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To cite this version:

E.K. Lindersson, P. Højrup, W.P. Gai, D. Locker, D. Martin, et al.. α-synuclein filaments bind the transcriptional regulator HMGB-1. NeuroReport, 2004, 15, pp.2735-2739. hal-00088420

HAL Id: hal-00088420

https://hal.science/hal-00088420v1

Submitted on 30 Jun 2021

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α-Synuclein filaments bind the transcriptional regulator HMGB-1

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Abnormal accumulation of α-synuclein filaments in Lewy bodies is a neuropathological hallmark of Parkinson's disease and sequestration of cellular protein into these protein aggregates may contribute to the degenerative process. We identified the transcriptional co-factor high mobility group protein 1 (HMGB-1) as a ligand for α-synuclein filaments by a filament spin-down technique, mass spectrometric peptide mapping and immunoblotting. HMGB-1 binds preferentially to aggregated α-synuclein and is present in α-synuclein filament-containing Lewy bodies isolated from brain tissue affected with dementia with Lewy bodies or Parkinson's disease. Our results demonstrate that α-synuclein filaments hold the potential for disturbing the cellular gene expression as they can sequester a component involved in cellular transcription regulation.

Key words: Filaments; HMGB-1; Lewy body; Parkinson's disease; α-Synuclein

INTRODUCTION

Deposition of α-synuclein aggregates as filaments in Lewy bodies and Lewy neurites represents a hallmark of Parkinson's disease and dementia with Lewy bodies. Two familial Parkinson's-disease-causing missense mutations, A30P and A53T, have been demonstrated to increase the aggregation of α-synuclein *in vitro* and this points to a central role for protein aggregation in the pathophysiology of Lewy-body-related disorders. Several ligands have been described for monomeric α-synuclein but ligands that preferentially bind α-synuclein in its aggregated states, e.g. 20S proteasomal subunits [1], and thus correlate with its pathological conformation have not been studied in detail. Proteins selectively binding aggregated α-synuclein may represent down-stream effectors of the gain of toxic function conferred by the pathogenic α-synuclein mutations and they may thus reveal novel disease-causing mechanisms relevant for Parkinson's disease and dementia with Lewy bodies. It has been suggested that alteration of signaling cascades due to mislocation of transcriptional factors into cytosolic α-synuclein inclusions may contribute to this effect [2,3].

Proteins preferentially binding α-synuclein filaments were identified by applying the α-synuclein filament spin-down technique [1,4] on rat brain cytosol. We identified the high mobility group protein 1 (HMGB-1) as a α-synuclein filament-binding protein. HMGB-1 is a nuclear non-histone protein present in all eukaryotic cells [5]. It plays a role in the transcriptional activity of e.g. steroid receptors [6]. We demonstrated that HMGB-1 preferentially binds filamentous α-synuclein and identified HMGB-1 as a novel component in Lewy bodies. Sequestration of newly transcribed HMGB-1 into cytosolic α-synuclein filaments may impact the normal cellular regulation of gene expression and represents a toxic gain of function that is obtained by aggregated α-synuclein.

MATERIALS AND METHODS

The α-synuclein proteins and the synthetic β-amyloid-(1-40) peptide were expressed and purified or purchased as explained [1]. Rat HMGB-1 and the recombinant human HMGB-1 peptides B (residues 88–164) and AB (residues 1–176) were prepared as previously described [7].

The preparation of the filaments, the α-synuclein filament spin-down assay, immunoelectron microscopy, labelling of HMGB-1 and the solid phase binding assay were performed as described elsewhere [1].

For the identification by mass spectrometry, the protein of interest was excised from Coomassie Blue stained gel and analysed as described previously [8–10]. For the electrophoretic mobility shift assay, the recombinant rat HMGB-1 in concentrations of 0 or 3.85 g/ml was incubated together with increasing concentrations of filamentous α-synuclein (0–0.7 mg/ml) followed by addition of ^{32}P-labelled platinated oligonucleotides (1 or 0.5 nM), produced as described [11].

Immunohistochemical staining of HMGB-1 was conducted with paraffin-embedded or free-floating human

brain sections as described [1]. Specific immunostaining was ascertained by the presence of neuronal cytoplasm and their processes that were abolished by omitting the primary antibodies as described [1].

Immunolabelling of isolated Lewy bodies and Lewy neuritis were performed as described [1] except for the use of polyclonal rabbit anti-rat HMGB-1 (Agro-Bio).

RESULTS

Filaments formed from recombinant human α-synuclein (Fig. 1f) were used as a model to investigate putative functions of α-synuclein filaments present in the pathological structures of diseased human brain tissue. The filaments were readily depolymerised by denaturation in 8 M urea, 4% SDS and, accordingly, did not contain *in vitro* formed oxidative cross-links (Fig. 1a). The α-synuclein filament spin-down technique was applied to rat brain cytosol to identify filament-binding proteins. Incubation of the filaments with cytosol prior to centrifugation resulted in the appearance of a few bands that were not present in any of the negative controls, among which a 25 kDa band was predominant (Fig. 1a). This Coomassie Blue stained band was excised from the gel and subjected to proteolytic peptide mapping, followed by MALDI mass spectrometry of the resulting peptides. Rat HMGB-1 was identified with the peptides covering 34.4% of the total protein. In order to establish an unambiguous assignment, several peptides were analysed by tandem MS (Fig. 1c). The sequence MSSYAFFVQTCR could be deduced from the generated fragments and it is identical to amino acids 13–24 in rat HMGB-1, which clearly establish the parent protein as rat HMGB-1. This result was verified by anti-HMGB-1 immunoblotting (Fig. 1d, upper panel). Co-incubation of

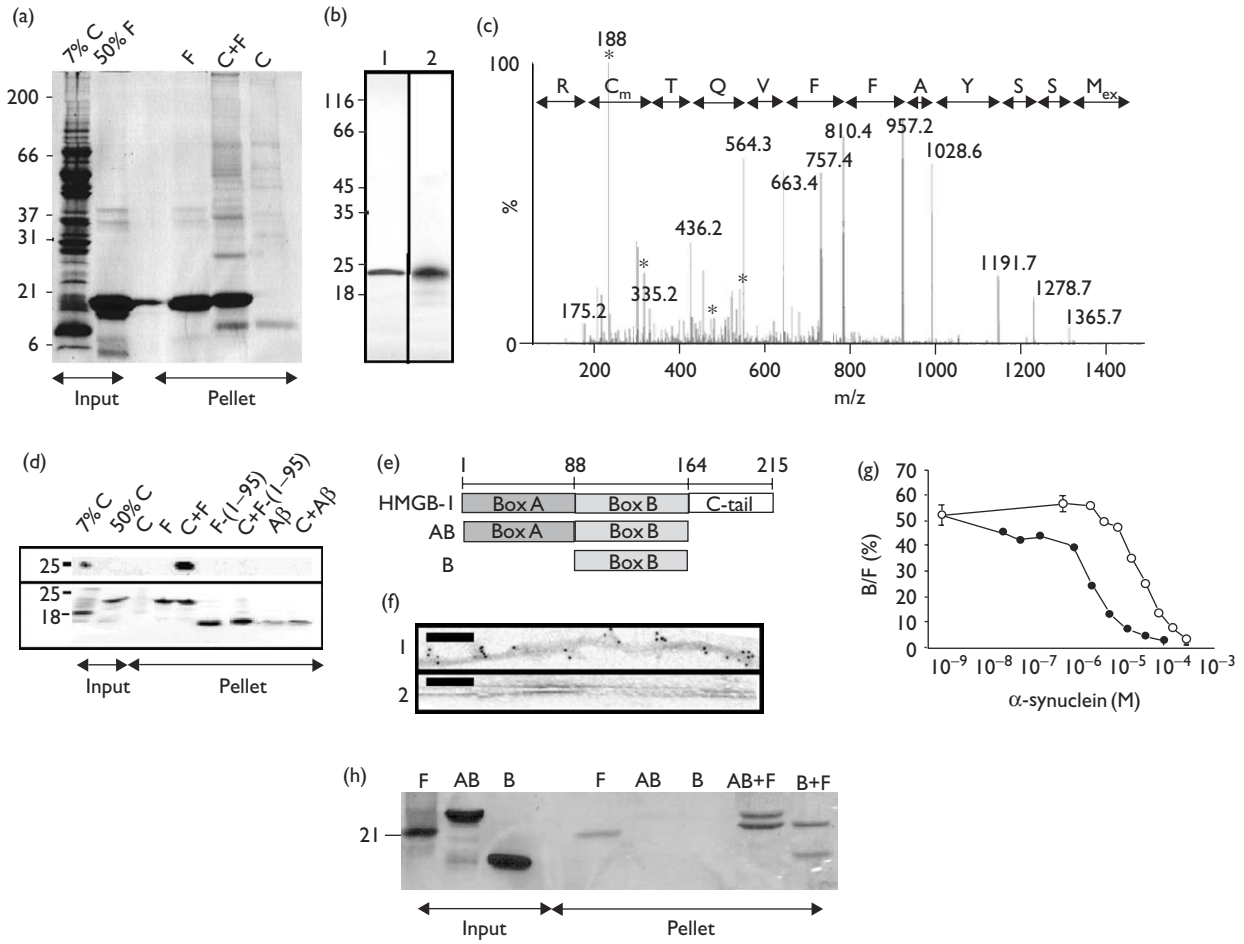

Fig. 1. (**a**) The binding of proteins from rat brain cytosol to α-synuclein filaments was investigated by co-sedimentation analysis. Figure presents total amount of pellet samples and fractions of input samples. Filament-cytosol pellet (C + F), cytosol (C) and filaments (F). Molecular size markers in kDa indicated to the left for a, b, d and h. (**b**) Purified rat HMGB-1 (2 μg) mixed with 20 000 c.p.m. [^{125}I]HMGB-1. Lane 1, silver staining; lane 2, autoradiogram of the same gel. (**c**) Collision-induced dissociation spectrum of a doubly charged parent ion (MH^{2+}) corresponding to 1510.6 Da (tryptic peptide 13–24 from HMGB-1). (**d**, upper panel) Association of HMGB-1 with α-synuclein filaments was verified by immunoblotting as accumulation of HMGB-1 in pellet (C + F) after co-sedimentation of filaments (F) and cytosol (C). (**d**, lower panel) Samples were analysed by Coomassie Blue staining. (**e**) Schematic representation of HMGB-1 and recombinant peptides. HMGB-box domains are grey, the acidic region are white. (**f**, panel 1) Immuno-electron microscopy of direct binding of HMGB-1 to filaments using rabbit anti-HMGB-1 antibody and gold-conjugated goat anti-rabbit antibody. (**f**, panel 2) Negative controls labelled with non-immune IgG. Bars=100 nm. (**g**) Binding of 5 nM [^{125}I]HMGB-1 to immobilized α-synuclein filaments. Concentrations of monomeric α-synuclein (○) and filamentous α-synuclein (●). Ordinate, bound/free ligand; abscissa, free ligand measured in monomeric building blocks. Value represents mean ± s.d. of three replicates in 1 of 3 independent experiments. (**h**) Binding of recombinant HMGB-1 peptides, B and AB to α-synuclein filaments (F) investigated by co-sedimentation analysis.

filaments and cytosol prior to the sucrose density centrifugation caused approximately 50% of the HMGB-1 immunoreactivity to appear in the pellet, whereas no immunoreactivity was detected in the pellet from the cytosol alone (Fig. 1d, upper panel). Accordingly, rat brain HMGB-1 is, indeed, a α-synuclein filament-binding protein. The structural requirements of the filaments necessary for the binding were investigated using filaments formed from α-synuclein-(1–95) peptides, which were truncated for the C-terminal 45 amino acid residues, and filaments formed from Aβ peptides, which were used as a model filament (Fig. 1d, lower panel). Neither of these filaments bound the HMGB-1 as shown by immunoblotting (Fig. 1d, upper panel). This demonstrates that the interaction does not depend on a general amyloid-type filament characteristic, but requires the C-terminal segments of α-synuclein.

Immunoelectron microscopy was used to determine whether the co-sedimentation of rat brain HMGB-1 and recombinant human α-synuclein filaments resulted from a direct interaction between the two protein structures or from HMGB-1 being part of a filament binding complex. The rat HMGB-1 used for the experiment was essentially pure, as seen by the single ~23 kDa band in the silver-stained gel (Fig. 1b, lane 1). Co-incubation of purified rat HMGB-1 and purified α-synuclein filaments followed by immunolocalisation of the HMGB-1 using a rabbit anti-HMGB-1 IgG as primary antibody and a gold-conjugated second antibody entailed strong gold labelling of the filaments (Fig. 1f, panel 1). No labelling was detected when non-immune IgG was used as primary antibody (Fig. 1f, panel 2). Accordingly, rat HMGB-1 binds directly to α-synuclein filaments. The interaction between filaments and HMGB-1 was analysed quantitatively by a solid phase binding assay using α-synuclein filaments immobilised in microtiter plates and ^{125}I-labeled rat HMGB-1 proteins. The ^{125}I-HMGB-1 tracer co-migrated with the unlabelled HMGB-1 protein as a single radiolabelled band (Fig. 1b, lane 2). The binding of the ^{125}I-HMGB-1 tracer could be inhibited by both soluble monomeric and filamentous α-synuclein (Fig. 1g). The binding of HMGB-1 to filaments displayed an IC_{50} of ~1 μM α-synuclein building blocks, whereas the IC_{50} was ~10-fold higher when monomeric α-synuclein was used. This reveals a selectivity of HMGB-1 for the binding to α-synuclein in its filamentous form. This selectivity is even higher as the competitive concentration on the abscissa represents the monomeric contents of the aggregates. The molar concentration of the filaments is likely to be 100- to 1000-fold lower.

HMGB-1 is a basic protein containing three domains: two basic HMG boxes (HMG domains A and B) and a highly acidic C-terminal domain (Fig. 1e). The HMG boxes mediate the binding of HMGB-1 to double-stranded DNA [5]. Recombinant human HMGB-1 was shown to bind to α-synuclein filaments through its DNA binding domain as both the recombinant B and AB peptides co-sediment with α-synuclein filaments through a sucrose cushion (Fig. 1h).

One of the functions of HMGB-1 is to bind double-stranded DNA and it thereby facilitates the subsequent DNA binding of certain transcription factors. We investigated the effect of filamentous α-synuclein on the binding of HMGB-1 to double-stranded DNA. We used a band shift assay with a small ^{32}P-labelled *cis*-platinated double-stranded DNA probe that was incubated with HMGB-1 in the presence or absence of filamentous α-synuclein. From this assay we conclude that binding of HMGB-1 to α-synuclein filaments *in vitro* inhibits its ability to interact normally with double-stranded DNA (data not shown).

HMGB-1 immunostaining was found in neuronal cell bodies and their dendrites (Fig. 2p,q). At the light microscope level, no obvious difference in either number or intensity of positive neurons was discernable between normal and Parkinson's disease cases (data not shown). Parkinson's disease and dementia with Lewy bodies are characterised by Lewy bodies that contain abundant α-synuclein filaments. Immunohistochemical staining of paraffin-embedded or free-floating brain sections has been widely used in the localisation of proteins in Lewy bodies. This method, though perceived as a gold-standard, is less reliable in detecting epitopes that are sensitive to modifications by fixative and delipidating chemicals (e.g. xylene, chloroform) [12]. The method is also in no way able to distinguish those proteins that tightly bind Lewy body filaments from those merely diffusing into Lewy bodies due to their high cytoplasmic concentrations surrounding Lewy bodies. For these reasons, we used individually isolated Lewy bodies from unfixed postmortem human brains

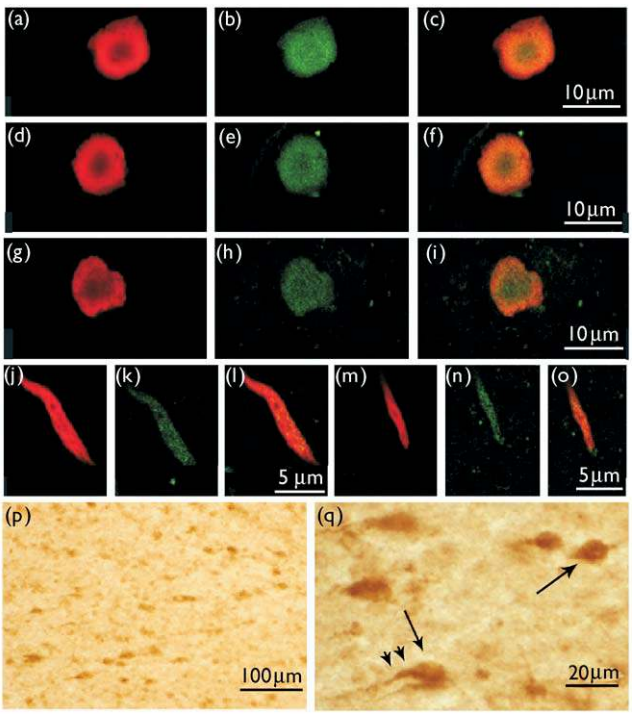

Fig. 2. Localization of HMGB-1 in Lewy bodies and Lewy neurites isolated from cortical tissue of dementia with Lewy bodies or from substantia nigra of Parkinson's disease. Lewy bodies and Lewy neurites were as determined by confocal laser scanning microscopy. (**a–c**) Lewy body from substantia nigra of Parkinson's disease. (**d–f**) and (**g–i**) Cortical Lewy body in two different cases of dementia with Lewy bodies. (**j–l**) A Lewy neurite from substantia nigra of Parkinson's disease. (**m,o**) A Lewy neurite from cortex of dementia with Lewy bodies. Panels a,d,g,j,m show α-synuclein immunoreactivity (red), panels b,e,h,k,n show HMGB-1 immunoreactivity (green) and merged images are shown in panels c,f,i,l,o. For panels a–i, the bars in the right panels=10 μm (applies to all nine panels). For panels j–o, bar in panels o and l=5 μm (applies to all six panels). (**p,q**) Immunohistochemical localization of HMGB-1 in neocortical neurons from a normal case. Arrows in q indicate positive neuronal cell bodies, and arrow heads, positive neuronal processes presumably dendrites.

affected by Lewy body pathologies, to analyse their chemical constituents [12]. Our confocal laser scanning microscopy analysis demonstrated unequivocally the presence of HMGB-1 immunoreactivity in typical Lewy bodies (Fig. 2a–c) and Lewy neurites (Fig. 2j–l) isolated from the substantia nigra of Parkinson's disease, and cortical Lewy bodies (Fig. 2d–f,g–i) or Lewy neurites (Fig. 2m–o) isolated from cingulated cortex of dementia with Lewy bodies.

DISCUSSION

α-Synuclein aggregation and filament formation are general characteristics of Parkinson's disease, dementia with Lewy bodies and the Lewy body variant of Alzheimer's disease. The formation of α-synuclein aggregates and filaments thus correlate closely with the neurodegenerative process and the α-synuclein aggregates and filaments presumably have toxic properties, as corroborated by the gain of toxic function in the familial Parkinson's disease cases with α-synuclein mutations.

We identified rat brain HMGB-1 as an α-synuclein filament-binding protein and demonstrated their direct interaction by immunoelectron microscopy. The interaction is supported by aggregate-dependent determinants in the C-terminus of α-synuclein and does not rely on amyloid-type characteristics. The filament-binding site in HMGB-1 resides in the DNA-binding HMGB-1 box motifs, and α-synuclein filaments were able to inhibit the normal binding of a radiolabelled double-stranded DNA probe to HMGB-1 *in vitro* (data not shown).

Aggregated α-synuclein thus hold the potential to disturb nuclear transcription factors as α-synuclein has been detected in oligodendroglial nuclear inclusions in patients with multiple system atrophy [13]. These nuclear inclusions of α-synuclein, along with the intracellular inclusions of α-synuclein, resemble mutant forms of the huntingtin protein associated with Huntington's disease. Such mutations result in huntingtin-immunopositive aggregates in both the cytoplasm and the nucleus [14]. Transcriptional dysregulation has been hypothesised to be an important pathogenic mechanism in this disease [15] and the binding of HMGB-1 to α-synuclein could resemble a similar mechanism. However, we detected no α-synuclein in the nucleus of cortical brain tissue affected by dementia with Lewy bodies or in substantia nigra brain tissue affected with Parkinson's disease. Instead, we found the presence of HMGB-1 in Lewy bodies and Lewy neurites isolated from human brain tissue affected by both dementia with Lewy bodies and Parkinson's disease. This indicates that aggregated α-synuclein can sequester HMGB-1 in the cytosol; an effect that is likely to occur after its cytosolic translation. Other reports have described mislocation of nuclear proteins into cytosolic α-synuclein inclusions, e.g. the transcriptional factors Elk-1 [2] and NF-kappa(κ)B [3], indicating an altered transcriptional regulation.

Estrogen has been demonstrated to play a protective role in several neurodegenerative models, however, the mechanisms of action have not been fully elucidated [16]. HMGB-1 stimulates the binding of the activated estrogen receptor to the DNA, the subsequent estrogen-receptor-mediated transcription [17,18] HMGB-1 gene knockout mice display impaired steroid response to glucocorticoids [19]. Accordingly, α-synuclein inactivation of HMGB-1 may contribute to the impairment of the neuronal response to estrogen in the group of neurodegenerative diseases characterised by α-synuclein aggregation.

CONCLUSIONS

The data demonstrate that HMGB-1 specifically binds to aggregated α-synuclein. This is further corroborated by the presence of HMGB-1 in Lewy bodies and Lewy neurites isolated from brains of dementia with Lewy bodies and Parkinson's disease. This indicates α-synuclein aggregates may sequester the transcriptional cofactor HMGB-1, thereby contributing to the neurodegenerative process.

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Acknowledgements: We wish to thank Professor Arvid Maunsbach and Karen Thomsen, Department of Cell Biology, University of Aarhus, Denmark for valuable guidance on the electron microscopy. Grants 22030314, 22020140 were from the Medical Research Foundation, The Lundbeck Foundation, The Danish Parkinson Foundation and Aarhus University Research Foundation.