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ORIGINAL ARTICLE

Identification of coronin-1a as a novel antibody target for clinically isolated syndrome and multiple sclerosis

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Abstract

Recently, we identified the mimotope UH-CIS6 as a novel candidate antibody target for clinically isolated syndrome (CIS) and relapsing-remitting (RR) multiple sclerosis (MS). The purpose of this study was to further validate UH-CIS6 as an antibody target for CIS and MS and to identify the *in vivo* antibody target of UH-CIS6. First, a UH-CIS6 peptide ELISA was optimized. Next, we investigated the antibody response toward UH-CIS6 in cerebrospinal fluid (CSF) from patients with CIS ($n = 20$), MS ($n = 43$) and other neurological diseases ($n = 42$). Immunoprecipitation of anti-UH-CIS6 antibodies on a normal human brain lysate was performed to identify the *in vivo* antibody target of UH-CIS6. The cellular expression of an *in vivo* candidate target was investigated by immunohistochemistry using MS brain tissue sections. Antibody reactivity toward UH-CIS6 was detected in a significantly increased proportion of CSF samples from CIS and RR-MS patients as compared with neurological controls ($p = 0.046$). We identified and confirmed coronin-1a as the *in vivo* antibody target for UH-CIS6. Furthermore, coronin-1a was expressed by T cells

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Abbreviations used: CIS, clinically isolated syndrome; EAE, experimental autoimmune encephalomyelitis; HRP, horse-radish peroxidase; IP, immunoprecipitation; MHC II, major histocompatibility complex class II; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NIND, non-inflammatory neurological disorders; OIND, other inflammatory neurological diseases; PBST, 0.05% Tween 20 in phosphate-buffered saline; PLP, proteolipid protein; RR, relapsing-remitting; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

and macrophages in an active MS lesion. Together, these results demonstrate that coronin-1a is a novel antibody target for CIS and MS.

Keywords: antibody target, clinically isolated syndrome, coronin-1a, multiple sclerosis.
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Multiple sclerosis (MS) is a chronic inflammatory neurological disorder of the central nervous system (CNS), characterized by inflammation-driven demyelination and neuro-axonal degeneration. In Europe, MS has a prevalence of approximately 0.1% (Pugliatti *et al.* 2006). The majority of MS patients display a relapsing-remitting (RR) disease course, in which disease exacerbations are followed by periods of remission. In 85% of the MS patients, the disease first manifests with a 'clinically isolated syndrome' (CIS), a single episode of neurological disturbance which should last at least for 24 h (Miller *et al.* 2012). Clinical symptoms and signs in CIS can be caused by a lesion in the optic nerve, spinal cord, brainstem, cerebellum or cerebral hemisphere (Miller *et al.* 2012). Thirty to 70% of CIS patients develop MS by a second clinical relapse or by new lesions on a follow-up MRI (Miller *et al.* 2005). An ongoing need exists for markers which allow early diagnosis.

The presence of antibodies in cerebrospinal fluid (CSF) and in peripheral blood is one of the inflammatory hallmarks of CIS and MS. However, targets of this antibody response are largely unknown. An example of an antibody target shared by CIS and MS is myelin oligodendrocyte glycoprotein (MOG) (Mayer and Meinl 2012). However, contradictory results on the anti-MOG antibody response have been obtained, which is reflected by the wide range of antibody positive CIS and MS patients that have been reported (Mayer and Meinl 2012). These discrepancies may be caused by the different types of assays, such as western blot analysis, ELISA and cell based assays that have been used. In addition, antibody reactivity toward linear and conformational MOG epitopes has been investigated (Mayer and Meinl 2012). Recently, the potassium channel KIR4.1 has been identified as a novel antibody target for CIS and MS (Srivastava *et al.* 2012). Serum antibodies to KIR4.1 were detected in 46.9% of CIS and MS patients, while these antibodies were present in serum from 0.9% of neurological controls and absent in healthy subjects. However, additional studies are needed to confirm these promising findings.

In a recent study, we identified UH-CIS6 as a novel candidate antibody target for CIS and RR-MS by using a phage display approach (Rouwette *et al.* 2012). Phage ELISA assays confirmed antibody reactivity toward UH-CIS6 in CSF from the CIS discovery cohort as well as in CSF samples from additional CIS and RR-MS patients. Furthermore, a significantly higher percentage of CIS and RR-MS patients displayed serum antibody reactivity toward UH-CIS6 as compared to neurological and healthy controls (21% vs. 7%). A disadvantage of phage display, however, is the identification of mimotopes. These peptides or proteins do not necessarily have the same or even a similar amino acid sequence as the natural epitopes, but mimic their binding properties (Cortese *et al.* 2001). UH-CIS6 is an example of a mimotope and therefore the *in vivo* antibody target is unknown.

As UH-CIS6 represents a promising candidate autoantibody target for CIS and MS, the goal of this study was to further characterize UH-CIS6. First, a UH-CIS6 peptide ELISA was developed to study the antibody response toward UH-CIS6 in CSF from CIS and MS patients. Secondly, as UH-CIS6 encodes a mimotope, we aimed to identify the *in vivo* antibody target and further characterize the expression of a candidate target in MS brain tissue.

Materials and methods

Patient material

Immunoreactivity toward UH-CIS6 was assessed in CSF samples from 20 CIS, 31 RR-MS, seven primary-progressive (PP) MS and five secondary-progressive (SP) MS patients. In the control group, 26 and 16 CSF samples were included from patients with non-inflammatory neurological disorders (NIND) and other inflammatory neurological diseases (OIND), respectively (Table 1). MS diagnosis and MS development in CIS patients was established according to McDonald criteria (McDonald *et al.* 2001; Polman *et al.* 2005). In general, CIS patients had a clinical follow-up for 2 years after sampling. This study was approved by the ethics committee of Hasselt University and fulfilled guidelines from the declaration of Helsinki. Samples were obtained after informed consent.

UH-CIS6 peptide ELISA assays

Two N-terminal biotinylated synthetic UH-CIS6 peptides (GL Biochem, Shanghai, China) with or without six additional amino acids of the phage vector (MPVVPATWEAETGESLEPGRRRLQ and QEFGTSMPVVPATWEAETGESLEPGRRRLQ) were used. An irrelevant peptide (WTKTPDGNFQLGGTEP) was purchased to correct for background reactivity.

To determine human IgG antibody reactivity toward the biotinylated UH-CIS6 peptide, ELISA plates coated with streptavidin (Nunc, Roskilde, Denmark) were used. First, ELISA plates were washed three times with 0.05% Tween 20 in phosphate-buffered saline (PBST). Subsequently, plates were coated with 0.5 μg/mL biotinylated UH-CIS6 peptide in PBST for 1 h at 22°C, while

Table 1 Characteristics of patients and controls used for anti-UH-CIS6 antibody reactivity analysis in CSF

Patient group	Gender (M/F)[a]	Mean age (years) ± SD	Mean disease duration[b] (years) ± SD	EDDS score[c] ± SD
CIS (n = 20)	7/13	30.8 ± 9.4	0.4 ± 0.7	1.6 ± 0.7
CIS-CIS (n = 5)	2/3	32.0 ± 11.4	0.1 ± 0.2	1.7 ± 0.6
CIS-MS (n = 15)	5/10	30.4 ± 9.0	0.5 ± 0.8	1.6 ± 0.7
RR-MS (n = 31)	9/22	38.7 ± 9.4	6.3 ± 7.1	2.8 ± 1.2
PP-MS (n = 7)	2/5	45.1 ± 13.8	8.2 ± 4.7	4.5 ± 1.4
SP-MS (n = 5)	2/3	50 ± 9.6	15.8 ± 12.3	4.2 ± 1.3
OIND (n = 16)	5/11	51.6 ± 14.7	NA[d]	NA
NIND (n = 26)	8/18	48.6 ± 15.1	NA	NA

[a]M, male; F, female.
[b]Data not available for three CIS (CIS-MS) patients.
[c]EDDS, expanded disability disease scale. Data available for 11 CIS (three CIS-CIS, eight CIS-MS), 29 RR-MS, seven PP-MS and three SP-MS patients.
[d]NA, Not available/not applicable.

shaking. Plates were blocked in 5% (w/v) skimmed milk powder in PBS (MPBS) for 2 h at 37°C, which was followed by washing of the plates. Subsequently, 100 μL 1 : 100 diluted serum or 1 : 1.33 diluted CSF was added to each well and incubated for 2 h at 22°C, while shaking. After washing steps were repeated, 100 μL of a rabbit anti-human IgG horse-radish peroxidase (HRP) labeled antibody (diluted 1 : 2000, Dako, Heverlee, Belgium) was added to detect the amount of bound human IgG (1 h, shaking at 22°C). Color development was started after the addition of 100 μL 3,3′,5,5′ tetramethyl-benzidine dihydrochloride solution (Perbioscience, Erembodegem, Belgium) and stopped with 50 μL 2N H_2SO_4. Plates were read at 450 nm in a Tecan plate reader (Tecan, Männedorf, Switzerland).

Competition assays

To confirm that UH-CIS6 phage particles and synthetic UH-CIS6 peptides contained identical epitopes, competition ELISA assays were performed as described previously (Rouwette *et al.* 2012). Briefly, 96-well ELISA plates (Greiner Bio-One, Wemmel, Belgium) were coated overnight at 4°C with 10 μg/mL anti-M13 filamentous phage antibody (GE healthcare, Diegem, Belgium). Plates were blocked for 2 h at 37°C with 2% MPBS. Next, 100 μL of polyethylene glycol purified UH-CIS6 phage particles (7×10^{11} colony forming units/mL) were added to each well and incubated for 1 h at 37°C, followed by 30 min at 22°C while shaking. Meanwhile, pre-incubation mixtures were prepared in a separate 96-well round bottom plate (Nunc). These mixtures consisted of 1 : 100 diluted UH-CIS6 antibody positive or negative serum sample [based on previously reported phage ELISA results (Rouwette *et al.* 2012)] and increasing concentrations of UH-CIS6 or irrelevant peptide (0–10 μg/mL). Serum antibodies and peptides were allowed to interact for 1.5 h (22°C, shaking). Subsequently, 100 μL of each pre-incubation mixture was transferred to the ELISA plate (1 h 37°C, 30 min 22°C shaking). The amount of bound IgG was then determined with 100 μL 1 : 2000 diluted goat-anti-human IgG HRP labeled antibody pre-adsorbed against mouse IgG (Life technologies, Merelbeke, Belgium). The remaining steps were performed as described for the UH-CIS6 peptide ELISA.

Purification of anti-UH-CIS6 antibodies and total IgG

To further study and characterize the target of UH-CIS6, a rabbit polyclonal anti-UH-CIS6 antibody was purchased (Eurogentec, Seraing, Belgium). A cysteine was added to the C-terminus of the UH-CIS6 peptide to direct the coupling of keyhole limpet hemocyanin (KLH) to the C-terminal end (MPVVPATWEAET-GESLEPGRRRLQC+KLH). Subsequently, this peptide was used for immunization according to the 28-day speedy immunization protocol of Eurogentec. Anti-UH-CIS6 antibodies were commercially purified from serum by affinity purification (Eurogentec). A Poros-A column (Life technologies) was used to purify total IgG from the pre-immune rabbit serum, which could be used as a negative control. To purify human anti-UH-CIS6 antibodies, UH-CIS6 peptide was coupled to a HiTrap NHS-Activated HP colum (GE healthcare), according to manufacturer's instructions. An Äkta Prime Plus device (GE healthcare) was used for purification. Rabbit and human serum was bound to the resin by addition of binding buffer (0.1 M glycine-sodium hydroxide, 3 M sodium chloride, pH 8.6 or 0.4 M sodium hydrogen carbonate, 1.0 M sodium chloride, pH 8.3), followed by elution of rabbit IgG or human anti-UH-CIS6 antibodies with elution buffer (0.1 M citric acid monohydrate, pH 3 or 100 mM glycine, pH 3). Positive elution fractions were pooled and rabbit IgG was concentrated using Pierce Protein Concentrators (Thermo Scientific, Erembodegem, Belgium). Subsequently, IgG concentration was determined with the BCA protein assay kit (Thermo Scientific) or with a Nanodrop 2000 (Thermo Scientific).

Preparation of human brain lysate

A human brain lysate was prepared by homogenization of normal human brain tissue with a rotor-stator in radio-immunoprecipitation assay buffer (150 mM sodium chloride, 1% NP-40 (Sigma-Aldrich, Bornem, Belgium), 0.5% sodium dioxycholate, 0.1% sodium dodecyl sulphate (SDS) 50 mM Tris, pH 8) supplemented with EDTA-free nuclease inhibitors (Roche Diagnostics, Vilvoorde, Belgium). Subsequently, the lysate was centrifuged and the protein concentration of the supernatant was determined with the BCA protein assay kit (Thermo Scientific), following manufacturer's instructions.

Immunoprecipitation

To identify the *in vivo* antibody target for UH-CIS6, an immunoprecipitation (IP) procedure was performed with a rabbit polyclonal anti-UH-CIS6 antibody on a normal human brain lysate. To this end, the Pierce Crosslink Immunoprecipitation Kit (Thermo Scientific) was used according to manufacturer's instructions with minor modifications. Briefly, 50 µg anti-UH-CIS6 antibody diluted in tris-buffered saline (TBS) (0.025 M Tris, 0.15 M sodium chloride, pH 7.2) was coupled to 20 µL Protein A/G Plus Agarose overnight at 4°C, followed by crosslinking of bound antibody. To remove aspecific binding, human brain lysates were pre-cleared prior to the IP procedure. For this purpose, 2 mg lysate was added to 160 µL control agarose resin (overnight at 4°C). Next, pre-cleared lysate was added to the UH-CIS6 antibody coupled column and incubated overnight at 4°C. The antibody coupled column was washed, followed by four sequential elution steps. Elution fractions were neutralized with 1 M Tris (pH 9.5).

Mass spectrometry

IP elution fractions were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and proteins were stained with the Silverquest Silver Staining Kit, according to manufacturer's recommendation (Life technologies). Protein bands were excised, separately trypsinized and resulting peptides were analyzed by nanoliquid chromatography mass spectrometry as previously described (Vanheel *et al.* 2012). Thus, obtained peptide fragmentation spectra were searched in Proteome Discoverer v1.2 using Sequest v1.2.0.208 and Mascot v2.4 against the International Protein Index Human database (v.3.87; 91464 entries). The output of both search engines was validated with Scaffold v3.6.1.

Western blot

Ten µg of a human coronin-1a over-expressing human embryonic kidney lysate (Novus Biologicals, Cambridge, UK) or 1 µg purified human coronin-1a protein expressed in human embryonic kidney cells (Origene, Rockville, MD, USA) was separated by SDS-PAGE. Next, proteins were blotted onto a polyvinylidene difluoride membrane (Millipore, Brussels, Belgium), followed by a blocking step for 2 h in 5% MPBS supplemented with 0.1% Tween. The rabbit anti-UH-CIS6 antibody (1 : 100), pre-immune rabbit IgG (1 : 100), and a commercial anti-coronin-1a antibody (1 : 250; Sigma-Aldrich) were diluted in blocking buffer and incubated overnight at 4°C. After the polyvinylidene difluoride membrane was washed several times with PBST, a swine anti-rabbit IgG HRP labeled antibody (diluted 1 : 1000; Dako) was added and incubated for 2 h at 22°C. Washing steps were repeated and bound antibody was visualized using the Pierce ECL Plus Western Blotting Substrate kit (Thermo Scientific).

Coronin-1a ELISA

Ninety-six well ELISA plates (Greiner Bio-One) were coated overnight at 4°C with 2 µg purified coronin-1a protein (Origene) in coating buffer (0.1 M sodium hydrogen carbonate pH 9.6). Plates were blocked with 2% MPBS for 2 h at 37°C. Next, plates were washed three times with 0.1% PBST and once with PBS. Subsequently, the commercial anti-coronin-1a antibody (Sigma-Aldrich) was diluted 1 : 690, the rabbit polyclonal anti-UH-CIS6 antibody 1 : 20 (stock 1 mg/mL), pre-immune rabbit IgG 1 : 100 (stock 5 mg/mL), and purified human anti-UH-CIS6 antibodies 1 : 5 (stock 0.2 mg/mL) and 100 µL diluted antibody was added to each well and incubated for 2 h at 22°C while shaking. Plates were washed and 100 µL swine anti-rabbit IgG HRP labeled antibody (diluted 1 : 1000; Dako) or rabbit anti-human IgG HRP labeled antibody (diluted 1 : 2000; Dako) was added (1 h, 22°C, shaking). The remaining steps were performed as described for the UH-CIS6 peptide ELISA.

Immunohistochemistry

Frozen tissue sections from an active MS lesion from one human MS brain and normal white matter from one non-demented neurological control were used to investigate coronin-1a expression. Sections were fixed with aceton and blocked with serum-free protein block (Dako). Next, sections were incubated with primary antibodies (coronin-1a: 1 : 1000; Sigma-Aldrich), proteolipid protein (PLP): 1 : 500 (Serotec, Düsseldorf, Germany), major histocompatibility complex class II (MHC II: HLA-DP, DQ, DR) 1 : 100 (Dako) for 1.5 h at 22°C. To detect binding, the Envision Dual Link System-HRP (Dako) was used, followed by staining with (3,3′-diaminobenzidine) DAB chromogen solution (Dako). Hematoxylin was used as counter-staining. In addition, a double staining was performed for coronin-1a and MHC II. Antibodies were incubated simultaneously overnight at 4°C. As secondary antibodies, a goat anti-rabbit Alexa 488 (1 : 250; Life Technologies) and a goat anti-mouse Alexa 555 (1 : 250; Life Technologies) fluorescent labeled antibody were used. A nuclear staining was performed with 4′, 6-diamidino-2-phenylindole (DAPI). Stained tissue sections were evaluated with a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and NIS-Elements Basic Research Software (Nikon).

Statistics

Graph Pad Prism 5 software (GraphPad software, La Jolla, CA, USA) was used for statistical analysis. ELISA data were analyzed with the Kruskall–Wallis test, followed by Dunn's multiple comparison test. The cut-off for a positive UH-CIS6 sample was set at three times the SD above the mean OD signal obtained for NIND and OIND samples. Putative associations between antibody reactivity and disease were analyzed using the Fisher's exact test. In addition, associations between antibody reactivity and clinical parameters were examined. An unpaired two-tailed Student's *t*-test or Mann–Whitney test was used for demographical variables, while the Fisher's exact test was used for categorical variables. A *p*-value smaller than 0.05 was considered significant.

Results

Development of a peptide ELISA for UH-CIS6

To further validate the potential of UH-CIS6 as an antibody target in CIS and MS, we aimed to screen both CIS and MS patients for anti-UH-CIS6 antibody reactivity in a peptide ELISA format. First, the peptide ELISA was optimized. Two biotinylated UH-CIS6 peptides, with and without linker were tested. This linker consisted of six additional amino acids of the phage vector and may further improve the accessibility of the epitope.

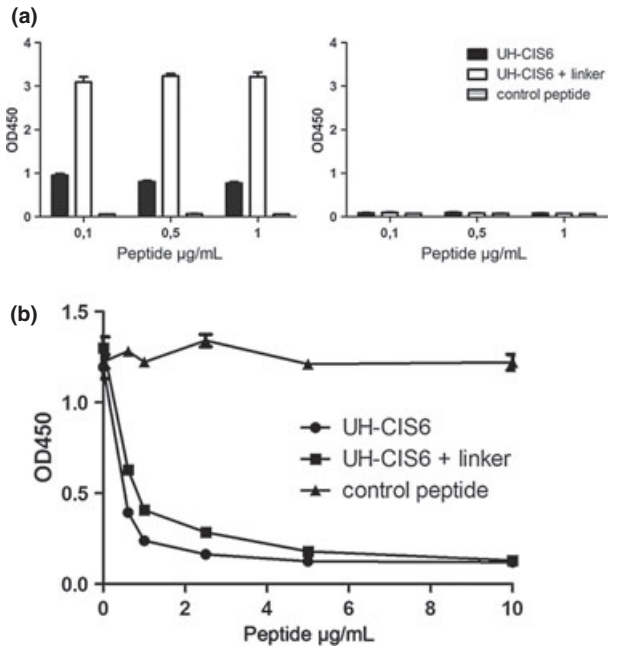

Fig. 1 Development of the UH-CIS6 peptide ELISA. An antibody positive and negative serum sample were selected based on UH-CIS6 phage ELISA results. (a) Antibody reactivity toward the UH-CIS6 peptides could be demonstrated for the antibody positive sample, while no anti-UH-CIS6 antibody reactivity was observed for the antibody negative sample. Addition of the linker markedly increased the observed OD signal for the antibody positive sample. (b) Both UH-CIS6 peptides were able to compete with UH-CIS6 phage particles and contained the epitope of the UH-CIS6 phage.

As shown in Fig. 1a, antibody reactivity toward UH-CIS6 could be detected with both UH-CIS6 peptides. A clear increase in the OD signal could be observed for the UH-CIS6 peptide with linker as compared with the UH-CIS6 peptide without linker. Moreover, OD signals increased two to six fold for the antibody positive sample with biotinylated UH-CIS6 peptides as compared with unbiotinylated UH-CIS6 peptides with and without linker (data not shown). Competition assays revealed that both UH-CIS6 peptides were able to compete with UH-CIS6 phage particles, which confirmed that the UH-CIS6 peptides and phage particles contain identical epitopes (Fig. 1b). Based on these results the biotinylated UH-CIS6 peptide with linker was used in further experiments.

To validate the UH-CIS6 peptide ELISA, 52 CSF samples (17 CIS and 17 MS patients, 18 neurological controls) were tested with both peptide and phage ELISA assays (data not shown). Two CIS patients and two neurological controls tested positive using the UH-CIS6 phage ELISA. These samples tested also positive when using the UH-CIS6 peptide ELISA. Interestingly, six additional UH-CIS6 antibody positive samples (three CIS and three MS patients) were identified using the UH-CIS6 peptide ELISA, demonstrating an increased sensitivity of the UH-CIS6 peptide ELISA.

CSF antibody reactivity toward UH-CIS6 in CIS and MS patients

Using the UH-CIS6 peptide ELISA, antibody reactivity toward UH-CIS6 was evaluated in an initial screening on CSF samples from 20 CIS, 31 RR-MS, 7 PP-MS, and 5 SP-MS patients. In addition, anti-UH-CIS6 antibody reactivity was analyzed in CSF samples from 26 NIND and 16 OIND patients. This cohort also contained CSF samples that were used for the validation of the UH-CIS6 peptide ELISA. Five CIS patients (25%), seven RR-MS patients (23%) and one SP-MS patient (20%) tested positive for anti-UH-CIS6 antibodies. On the other hand, three neurological controls (7%), diagnosed with epilepsy (n = 2) and papilla edema (n = 1), also displayed antibody reactivity toward UH-CIS6 (Fig. 2a). As shown in Fig. 2b, the number of antibody positive CIS and RR-MS patients was significantly increased as compared with controls (24% vs. 7%, Fisher's exact test: p = 0.046). No correlation between antibody reactivity toward UH-CIS6 and clinical parameters, including CSF IgG concentration, was detected (data not shown). Furthermore, CSF IgG concentrations of patients and controls were in a similar range (1–10 mg/dL). In conclusion, antibody reactivity toward UH-CIS6 could be primarily detected in CSF from CIS and RR-MS patients. Thus, these findings confirm that UH-CIS6 is a candidate antibody target for CIS and RR-MS.

Clinical follow up revealed that 15 of 20 CIS patients developed MS. Interestingly, all CIS patients that tested positive for antibody reactivity toward UH-CIS6 progressed to MS, which may indicate that UH-CIS6 could serve as a prognostic marker. However, the number of CIS patients should be expanded in the future to further investigate whether antibody reactivity toward UH-CIS6 has prognostic potential to predict conversion to MS.

Identification of coronin-1a as the antibody target for UH-CIS6

To identify the *in vivo* antibody target corresponding to UH-CIS6, a polyclonal rabbit anti-UH-CIS6 antibody was generated. First, we tested whether this polyclonal rabbit anti-UH-CIS6 antibody bound the same epitope as human anti-UH-CIS6 antibodies. Competition assays revealed that the anti-UH-CIS6 polyclonal rabbit antibody and human anti-UH-CIS6 antibodies indeed bound the same epitope (Fig. 3). Therefore, the polyclonal rabbit anti-UH-CIS6 antibody was suitable to characterize and identify the *in vivo* antibody target of UH-CIS6.

Next, to identify the *in vivo* antibody target for UH-CIS6, a normal brain lysate was immunoprecipitated with the rabbit anti-UH-CIS6 antibody. SDS-PAGE followed by mass spectrometric analysis of obtained precipitates identified the peptide 'AAPEASGTPSSDAVSR' with a peptide probability of 95% corresponding to amino acids 417–432 of coronin-1a (Fig. 4).

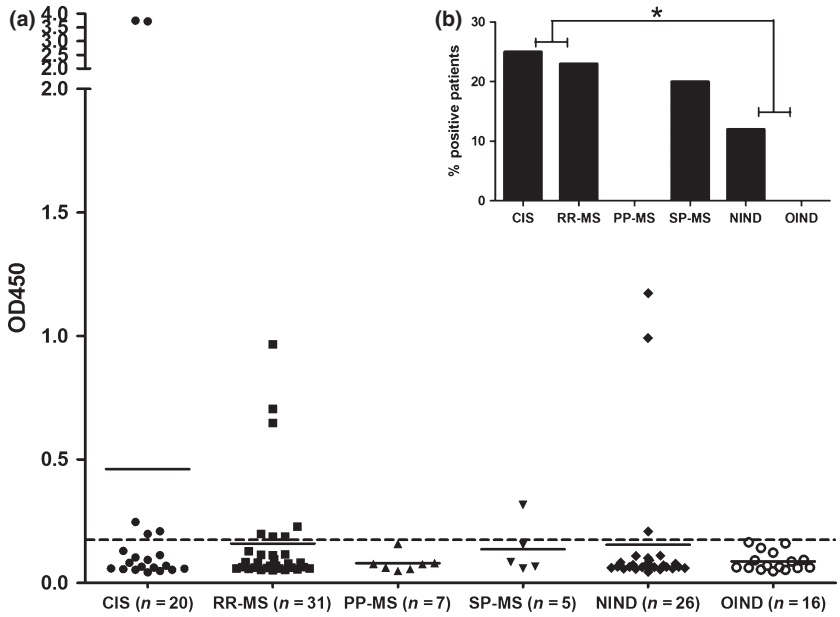

Fig. 2 Increased CSF antibody reactivity toward UH-CIS6 was primarily present in clinically isolated syndrome (CIS) and relapsing-remitting multiple sclerosis (RR-MS). (a) The UH-CIS6 peptide ELISA was used to detect anti-UH-CIS6 antibody reactivity in CSF from patients with CIS, MS and other (inflammatory) neurological diseases (NIND and OIND). Measurements were performed in duplicate and mean values are shown. Lines indicate mean antibody levels. The cut-off for a positive sample (dash line) was set at three times the SD above the mean OD signal obtained for NIND and OIND samples. (b) CSF samples from a significant larger proportion of CIS and RR-MS patients tested positive for UH-CIS6 as compared with neurological controls. * indicates a p-value < 0.05.

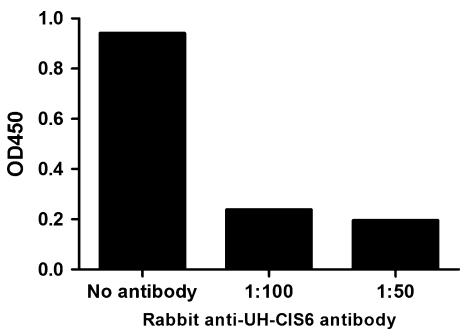

Fig. 3 Rabbit and human anti-UH-CIS6 antibodies contained an identical epitope. A human serum sample positive for UH-CIS6 was pre-incubated with increasing amounts of the rabbit anti-UH-CIS6 antibody. The rabbit anti-UH-CIS6 antibody competed with the human anti-UH-CIS6 antibodies as increasing amounts of the rabbit anti-UH-CIS6 antibody led to a strong decrease in bound human anti-UH-CIS6 antibodies. Measurements were performed in duplicate and mean values are shown.

Confirmation of coronin-1a as an antibody target in CIS and MS

To confirm coronin-1a as the *in vivo* antibody target for UH-CIS6, an *in silico* alignment of the amino acid sequence of UH-CIS6 and coronin-1a was performed (http://blast.ncbi.nlm.nih.gov/). This led to the identification of a shared protein motif, which could represent the UH-CIS6/coronin-1a epitope (Fig. 5a). Furthermore, binding of the rabbit anti-UH-CIS6 antibody to coronin-1a could be demonstrated by western blot analysis on purified coronin-1a protein and a coronin-1a over-expressing lysate (Fig. 5b and c). In addition, these findings could be confirmed by ELISA (Fig. 5d). To investigate whether human anti-UH-CIS6 antibodies also displayed reactivity toward coronin-1a, anti-UH-CIS6 antibodies were purified from a UH-CIS6 positive human serum sample and tested by ELISA. As shown in Fig. 5d, the human anti-UH-CIS6 antibodies were also positive for coronin-1a. In conclusion, UH-CIS6 was found to correspond to coronin-1a, which represents a novel antibody target for CIS and MS. In the future, the antibody response toward coronin-1a should be analyzed into more detail by analysis of anti-coronin-1a antibody reactivity in serum and CSF samples from patients with CIS and MS.

Expression of coronin-1a in MS brain

Immunohistochemistry was performed to investigate the expression pattern of coronin-1a in human brain samples. An active MS lesion, characterized by reduced PLP and increased MHC II expression (Fig. 6a and b), contained many coronin-1a immunopositive cells (Fig. 6c). Coronin-1a expression was markedly increased inside the demyelinated MS lesion as compared with surrounding normal appearing white matter (NAWM). In NAWM, microglia were decorated with anti-coronin-1a expression (Fig. 6d), which was similar to normal white matter from a non-demented neurological control (data not shown). Within the active MS lesion, coronin-1a expression was restricted to macrophages and T cells (Fig. 6e). Moreover, double positive coronin-1a and MHC II cells were identified (Fig. 6f). These results demonstrate that coronin-1a immunoreactivity is enhanced and predominantly expressed by infiltrated immune cells in active MS lesions.

Discussion

In a previous study, we identified UH-CIS6 as a novel candidate antibody target for CIS and MS. Therefore, the

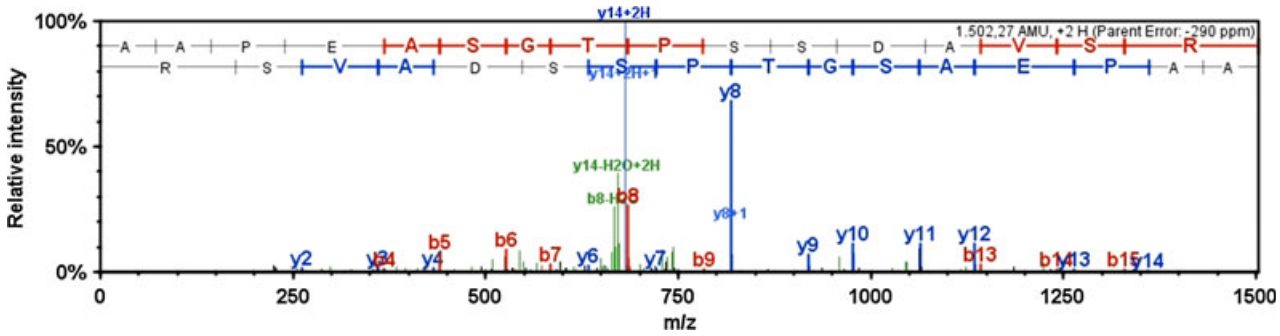

Fig. 4 Peptide mass spectrum for coronin-1a. Immunoprecipitation output was analyzed by mass spectrometry. N-terminal b and C-terminal y fragment ions are indicated as well as corresponding amino acids. This revealed the identification of the peptide 'AAPEASGTPSSDAVSR' identical to amino acid 417–432 of coronin-1a.

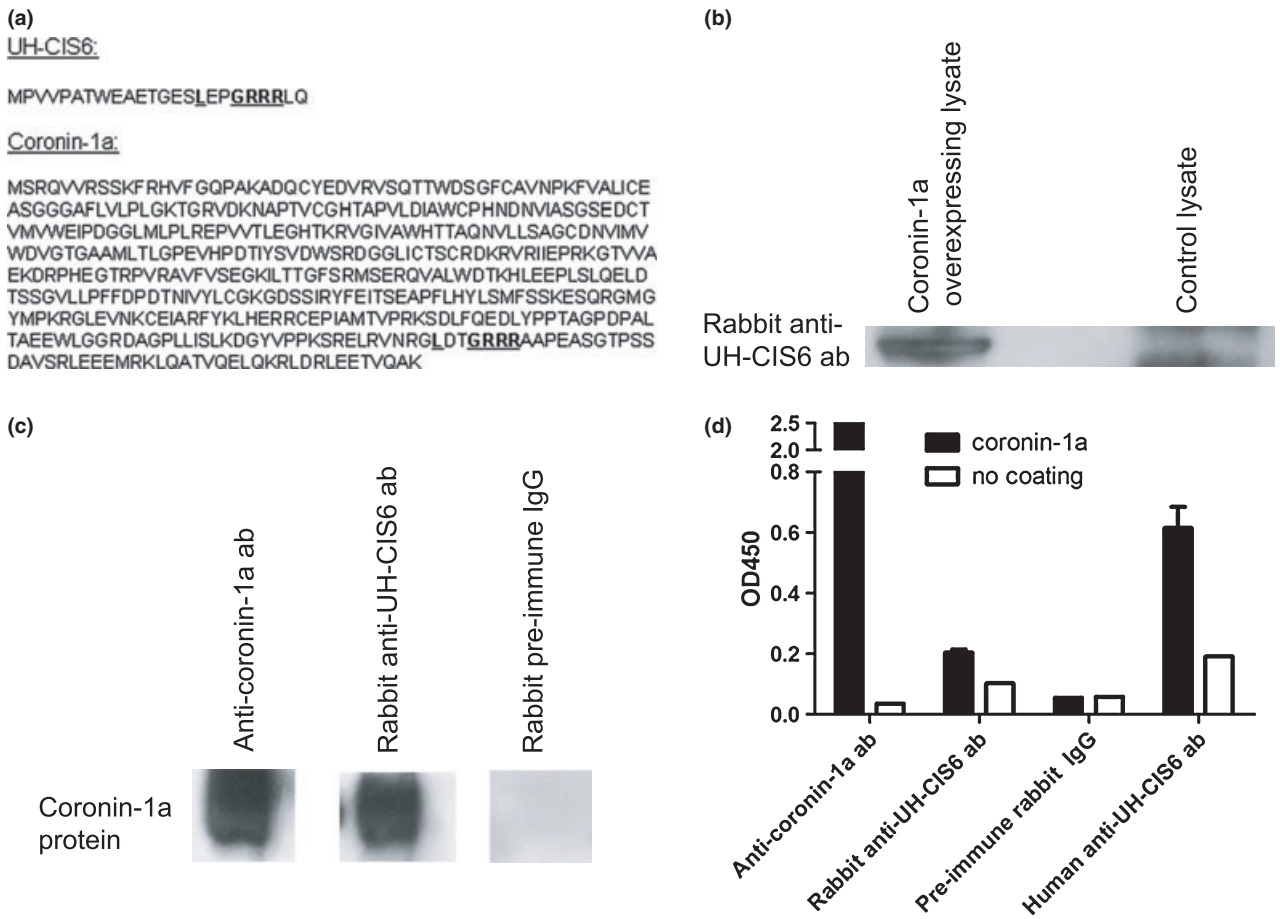

Fig. 5 Confirmation of anti-UH-CIS6 antibody binding toward coronin-1a. (a) Alignment of UH-CIS6 and coronin-1a protein sequences (Swiss prot accession number P31146) led to the identification of a shared protein motif (indicated in bold). (b) Western blot analysis with the rabbit anti-UH-CIS6 antibody on a human coronin-1a over-expressing lysate revealed the presence of a band with a molecular weight similar to coronin-1a. This band was absent in western blot analysis with the anti-UH-CIS6 antibody on a control lysate without coronin-1a over-expression. (c) Binding of the rabbit anti-UH-CIS6 antibody to coronin-1a could be confirmed by western blot analysis on purified human coronin-1a protein. A commercial anti-coronin-1a antibody was used as a positive control. Pre-immune IgG from the rabbit used for UH-CIS6 immunization was used as a negative control (d). Binding to coronin-1a and anti-UH-CIS6 antibodies could also be demonstrated by ELISA. Moreover, purified human anti-UH-CIS6 antibodies were also positive for coronin-1a.

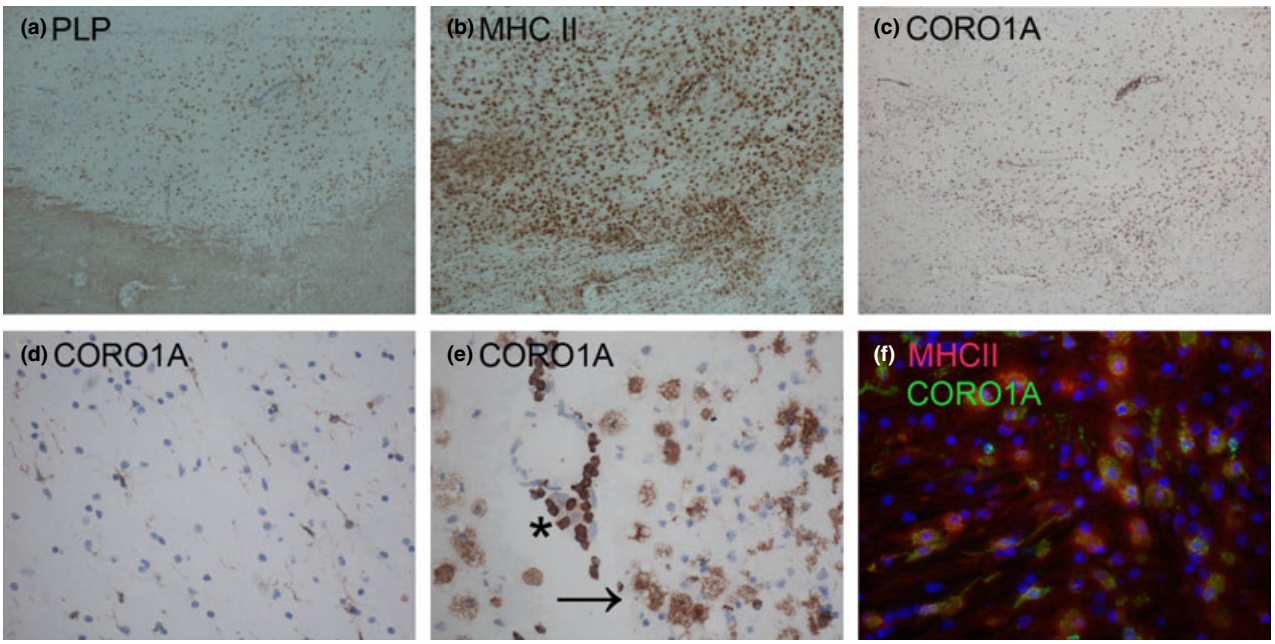

Fig. 6 Expression of coronin-1a in multiple sclerosis (MS) brain. An active MS lesion is shown, reflected by reduced proteolipid protein (PLP) expression (a) and a strong increase in MHC II immunoreactivity (b). Coronin-1a expression was increased inside the active lesion as compared to the surrounding NAWM (c). When the primary antibody was omitted no staining was visible (data not shown). Resting microglia in NAWM were positive for coronin-1a (d), whereas inside the lesion coronin-1a expression was found in macrophages (arrow) and T cells (asterisk) (e). Furthermore, double positive coronin-1a (green) and MHC II (red) cells were identified (f). Representative images are shown. Images are 40X (a–c) and 400X (d–f) magnified.

goal of this study was to further characterize UH-CIS6. First, we developed a sensitive peptide ELISA to detect antibody reactivity toward UH-CIS6. Using this ELISA, anti-UH-CIS6 antibody reactivity could be detected in CSF samples from a significantly increased proportion of CIS and RR-MS patients as compared with neurological controls. As UH-CIS6 encodes a mimotope, we also aimed to identify the *in vivo* antibody target for UH-CIS6. To this end, immunoprecipitation of a rabbit anti-UH-CIS6 antibody on a normal human brain lysate was performed, which led to the identification of coronin-1a as the *in vivo* antibody target for UH-CIS6. Binding of the rabbit anti-UH-CIS6 antibody to coronin-1a could be confirmed by western blot analysis and ELISA. Moreover, purified human anti-UH-CIS6 antibodies also bound coronin-1a. In addition, coronin-1a positive T cells and macrophages were identified inside an active MS lesion which demonstrates the presence of coronin-1a expression in MS brain. Therefore, coronin-1a represents a novel antibody target for CIS and MS.

In this study, a sensitive UH-CIS6 peptide ELISA was developed. The improved sensitivity of the peptide ELISA as compared with the phage ELISA was demonstrated by the identification of a higher number of UH-CIS6 antibody positive samples (10 vs. 4). This may also clarify why the percentage of UH-CIS6 antibody positive CSF samples from CIS and RR-MS patients was increased as compared to findings reported in our previous study (25% vs. 7% and 23% vs. 2%) (Rouwette *et al.* 2012).

Antibody reactivity toward UH-CIS6 was detected in a significantly larger proportion of CSF samples from CIS and RR-MS patients as compared with neurological controls. Interestingly, all CIS patients that tested positive for antibody reactivity toward UH-CIS6 progressed to MS. These findings further support the possible prognostic potential of UH-CIS6 to predict conversion to MS as was demonstrated in our previous study (Rouwette *et al.* 2012). However, the number of CIS patients should be expanded in the future, to further investigate the prognostic potential of anti-UH-CIS6 antibody reactivity. In this way, it would be relevant to investigate whether antibody reactivity toward UH-CIS6 associates with disease activity and if a higher anti-UH-CIS6 antibody response correlates with a more rapid conversion to MS.

Coronin-1a was identified as the *in vivo* antibody target for UH-CIS6. Coronin-1a, primarily expressed in hematopoietic cells, has been proposed to be an actin regulatory protein, which functions as a bridging protein between plasma membrane domains and the dynamic actin cytoskeleton of leukocytes and may allow for the remodeling of the cytoskeleton in response to outside signals transmitted into leukocytes (Gatfield *et al.* 2005). However, coronin-1a may also have actin-independent functions (Mueller *et al.* 2008). In T cells, a role for coronin-1a has been recognized in

survival through promotion of Ca^{2+} mobilization form intracellular stores (Mueller *et al.* 2008) and in T cell receptor induced immunological synapse formation and signaling (Mugnier *et al.* 2008). In addition, coronin-1a is expressed in macrophages, where it has been associated with survival of mycobacteria within phagosomes (Ferrari *et al.* 1999), Ca^{2+} dependent signaling processes (Jayachandran *et al.* 2007) and with lipoprotein uptake and degradation (Holtta-Vuori *et al.* 2012). Finally, coronin-1a has been implicated in phagocytosis in neutrophils (Grogan *et al.* 1997). Mutations in coronin-1a have been described in an individual with severe combined immunodeficiency (Shiow *et al.* 2008, 2009) and in three siblings with immunodeficiency and EBV-associated B-cell lymphoproliferation (Moshous *et al.* 2013), which suggest a key role for coronin-1a in the immune system. Furthermore, coronin-1a deficient mice did not develop experimental autoimmune encephalomyelitis (EAE), the animal model of MS after immunization with MOG_{35-55} (Siegmund *et al.* 2011), although after re-immunization coronin-1a deficient mice exhibited enhanced EAE signs that correlated with increased number of IL-17 producing CD4+ cells in the CNS (Kaminski *et al.* 2011).

Until now, coronin-1a has not been associated with antibody reactivity in humans. On the other hand, antibody reactivity toward coronin-1a has been observed after T-cell vaccination in mice (Xu *et al.* 2011). To our knowledge, antibody reactivity toward proteins which are primarily expressed in immune cells, such as coronin-1a, has not been described yet in CIS and MS. In other diseases, however, antibody reactivity toward such antibody targets has been reported. For example anti-neutrophil cytoplasmic antibodies (ANCAs) directed toward antigens expressed in neutrophils and monocytes, have been described in diseases such as vasculitis, rheumatoid arthritis, and inflammatory bowel disease (Roozendaal and Kallenberg 1999; Kurita *et al.* 2010; Kallenberg 2011). Once ANCAs have bound their target antigen, intracellular molecules are recruited to activate the neutrophil, which will lead for example to degranulation, super-oxide generation, and cytokine production (Kettritz 2012). Apart from these pathogenic effects, antibodies may have protective effects as well. For instance, IgM antibodies toward neurons have been shown to promote axonal outgrowth and improve functional recovery in a mouse model of MS (Xu *et al.* 2013). Whether anti-coronin-1a antibodies have pathogenic effects and mediate activation of immune cells or have protective properties by blocking the inflammatory response, needs to be examined in future studies. Alternatively, the presence of anti-coronin-1a antibodies may represent an epiphenomenon caused by the infiltration of coronin-1a expressing immune cells in MS brain. To this end, anti-coronin-1a antibodies may be injected into animals with EAE. It may be also interesting to investigate how antibodies can be generated toward an intracellular protein such as coronin-1a. Several hypotheses on the generation of an antibody response toward intracellular proteins have been proposed (Racanelli *et al.* 2011). Such antibodies may be generated by dysregulation of the immune system, which causes expansion of polyreactive natural antibodies. Apoptosis may result in exposure of intracellular antigens on the cell surface or release into the extracellular environment. Furthermore, alterations in epigenetic modifications, such as DNA methylation, histon acetylation, and microRNA expression, as well as molecular mimicry to extracellular or foreign antibody targets may cause an antibody response toward intracellular targets. Moreover, antibody reactivity toward other intracellular targets has already been described in MS (Silber *et al.* 2002; Kolln *et al.* 2006; Lovato *et al.* 2008; Svarcova *et al.* 2008).

In summary, antibody reactivity toward UH-CIS6 was detected in CSF from a significantly increased proportion of CIS and RR-MS patients as compared with neurological controls. Coronin-1a was identified as the *in vivo* antibody target for UH-CIS6, which represents a novel antibody target for CIS and MS. Future research is needed to fully explore the role of an anti-coronin-1a antibody response in the pathogenesis of CIS and MS. In addition, studies should be performed to investigate the prognostic potential of anti-coronin-1a antibody reactivity to predict conversion to MS in CIS patients.

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