Prevention of oculopharyngeal muscular dystrophy-associated aggregation of nuclear poly(A)-binding protein with a single-domain intracellular antibody

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Oculopharyngeal muscular dystrophy (OPMD) belongs to the group of protein aggregation disorders and is caused by extensions of the N-terminal polyalanine stretch of the nuclear poly(A)-binding protein 1 (PABPN1). The presence of PABPN1-containing intranuclear aggregates in skeletal muscle is unique for OPMD and is also observed in transgenic mouse and cell models for OPMD. These models consistently support a direct role for the protein aggregation in OPMD pathogenesis. We have isolated and characterized a diverse panel of single-domain antibody reagents (VHH), recognizing different epitopes in PABPN1. The antibody reagents specifically detect endogenous PABPN1 in cell lysates on western blot and label PABPN1 in cultured cells and muscle sections. When expressed intracellularly as intrabodies in a cellular model for OPMD, aggregation of PABPN1 was prevented in a dose-dependent manner. More importantly yet, these intrabodies could also reduce the presence of already existing aggregates. Given the domain specificity of VHH-mediated aggregation interference, this approach at least allows the definition of the nucleation kernel in aggregation-prone proteins, thus facilitating etiological insight into this and other protein aggregation disorders, and ultimately, it may well provide useful therapeutic agents.

INTRODUCTION

Oculopharyngeal muscular dystrophy (OPMD) (MIM164300) is a late-onset disease, clinically characterized by slow progressive ptosis, dysphagia and limb girdle weakness (1). OPMD is usually inherited as an autosomal-dominant trait and caused by a trinucleotide repeat expansion in the coding region of the nuclear poly(A)-binding protein 1 (PABPN1) gene (2). The alanine stretch that is encoded by this trinucleotide sequence contains 10 alanines in the non-affected protein, but is expanded to 12–17 alanines in the mutant protein in autosomal-dominant OPMD.

PABPN1 is ubiquitously expressed and is involved in poly(A)-tail synthesis and poly(A)-tail length control (3). One of the pathological hallmarks of OPMD is the presence of PABPN1-containing fibril-like aggregates in 2–5% of myonuclei in affected muscle (4–6). The roles of the formation of the intranuclear inclusions in the progression of OPMD are poorly understood (1,7,8).

Recently, a doxycycline-based treatment for OPMD was proposed on the basis of the animal studies with transgenic mice (8). These mice develop OPMD-like muscle defects (8). These mice develop OPMD-like muscle defects and also show intranuclear aggregation of mutant PABPN1. Upon doxycycline treatment, the muscle defects improved
phage selection rounds were employed against *Escherichia coli* to isolate antibody reagents against PABPN1. Two successive antibody selections led to the isolation of VL antibody reagents (16), it was possible to inhibit huntingtin aggregation by intracellular expression of single-chain Fv (scFv) (15) and several approaches have been applied to reduce aggregation. Instead, many of these agents were tested in analogy to reducing agents, none of them is specific for PABPN1 aggregation and aggregate formation was reduced, suggesting a direct role for protein aggregation in OPMD pathogenesis.

PABPN1 aggregation has also been studied in cellular models, using transient expression of wild-type and expanded PABPN1. Aggregate formation was inhibited in these cellular models for OPMD by doxycycline, Congo red and over-expressed chaperones (9–11). These studies also resulted in increasing knowledge of the toxicity of the intranuclear inclusions (12), proteins (9,13,14) and nucleic acids included in the formed inclusions, and the dynamic nature of the intranuclear inclusions (14). For example, it was shown that reduction of aggregate formation leads to increased cell survival (11).

Despite the effectiveness of some of these aggregation reducing agents, none of them is specific for PABPN1 aggregation. Instead, many of these agents were tested in analogy to other protein aggregation conditions and operate through poorly understood mechanisms. In parallel, also antigen-specific approaches have been applied to reduce aggregation. By intracellular expression of single-chain Fv (scFv) and *V*<sub>L</sub> antibody reagents (16), it was possible to inhibit huntingtin aggregate formation in cellular models for Huntington’s disease.

The advantage of single-domain antibody reagents over the approaches just described lies in their single-domain character, ease of generation, small size and stability (17). The immediate availability of their sequence makes these antibody fragments amenable to genetic modification and intracellular expression using conventional expression vectors. This also facilitates their structural and functional analysis. Thus, single-domain antibodies were used to immuno-modulate several antigens (18–20).

We aimed at isolating single-domain antibody reagents against PABPN1 to investigate whether we could inhibit intranuclear inclusion formation in OPMD cell models. Using a combination of selections for single-domain antibody reagents, we obtained different sets of antibody reagents. By intracellular expression of some of these antibody reagents, we were able to inhibit aggregation in situ in a dose-dependent manner. Experiments with serial expression of mutant PABPN1 and PABPN1-specific intrabody indicate that even existing aggregates could be cleared.

**RESULTS**

**Antibody selections**

To isolate antibody reagents against PABPN1, two successive phage selection rounds were employed against *Escherichia coli*-produced and affinity-purified full-length human PABPN1. In pilot experiments, we found that a combination of capturing the antigen in the first round (by its T7 tag) followed by direct immobilization in the second round yields the most diverse set of binders (P. Verheesen et al., manuscript in preparation). To ensure that different epitopes were recognized by the selected antibody reagents, we performed several epitope-masking selections in which antibody reagents obtained in earlier selection rounds were used to capture PABPN1. The enriched sub-libraries were screened for monoclonal antibody reagents with specificity for PABPN1. From the different selections, a total of six different antibody reagents were identified with affinities for PABPN1 between 5 and 57 nm (data not shown).

**Epitope mapping**

Three selected antibody reagents were further characterized by epitope mapping with western blotting, using a panel of truncated recombinant PABPN1 proteins. All mapped to epitopes within the N-terminal 155 amino acids. One of the VHH, coded 3F5, obviously showed the strongest signals on western blot. As this antibody reagent also exhibited the highest affinity (5 nM) for both the recombinant-produced PABPN1 and the native PABPN1, we continued with this antibody reagent for a more detailed epitope mapping. From these epitope mappings (Fig. 1), we conclude that the epitope for 3F5 is situated between amino acids 113 and 133, which largely overlaps with the part of PABPN1 that was predicted to form an amphipathic α-helical or coiled-coil domain (amino acids L119–Q147) (21). Using a series of different point mutants of PABPN1, we could demonstrate that residues at positions 126, 129 and 131 are essential for the binding of the antibody reagent (Fig. 1).
Detection of endogenous PABPN1

3F5 was subsequently used for western blotting with cytosolic and nuclear fractions from HeLa cells (Fig. 2A). A single band with an expected molecular weight of 50 kDa was specifically detected in the nuclear protein fraction. Next, in two independent cell lines, HeLa and COS-1 cells, we observed an expected predominant nuclear staining with denser fluorescent signal in a speckle-like pattern by immunofluorescence microscopy (Fig. 2B–E) (22). In addition to cultured cells, cryosections of control human muscle were stained with 3F5 as well (Fig. 1F and G). Nuclear localization with accumulation in a speckle-like pattern was observed, indicating successful detection of PABPN1 in muscle.

Cell model for PABPN1 aggregation

To discriminate between endogenous and over-expressed mutant PABPN1, we transfected mPABPN1-ala17 in fusion with the vesicular stomatitis virus (VSV) glycoprotein tag in COS-1 and HeLa cells. Intranuclear aggregation of PABPN1 was observed (Fig. 3A and B). Incubation of these cells with fluorescent oligo(dT), anti-HSP70 and anti-ubiquitin antibodies showed that poly(A)-RNA, HSP70 and ubiquitin were present in the aggregates (data not shown), as has been reported previously (4,9).

We assessed whether transfected mPABPN1-ala17 was localized differently compared with endogenous PABPN1. To this end, HeLa cells were transfected with mPABPN1-ala17, and the transfected mPABPN1-ala17 was visualized by incubation with anti-VSV antibodies, whereas total PABPN1 was visualized with 3F5. No differences in localization of both signals were observed.

Prevention of aggregation by intrabody 3F5

The effect of intracellular expression of 3F5 was investigated in cellular PABPN1 aggregation models. Intrabody 3F5 was transfected in fusion with a nuclear localization signal (NLS) and green fluorescent protein (GFP) (3F5–NLS–GFP) in COS-1 and HeLa cells. The GFP signal was exclusively observed in the nucleus, indicating both a successful expression of intrabody and its targeting to the nucleus (Fig. 3C and D).

Subsequently, 3F5–NLS–GFP was co-transfected with mPABPN1-ala17 (Fig. 3E–G) in different ratios. In a dose-dependent manner in which mPABPN1-ala17 was kept constant and increasing concentrations of 3F5–NLS–GFP were used, the intrabody could completely prevent aggregation (Fig. 4). The expression levels of mutant PABPN1 and intrabody 3F5 or NLS–GFP control were analyzed by western blotting (Fig. 5). This showed that expression of the intrabody did not affect the expression levels of its antigen mPABPN1-ala17.
To explore the putative cytotoxic effects of the intracellular expression of 3F5, cells were singly transfected with the intrabody construct and analyzed at different time points by western blotting. A gradual increase in the intrabody expression was observed in time, without a detectable effect on endogenous PABPN1 levels. Cells that were singly transfected with the intrabody construct were also microscopically investigated. PABPN1 was labeled with one of the selected antibody reagents, which recognizes a distinct epitope from the binding place of 3F5. The PABPN1 localization in intrabody-expressing cells was indistinguishable from its localization in non-transfected cells. With a cell proliferation assay, the metabolic activity in intrabody-transfected cells was compared with non-transfected cells. No difference in metabolic activity was observed between these cells. Therefore, intracellular expression of 3F5 did not cause any detectable cytotoxic effects based on the analysis of endogenous antigen levels and localization and cell viability.

Clearing of existing aggregates

To explore the putative cytotoxic effects of the intracellular expression of 3F5, cells were singly transfected with the intrabody construct and analyzed at different time points by western blotting. A gradual increase in the intrabody expression was observed in time, without a detectable effect on endogenous PABPN1 levels. Cells that were singly transfected with the intrabody construct were also microscopically investigated. PABPN1 was labeled with one of the selected antibody reagents, which recognizes a distinct epitope from the binding place of 3F5. The PABPN1 localization in intrabody-expressing cells was indistinguishable from its localization in non-transfected cells. With a cell proliferation assay, the metabolic activity in intrabody-transfected cells was compared with non-transfected cells. No difference in metabolic activity was observed between these cells. Therefore, intracellular expression of 3F5 did not cause any detectable cytotoxic effects based on the analysis of endogenous antigen levels and localization and cell viability.

In a 1:1 ratio of 3F5:mPABPN1-ala17, a reduction to 70 ± 4% (P < 0.05) was observed for HeLa cells, whereas a reduction to 89 ± 10% was observed in COS-1 cells.

DISCUSSION

By a combination of antigen capturing, panning and epitope masking, we have selected various antibody reagents against PABPN1. Among these is an antibody reagent that may have potential in the treatment of OPMD, as we demonstrate that it can reduce aggregate formation as well as clear already existing aggregates in a cell model for OPMD.

To date, the mechanism of specific development of muscle defects in OPMD remains unclear, but a direct role for the protein aggregation in OPMD pathogenesis has been suggested. On average, 2–5% of the nuclei in affected OPMD muscle contain visible intranuclear inclusions, and in some cases, intranuclear inclusions appear mostly in myonuclei of affected tissue (1,23). Moreover, homozygotes for OPMD show a more severe phenotype and have significantly more intranuclear inclusions (24). Aggregates of mutant PABPN1 are present in post-mitotic long-living myonuclei of OPMD patients (4–6), which may indicate a relationship between the differentiation state of the cell and the appearance of detectable inclusions (1). It was shown that mutant PABPN1 aggregates contain high concentrations of poly(A)-RNA, and it was suggested that poly(A)-RNA entrapment in aggregates may play a role in OPMD pathogenesis (4). The muscle-specific phenotype may be further explained by sequestration of ski-interacting protein (SKIP) in the aggregates, as it is known that PABPN1 and SKIP synergistically activate MyoD (25). Although the exact pathological mechanism underlying OPMD is only partly understood, cellular and animal model studies of OPMD are consistent with the view that the aggregation process, and more specifically early oligomeric or microaggregate mutant proteins, is toxic.
The selected antibody reagents may thus prove useful in further studies of the role of PABPN1 aggregation in OPMD pathogenesis.

Currently, over-expression of mPABPN1 in COS-1 and HeLa cells is the only cellular reporter system that has been demonstrated to show mPABPN1 aggregation that leads to cell death (11). Inhibition of aggregate formation with chaperones, doxycycline and Congo red has been described (9–11). However, these chaperones and chemicals are not specific for PABPN1 aggregates, but rather recognize a large number of misfolded or aggregated proteins and may thus have undesired side effects. In contrast, antibodies that specifically bind their target can be used for specific intervention. We have therefore selected a PABPN1-specific monoclonal antibody reagent for which we show that we are able to prevent aggregate formation by mutant PABPN1 in a dose-dependent manner. Intracellular expression of this antibody reagent did not yield any detectable deleterious side effects as assayed by normal antigen levels, localization or cell viability. The observation that endogenous and transient PABPN1 protein levels in transfected cells are not influenced by the expression of PABPN1-specific intrabodies indicates that reduction of aggregate formation by the intrabody is a direct effect of the intrabody and not the level of the mutant protein (Fig. 5). This concerns a very specific interaction, as intrabodies against other epitopes on PABPN1 were not able to prevent aggregate formation (data not shown). Potential oligomerization domains (ODs) have been identified in PABPN1 that were shown to play a role in aggregation (12). Binding of 3F5 to these or yet unidentified regions may prevent aggregation by shielding off these regions for other similar interactions, as was shown with deletion constructs (12).

In consecutive transfections of mPABPN1 and 3F5, we showed that the intrabody not only prevents aggregation, but also clears already existing aggregates. Three independent transfection experiments in two different cell lines showed a significant reduction of aggregate-containing cells when a surplus of 3F5 was expressed in cells in which aggregates were already formed (P < 0.05). This observation could suggest a dynamic nature of the aggregates formed in this cell model consistent with earlier photobleaching experiments, which revealed that both normal and expanded PABPN1 molecules are not irreversibly sequestered into aggregates, and with the presence of reversible non-pathological PABPN1 aggregates in normal magnocellular neurosecretory neurons of the rat hypothalamus (7,14). Furthermore, in a transgenic mouse model for OPMD, it was demonstrated that aggregate-reducing agents delayed the muscle pathology (8). Therefore, it is feasible that in OPMD patients, reagents that act on the equilibrium between soluble and oligomeric or aggregated PABPN1 may also reduce already formed aggregates.

In conclusion, we have shown that single-domain antibody reagents from Camelidae can highly selectively block or revert pathological processes. Efficient in vivo immunomodulation by single-domain antibody reagents was recently reported in plants (18). Therefore, in further studies, we will improve the efficient delivery of the single-domain antibody reagents in cells of affected OPMD tissues to evaluate whether, also in affected tissues, 3F5 will have preventive and curative properties.

Figure 6. Reduction of mPABPN1-ala17 aggregate formation in situ by consecutive transfection of 3F5. 3F5 was consecutively transfected in different intrabody:mPABPN1-ala17 plasmid ratios 24 h after transfection with mPABPN1-ala17. HeLa and COS-1 cells transfected with mPABPN1-ala17 contained 38 ± 4% and 33 ± 8% intranuclear aggregates 24 h post-transfection, respectively. The aggregation at the moment of secondary transfection was set to 100%. Doubly transfected cells were scored for the presence of intranuclear aggregates by microscopy 48 h after serial transfection. A dose-dependent decrease in the number of cells with intranuclear aggregates was observed. *P < 0.05 and NS (not significant) P > 0.05.

MATERIALS AND METHODS

Antibody selections

The human cDNA sequence of PABPN1 was cloned into the prokaryotic expression vector pET28a (Novagen). Recombinant protein was produced in BL21(DE3)-RIL E. coli (Stratagene). The protein was purified by means of the attached His-tag, using TALON (BD Biosciences). Two rounds of selection were performed with a large (5 × 109) non-immune llama single-domain antibody reagent library (kindly provided by Unilever Research Vlaardingen, The Netherlands), using standard procedures (26). Briefly, with differences as described, monoclonal antibody against the T7-tag (Novagen) [10 μg/ml in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4 and 1.5 mM KH2PO4)] was coated to maxisorp 96-well plates (Nunc). After blocking with 4% skimmed milk in PBS (4% MPBS), the purified T7-tagged PABPN1 (10 μg/ml in 0.1% MPBS) was captured. About 1011 phages [in 2% MPBS, 1% bovine serum albumin (BSA), 10% normal mouse serum (NMS)] were added and incubated for 90 min at room temperature (RT). After extensive washing with 0.05% Tween-20 in PBS (PBST) and PBS, bound phages were eluted with 100 mM triethylamine (TEA) for 10 min at RT. Phages were prepared for the second selection round as described. Bound phages were eluted with high pH. These phages were multiplicated and used for a second selection round. This second selection round was performed similar to round 1, except that PABPN1 (10 μg/ml in PBS) was directly coated to maxisorp plates and 107 phages were used. For epitope-masking selections (27,28) against PABPN1, 10 μg/ml VHH3F5 was coated to polystyrene plates and bovine PABPN1 from calf thymus (kindly provided by Antje Ostareck-Lederer, Martin-Luther-University Halle, Germany) was captured as described before. After the second selection round, single colonies were picked in 96-well plates and c-myc-tagged VHH
(VHH-myc) were produced by overnight induction in 96-well plates. Culture supernatants containing VHH-myc were tested with enzyme-linked immunosorbent assay for binding to directly coated PABPN1 (10 μg/ml in PBS). VHH-myc were detected with mouse anti-c-myc antibodies (kind gift from P.W. Hermans, Biotechnology Application Centre, The Netherlands) and anti-mouse peroxidase-conjugated antibodies (Jackson, West Grove, PA, USA). Single-colony polymerase chain reaction (PCR) was performed as described, and PCR fragments were cut with Hinfl (New England Biolabs) and analyzed on 2% agarose gels.

**Epitope mapping**

The following PABPN1 domains were PCR amplified from the full-length PABPN1 cDNA and cloned in a derivate of GST-fusion vector pGEX-3X (Amersham): OD 264–306 (12); OD 155–294 (12); amino acids 173–244 that contain most of the RNP domain that stretches from amino acids 161–257 (29); and amino acids 271–291 that contain a cluster of methylated arginines (29). Deletion constructs ΔN10, ΔN49, ΔN92 and ΔN113, an N-terminal protein fragment encoding amino acids 1–125 and point mutation constructs V126S, M129A, E131A, A133S, K135A, L136S and V143A were used for fine epitope mapping and were described before (21) (kindly provided by Uwe Kühn, Martin-Luther-University Halle, Germany) and supplied ready-to-use as purified proteins for western blotting.

**Affinity measurements**

Association and dissociation constants ($k_{on}$ and $k_{off}$, respectively) for the binding of 3F5 to PABPN1 were measured using a Biacore3000 (Biacore) and covalently coupled recombinant PABPN1 and 3F5 to a CM5 sensor chip. Affinities were calculated using Biacore evaluation software.

**Cell culture and immunofluorescent labeling**

HeLa and COS-1 cells were cultured according to standard protocols. Cells were grown on cover slips for 24 h, washed with PBS and fixed with 4% formaldehyde in PBS for 15 min at RT. Triton X-100 was added to a final concentration of 0.1%, and cells were permeabilized for 15 min at RT. Cells were blocked with 100 mM glycine in PBS and 1% BSA in PBS for 15 min at RT and incubated with 3F5 (1 μg/ml in 1% BSA/PBS) for 90 min at RT. VHH were detected with anti-c-myc monoclonal antibody and Alexa Fluor 488-labeled anti-mouse antibody (Molecular Probes) in 1% BSA/PBS, each for 1 h. Cells were incubated with 0.2 μg/ml DAPI (Roche), together with the last antibody incubation to visualize nuclei. Cross-sections of 6 μm from a control human muscle biopsy were air-dried for 30 min, fixed and labeled with 3F5 as described for cultured cells.

**Transfections and quantification of aggregates**

mPABPN1-ala17 was cloned into an eukaryotic expression vector (pSG8) (30) adjacent to the C-terminus of the VSV glycoprotein tag (VSV-tag). The VSV-tag allows specific immunological detection of the transfected mutant protein. The cDNA encoding 3F5 was cloned into an eukaryotic expression vector (modified pSG8) in fusion with the SV40 T-antigen NLS and the GFP.

As a model for PABPN1 aggregate formation, COS-1 and HeLa cells were transfected with plasmid encoding VSVG-tagged mutant PABPN1 with 17 alanines (mPABPN1-ala17) using FuGENE 6 (Roche, Indianapolis, IN, USA). Cells were fixed and permeabilized 24 and 48 h post-transfection as described earlier. The transfected PABPN1 was detected with mouse anti-VSV antibody (clone P5D4, Roche), followed by incubation with anti-mouse Cy3-conjugated goat antibody (Jackson). Cell nuclei were stained with DAPI (Roche, Mannheim, Germany).

Intrabody constructs with VHH in fusion with the SV40 T-antigen NLS and GFP were co-transfected and serially transfected in 0.5:1, 1:1, 2:1 and 4:1 intrabody:mPABPN1-ala17 ratios. mPABPN1-ala17 was visualized with anti-VSV antibody as described earlier, and the intrabody was readily visible by virtue of the fusion with GFP. An unrelated intrabody and NLS–GFP were transfected together with mPABPN1-ala17 in 1:1 ratio as controls. For the serial transfections, 3F5, control intrabody and NLS–GFP alone were serially transfected in cells that already expressed mPABPN1-ala17 for 24 h. Forty-eight hours after this second transfection, mPABPN1-ala17 was visualized as described earlier. Three independent experiments were performed for assaying aggregate prevention and gene dosage effects. From the co-transfection and serial transfection experiments, at least 200 and 100 transfected cell nuclei were scored for the presence of intranuclear aggregation, respectively.

With the SPSS package, the dose dependence could be described by logistic regression according to the formula $b_1 + \ln(1 + b_2 \times \exp(b_3 \times [ag]))$, in which $b_1$, $b_2$ and $b_3$ are the parameters to be estimated and [ag] the ratio of aggregated/ non-aggregated cells in co-transfection.

**Cell proliferation assay**

Possible toxic effects of the transient expression of our mPABPN1 and intrabody constructs were investigated with an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (Sigma-Aldrich) cell proliferation assay that discriminates between dead and living cells on the basis of their metabolic activity. MTT was dissolved in culture medium in 1 mg/ml concentration.

**Western blotting**

Cytosolic and nuclear fractions of HeLa cells were prepared as described (31), loaded on 12% sodium dodecyl sulfate (SDS)—polyacrylamide gel electrophoresis (PAGE) gels and transferred to PVDF western blotting membranes (Roche). Membranes were incubated with 3F5 (1 μg/ml in 2% MPBS) overnight at 4°C, followed by incubation with anti-c-myc and anti-mouse peroxidase-conjugated antibodies. Co-transfected cells were trypsinized and lysed in Laemmi buffer. Lysates were loaded on 12% SDS–PAGE gels, transferred to PVDF western blotting membranes and incubated with anti-VSV, anti-GFP (Roche) and anti-actin (ICN) antibodies.
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Conflict of Interest statement. None declared.

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