxycycline was omitted for at least 72 hours to recover the wildtype (Wt) genotype. Eukaryotic elongation factor 2α was used as a loading control for total fractions and calnexin was used as a loading control for membrane fractions. Shown are blots from representative experiments. Quantification shows the mean and SD of the relative increase of Rab6 (in arbitrary units, AU) of n=4 independent experiments.

**Results and discussion**

To determine if Rab6 is degraded by autophagy, we employed inducible Atg5-/- MEFs. These cells are autophagy proficient in the presence of doxycycline and deficient in the absence of doxycycline.256 We determined Rab6 levels in the absence and presence of doxycycline. The total levels of Rab6 were unaffected by autophagic capacity (fig. 1). Interestingly, the membrane-bound fraction of Rab6, which represents the active form181,290, increases almost two-fold if autophagy is rendered inactive (fig. 1). This indicates that at least part of the Rab6 pool is degraded by the ALP.

To address the connection between increased Rab6 levels and the dysfunctional ALP in AD neurons, we performed double immunohistochemical stainings with Rab6 and the autophagic marker LC3 (fig. 2A) and the lysosomal marker cathepsine D (CathD;
Rab 6 accumulates in lysosomes in Alzheimer’s Disease.

A

Control

Alzheimer’s Disease

IHC

Pseudo-F

Rab6

LC3

Merge

B

Control

Alzheimer’s Disease

IHC

Pseudo-F

Rab6

CathD

Merge
fig.2B) in the hippocampus of control and AD patients. Spectral imaging was performed and images were converted to pseudo fluorescence. In human hippocampus Rab6 positivity is found throughout the neuronal soma, some material appears to be associated with structures inside the cell, some appears more diffusely distributed (fig. 2A,B). In AD hippocampus, Rab6 levels are strongly increased as expected.

Both LC3 and cathepsine D positivity is found throughout the neuronal cell body as punctate structures that are enriched in the perinuclear region (fig. 2A,B, control). In addition, cathepsin D can be observed in tubular structures. In AD hippocampus, both markers are strongly increased (fig 2A,B, Alzheimer’s Disease), reflecting dysfunction of the ALP that occurs in the course of AD. Prominent colocalization of Rab6 is found with cathepsine D and to lesser extent with LC3 as well. This suggests that a subpool of Rab6 accumulates in ALP structures in AD neurons, although confocal microscopy is required to draw a definitive conclusion.

The colocalization with LC3 and cathepsine D positive structures supports the in vitro data that Rab6 itself is a substrate for autophagic clearance. Our results in the Atg5\(^{\text{-/}}\) MEFs indicate that only active Rab6 is degraded by autophagy, which could explain why also a large part of Rab6 does not co-localize with the ALP but rather accumulates diffusely outside the LC3 or cathepsine D positive structures, suggestive of cytosolic localization. This may represent the non-membrane bound pool of inactive Rab6-GDP. This selective degradation of the active form may entail an additional level of control of Rab6 activity, besides the GTP/GDP switch function inherent to the small GTPases. It is tempting to speculate that this provides feedback on the UPR modulation by Rab6, because the UPR also induces autophagy\(^{169,233}\) (see model Chapter 7). In support of this, we have previously reported that the levels of Rab6 are decreased by activation of the UPR\(^{226}\).

In AD neurons, UPR activation is associated with high Rab6 levels therefore Rab6 is apparently inactive or at least unable to perform its UPR-downregulating function. This may be because Rab6 requires an intact ALP for this function and this is defective in AD. It is also possible that a defect in the Rab6 pathway contributes to the ALP dysfunction. Interestingly, the cathepsine D positivity in AD neurons is concentrated in the perinuclear region. This organization is reminiscent of the relocated lysosomal clusters that we observe under stress conditions in cell models (Chapter 4 and unpublished data) as well as in Rab6 knockdown cells (Chapter 5). Although this remains speculative at this time, it is in agreement with the hypothesis that Rab6 may be inactive or ineffective in AD.

Our data support the important role of the dysfunction of the ALP system in AD pathogenesis. The interplay between Rab6 and ALP function during UPR activation therefore potentially presents a novel opportunity to intervene early in the disease process.
Acknowledgements

We thank Anna Nölle and Judith van der Harg for participating in stimulating discussions concerning this manuscript. This study was financially supported by grants from the Internationale Stichting Alzheimer Onderzoek Nederland (ISAO #07506) and the Netherlands Organisation for Scientific Research (NWO) to WS.