Alpha actinin is specifically recognized by multiple sclerosis autoantibodies
isolated using an N-glucosylated peptide epitope

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RUNNING TITLE

Alpha actinin is recognized by Multiple Sclerosis Autoantibodies

ABBREVIATIONS USED

Ab: antibody
Ag: antigen
AutoAbs: autoantibodies
CNPase: 2’,3’-cyclic-nucleotide 3’-phosphodiesterase
CNS: central nervous system
MuS: multiple sclerosis
MS/MS: tandem mass spectrometry
RR-MuS: relapsing remitting MuS.
SUMMARY
Sophisticated approaches have recently led to the identification of novel autoantigens associated with Multiple Sclerosis (MuS), e.g. neurofascin, contactin, CNPase and other membrane anchored proteins. These putative antigens, although differing from the conventional myelin derivatives, are conceptually based on an animal model of experimental autoimmune encephalomyelitis. In this report we describe the identification of putative antigens based on their recognition by autoantibodies isolated from MuS patient serum. In a previous work from this laboratory we have shown that a peptide probe, named CSF114(Glc), specifically identifies serum autoantibodies in a subset of MuS patients, representing approximately 30% of the patient population. The autoantibodies, purified from MuS patients’ sera (six), through CSF114(Glc) affinity chromatography, detected three immunoreactive protein bands present in the rat brain. Proteomic analysis of the immunoreactive bands, involving MALDI and MS/MS techniques, revealed the presence of four proteins distinguishable by their mass: alpha fodrin, alpha actinin 1, creatine kinase, and CNPase.

The immunoreactive profile of these rat brain proteins was compared with that of commercially available standard proteins by challenging against either CSF114(Glc) purified MuS autoantibodies, or monoclonal antibodies. Further discrimination among the rat brain proteins was provided by the following procedure: whereas monoclonal antibodies recognized all rat brain proteins, isolated MuS specific antibodies recognize only Alpha actinin 1 as a putative antigen. In fact, Alpha actinin 1 displayed a robust immunoreactive response against all MuS patients’ sera examined, whereas the other three bands were not consistently detectable. Thus, alpha actinin 1, a cytoskeleton protein implicated in inflammatory/degenerative autoimmune diseases (lupus nephritis and autoimmune hepatitis) might be regarded as a novel MuS
autoantigen, perhaps a prototypic biomarker for the inflammatory/degenerative process typical of the disease.

INTRODUCTION

The development of serum antibody (Ab) biomarkers employed for the diagnosis, monitoring, and prognosis of multiple sclerosis (MuS) has been a challenge because of the ambiguous identification of antigens (Ags) implicated in the disease.

Most of the putative Ags belong to the myelin family (myelin basic protein, proteolipid lipoprotein, and myelin oligodendrocyte glycoprotein). However, the disappointing results obtained in extensive studies attempting to develop immunological assays employing these Ags, have led to the conclusion that these assays have only limited clinical value due to low sensitivity that compromises their ability to discriminate between various inflammatory central nervous system (CNS) diseases (1). In fact, the recently revised criteria for the diagnosis of MuS (issued by the International Panel on Diagnosis of MuS) are essentially based on neurologist’s clinical observation, supported by magnetic resonance imaging (MRI), and do not include any confirmation derived from immunological assay (2).

Recently, a number of non-myelin Ags have been reported as putative biomarkers of MuS. These findings highlighted the complexity of the disease and were instrumental in the advancement of novel interpretations of this pathology. For example, by applying a proteomic approach, Mathey and colleagues have identified neurofascin and contactin-2 as candidate Ags for MuS, demonstrating that Abs against each of these proteins cause axonal injury and demyelinating lesions in the cortex (3, 4). Since high neurofascin expression occurs predominantly in the brain
of MuS patients characterized by chronic progressive disease, it appears that these Ags might be implicated mainly in the degenerative phase of the pathology.

Similarly, an exhaustive search for Ags recognized by IgG autoAbs derived either from sera or cerebrospinal fluid of MuS patients, revealed the presence of both oligodendroglia (transketolase, CNPase) and cytoskeletal proteins (radixin, actin interactin protein 1) in human brain (5).

A novel approach, which has shown promising results in terms of predictive value for the MuS pathology, is based on the documented post-translational modifications of native Ags present in the CNS. Pathophysiologial post-translational glycosylation of native Ags generates neoAgs that trigger the immune system to generate autoAbs, which escape conventional diagnostic procedures (6).

Based on these premises, others and we have developed a unique synthetic glycopeptide, CSF114(Glc) (7, 8) or a glycan complex Glc(alpha1,4)Glc(alpha) (9, 10) as neoAg surrogates that serve as probes for the detection of disease-related autoAbs present in the serum of MuS patients. These probes are capable of identifying and measuring MuS-related autoAbs whose levels are correlated with clinical assessment of MuS activity and MRI profile of brain lesions. Furthermore, CSF114(Glc) affinity-purified autoAbs from MuS serum specifically stained myelin and oligodendrocytes Ags in human brain histological specimens (8).

The CSF114(Glc) peptide represents an unconventional approach since its structure is completely unrelated to myelin oligodendrocyte glycoprotein or any other myelin derivative and is not linked to any particular pathogenetic hypothesis. The main characteristic of CSF114(Glc) is its conformational propensity to form a β-turn that exposes the sugar moiety, perhaps the key element, for recognition by the MuS-relevant autoAb (11, 12).
Given this background we wondered whether CSF114(Glc) affinity-purified Abs from MuS patients’ sera, could be useful in back-tracking CNS Ags implicated in the MuS-related autoimmune response. This report describes the identification of putative Ags present in rat brain, which recognize serum MuS autoAbs purified through CSF114(Glc) affinity columns, hereafter termed “CSF114(Glc) Abs”. MALDI mass spectrometry analysis of the isolated Ags led to the identification of alpha actinin, a cytoskeletal protein abundant in CNS, as a principal MuS-related Ag.

EXPERIMENTAL PROCEDURES

CSF114(Glc)-Sepharose Coupling. CSF114(Glc)-Sepharose column was prepared by using Cyanogen-Bromide Activated resin (CNBr-Sepharose® 4B, Sigma). CNBr-Sepharose beads (60 mg, coupling capacity 30-40 mg of ligand per ml) were washed thoroughly with 1 mM HCl, equilibrated with coupling buffer (0.1 M NaHCO₃ containing 0.5 M NaCl, pH 8.3) and coupled to 1 mg of glycopeptide CSF114(Glc). Unreacted ligand was washed and unreacted sites were blocked with 0.2 M glycine (pH 8). Column was washed thoroughly and equilibrated with PBS (8).

Affinity Purification. Multiple Sclerosis patients’ serum from six individual patients (MuS1 – MuS6) was diluted in PBS (1:10) and filtered through 0.22 μm filter (MILLEX®, GS). The study was approved by the institutional ethic committee. All patient included corresponded to relapsing-remitting MuS and had no comorbidities. Filtered serum was loaded onto the column and incubated for one h at room temperature. Column was washed with PBS buffer to remove non-specific Abs, after washing specific Abs were eluted with 0.1 M glycine buffer, pH 2.6. Eluted fractions were neutralized with 0.1 M NaHCO₃ pH 8.3 and concentrated on Amicon
Ultra-0.5 ml, 50K (Millipore). Immunoreactivity of anti-CSF114(Glc) IgGs was examined by ELISA on CSF114(Glc) coated plates (7).

**Extraction of Brain Protein from Rat.** 120 mg of frozen rat brain was excised and minced into small pieces. Sample was dissolved in 1 ml PBS and sonicated (Brandelien SONOPLUS Probe KE-76, Sigma) for 2 min in ice. Homogenate was centrifuged at 10,000 g for 15 min and debris were removed. Supernatant was re-centrifuged at 31,870 x g for 90 min at 4°C. Pellet was solubilized in 0.01% Triton X-100 buffer containing a cocktail of protease inhibitors (Complete™ Mini tablets, Roche Applied Science) and NaN₃ (0.05%) at 37°C for one h. Total protein concentration was 17.9 mg/ml, measured by spectrophotometry (NanoDrop 1000, Thermo Scientifics). 10 µl of sample were loaded and examined on SDS-PAGE 12%.

**SDS-PAGE and Western Blot Analysis.** Protein samples were subjected to SDS-PAGE 12% under reducing condition (2% v/v β-mercaptoethanol). The following standard proteins were obtained from commercial sources: recombinant human alpha fodrin (kindly provided by Aesku Diagnostics GmbH, Germany), alpha actinin isoform 1 (brain), extracted from chicken gizzard (Sigma, Italy), recombinant human CNPase expressed in *E. coli* (Abcam, USA), recombinant human creatine kinase expressed in *Pichia pastoris* (Sigma, Italy). Gels were either stained with Coomassie blue or used to transfer onto nitro cellulose membrane (0.45 µm; BIO-RAD) at 220 mA for two h at room temperature. Membrane was blocked with milk powder 2% in TBST (0.1%) and incubated with affinity purified anti-CSF114(Glc)-IgGs (MuS patients and normal blood donors) for 1 h at room temperature. Membrane was washed thoroughly with TBST (0.1%) and incubated with alkaline phosphatase conjugated anti-IgGs (1:500; Sigma) for 1 h at
room temperature. Membrane was washed as above with TSBT (0.1%) and blots were developed using AP conjugated substrate kit (BIO-RAD).

**Excision and digestion.** The samples were obtained by excision and digestion of the bands from SDS-PAGE 12% of rat brain homogenate, after comparison with the Western blot results to identify the bands of interest. The bands were excised and minced into small pieces, washed with NH$_4$HCO$_3$ 50 mM and shrunk with acetonitrile. Reduction and alkylation were performed with βME 20 mM (56°C, 30 min) followed by 2-iodoacetamide 55 mM (20°C, 20 min, in the dark). Proteins were digested in a buffer containing 25 mM NH$_4$HCO$_3$, 5 mM CaCl$_2$ and ~20 ng/µl of trypsin (37°C, overnight). Peptides were extracted from gel in two steps by adding 25 mM NH$_4$HCO$_3$ and 5% formic acid; each step was followed by addition of acetonitrile to shrink the gel and maximize the peptide recovery.

**MALDI Analysis.** MALDI-MS measurements were performed using an Ultraflex III TOF-TOF instrument (Bruker Daltonics, Bremen, Germany), equipped with smartbeam I laser (λ = 355 nm) and operating in reflectron positive ion mode. The instrumental conditions were: UIS1 = 25 kV; UIS2 = 21.65 kV; reflectron potential: 26.3 kV; delay time = 0 nsec. The samples were dried, re-suspended in acetonitrile and desalted by Zip-Tip C18 pipette tips (Millipore). Zip-tipped samples were spotted on the stainless steel sample plate with α-cyano-4-hydroxycinnamic acid matrix (5 mg in H$_2$O/Acetonitrile/0.1% TFA). 1µL of the peptide eluted solution was deposited on the stainless steel sample holder, and allowed to dry before introduction into the mass spectrometer. External mass calibration was done using the Peptide Calibration Standard, basing on the monoisotopic values of [M+H]$^+$ of Angiotensin II, Angiotensin I, Substance P,
Bombesin, ACTH clip (1-17), ACTH clip (18-39), Somatostatin 28 at m/z 1046.542, 1296.685, 1347.736, 1619.823, 2093.087, 2465.199 and 3147.471, respectively. MALDI-TOF-TOF experiments were carried out using the LIFT device. The instrumental parameters were: UIS1=8 kV; UIS2=7.2 kV; ULIFT1=19 kV.

The resulting data were processed using the FlexAnalysis 2.4 software (Bruker Daltonics) and optimized for databank search. Searches were performed using the Mascot Server 2.3 search engine against the Swiss-Prot database (release 2012_8, subset Rattus, 7800 entries) considering up to one missed tryptic cleavage, monoisotopic peptide mass tolerance of 500 ppm, and fragment ion mass tolerance of 0.3 Da. Carbamidomethyl modifications of cysteine and oxidation of methionine were considered as appropriate.

RESULTS

Isolation and Purification of AutoAbs from MuS Patients’ Sera. As the primary aim of this study was to investigate whether Abs detected by CSF114(Glc) glycopeptide might reveal specific Ags present in brain tissue, we first isolated autoAbs responding to CSF114(Glc) in sera from six individual MuS patients and for comparison from healthy blood donors. Six MuS patient sera were taken from those (n = 250) previously analyzed for autoAbs by ELISA with the glycopeptide (7). All patients were in relapsing remitting MuS disease stage. Isolation and purification of Abs was performed through the well established affinity chromatography used in our laboratory (8). The purified Ab titer of each serum, evaluated by ELISA, indicated a wide range of Ab levels (Fig. 1), from 4- to 20-fold that present in pooled control serum, taken from blood donors of matching age.
Detection of Immunoreactive Proteins in Rat Brain Homogenate. To investigate the presence of Ags responding to the above described purified CSF114(Glc) Abs, we used soluble proteins isolated from rat brain homogenate following centrifugation and solubilization with Triton X-100. Soluble proteins (approx. 180 µg) were separated, through SDS-PAGE 12%, yielding a protein pattern with a relatively wide range of Mr (Fig. 2, lane B).

Western blot analysis of the separated proteins through affinity purified Abs against CSF114(Glc) from each MuS patient, revealed the presence of three major bands characterized by different Mr, i.e. 47, 98 and 130 kDa, the lowest Mr band containing 2 distinct proteins (Fig. 2, lane C). The 98 kDa band was recognized by Abs purified from all patients examined (6/6), while recognition of the other bands occurred only in 3 patients sera.

Identification of Ags through Proteomic Approach. To identify the nature of proteins present in rat brain homogenate detectable by CSF114(Glc) Abs, a thorough proteomic approach was applied to the immunoreactive bands above described. After excision and trypsinization of individual bands we performed MALDI analysis obtaining Peptide Mass Fingerprint data for each protein (Fig. 3). The search for these fingerprint signatures in the Mascot database, enabled to identify the rat brain proteins sensitive to recognition by human Abs. The identification was confirmed by MS/MS analyses of selected peptides for each protein (Table 1). Among the four protein identified, two, namely, alpha fodrin (130 kDa) and alpha actinin (98 kDa), belonging to the spectrin family, are associated with the cytoskeleton, while the lower Mr proteins, i.e. CNPase (47 kDa) and creatine kinase (47 kDa) are enzymes involved in cellular metabolic processes.

INSERT FIGURE 1 AND 2 HERE

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Recognition of Rat Brain Ags and the Corresponding Standard Proteins by either mAbs or CSF114(Glc) Abs. To rule out the possibility that the mass spectrometry analysis might identify proteins of similar Mr co-localizing in the same band (false positive), we compared side by side the four identified bands, (i.e. alpha fodrin, alpha actinin 1, CNPase and creatine kinase) and their standard counterparts against both IgGs mAbs and CSF114(Glc) Abs. A sequence homology analysis indicated that each standard protein exhibits a significant level of sequence overlapping with the corresponding rat sequence (86.6% – 95.4%), and in the case of alpha actinin, with the corresponding human form (96.9%, see supporting information). Note that protein co-localization was unavoidable given the use of monodimensional electrophoresis as a protein separation technique. The use of more powerful separation techniques, such as 2D electrophoresis, was hampered by the limited quantity of CSF114(Glc) Abs available, due to the low yield of the purification procedure from MuS sera.

Differences emerged when the rat brain Ags and the standard proteins were challenged either by mAbs or CSF114(Glc) Abs. In fact, the mAbs recognized all extracted proteins in similar fashion (Fig. 4, panel A). Similarly, mAbs recognize all standard proteins except alpha fodrin, possibly due to a faulty protein (Fig. 4, panel B). In contrast, when the standard proteins were challenged by CSF114(Glc) Abs, only alpha actinin provided a positive band, while all other proteins failed to respond to the Ab challenge (Fig. 4, panel C). The positive response obtained with alpha actinin is consistent with the results showing the presence of specific autoAbs in all patient sera.

DISCUSSION

This report describes the identification of a rat brain protein, alpha actinin, recognized by its immunoreactivity with autoAbs isolated from MuS patients’ sera. Our experimental approach for protein identification, primarily based on the hypothesis that serum autoAbs ought to recognize
the presence of corresponding Ag(s), was underpinned by the use of an autoAbs population proven to be relevant for the MuS pathology (8). Experiments were designed to include four sequential stages: purification of autoAbs from MuS sera, detection of Ags in the rat brain displaying immunoreactivity toward the MuS autoAbs, MALDI identification of putative Ags, and the crossover assessment of immunoreactivity of putative Ags and their standard protein counterparts against either specific mAbs or the CSF114(Glc) Abs. A key element of this study is represented by the use of autoAbs purified and enriched through affinity chromatography to CSF114(Glc). Indeed, a body of experimental evidence shows that CSF114(Glc) is capable of detecting serum IgM autoAbs in a subset of MuS patients (approx 30%), and that its immunoreactive responses correlate with the relapsing remitting phase of the disease (7). As a source of putative Ags we chose the brain of adult rats, a species in which the above mentioned proteins are conserved as compared to human, and which for its availability, avoids problems of protein degradation. In addition, brain homogenization might disclose proteins (putative autoantigens), which are hidden by the integrity of brain tissue.

The CSF114(Glc) Abs detected three distinct protein bands in the rat brain, which upon MALDI and MS/MS analyses led to the identification of four proteins, i.e. alpha fodrin, alpha actinin 1, CNPase and creatine kinase. Among these proteins, autoAbs to CNPase, a molecule associated with oligodendrocytes, has been reported to be present with variable frequency (40 to 70%) in MuS sera using a proteomic approach (5, 13). However, with the exception of alpha actinin 1, CNPase, alpha fodrin and creatin kinase, in their authentic form, failed to be recognized by CSF114(Glc) Abs, as they consistently provided a negative immunoreactive response when challenged with the MuS patient derived Abs. This failure might be attributed to post-translational modifications of antigens, well documented particularly for CNPase (13).
The distinct advantage of this study lies in the use of purified autoAbs specific for the N-glucosylated peptide CSF114(Glc), which recognize a limited repertoire of relevant Ags. In contrast, the extensive use in other studies of unselected Ig fractions may account for the detection of antigens having remote relevance for the disease. In our view the observed cross-reactivity between the N-glucosylated peptide and alpha actinin 1 fits the well-known hypothesis that common pathogens are associated with the onset of MuS (14), suggesting that molecular mimicry may cooperate with the adaptive immune system leading to recognition of brain antigens.

The salient finding of this work was the identification of alpha actinin 1 as the only molecule, which provided immunoreactive responses to the CSF114(Glc) Abs for both the extracted rat brain protein and the reference standard form. Alpha actinin 1 is an ubiquitous cytoskeletal protein, which in its isoforms 1 and 4, both termed non-muscle, has been reported to be involved in autoimmune diseases such as systemic lupus erythematosus and autoimmune hepatitis (15). To our knowledge, this is the first time that alpha actinin 1 has been identified as one of the targets recognized by specific autoAbs associated with MuS. Alpha actinin 1 appears to be implicated in the organization of the cytoskeleton and through its proximity to adherens junctions, might be involved in T-cells migration.

Anti alpha actinin 1 autoAbs have been found in lupus nephritis and in autoimmune hepatitis (AIH), and their detection in the latter pathology is an important diagnostic tool. Because these diseases are characterized by inflammatory and degenerative components, prevailing features also of MuS, the identification of alpha actinin 1 as a major autoAg in MuS suggests that the underlying mechanisms producing these derangements are similar for a number of autoimmune pathologies.
The cytoskeletal protein alpha actinin 1, a component of adherence and tight junctions, modulates blood brain barrier functions. Its detection by autoAbs from MuS patients, besides signaling its involvement in the pathology, implies a derangement of the barrier shield, leading to exposure of the neurovascular niche to further degenerative injuries.

It is of interest that the use of CSF114(Glc) Abs approach, which is not linked to any particular pathogenetic mechanism, discloses Ags undetected by conventional approaches, e.g. alpha actinin 1. It appears that the “a priori” designation of a specific pathogenetic autoimmune model for MuS, poses limitations as it reveals Ags confined to that specific mechanism. Recent reports have described an approach for the identification of diagnostically useful Abs, which bears similarities with our approach that is not based on predefined knowledge of Ags (16, 17). Both studies successfully employ peptide libraries, either synthetic or phage displayed, for capturing autoAbs involved in immune mediated disorders of the CNS.

Although it is difficult to assess, at this point, what is the role of alpha actinin 1 in the pathogenesis of MuS, its detection in several autoimmune diseases, which underscores its involvement in inflammatory/degenerative pathologies, warrants further investigations.

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FIGURE LEGENDS

Figure 1. Affinity purified IgG Ab titers to CSF114(Glc). ELISA absorbance at 405 nm of autoAbs purified by affinity column functionalized with CSF114(Glc) glycopeptide [six MuS patients and pooled control serum, taken from blood donors (BD)] of matching age.

Figure 2. Rat brain proteins are selectively recognized by CSF114(Glc) specific IgGs from MuS patients. SDS-PAGE 12% of rat brain proteins and Western Blot using anti-CSF114(Glc) purified from individual MuS patients’ sera. The circled bands in lane C indicate as the prevailing band at 98 kDa, detected in all patients examined (6/6) whereas the others bands (47 and 130 kDa) were recognized only in three out of six patients’ sera. (A) Mr markers; (B) brain proteins SDS-PAGE 12%; (C) WB of brain proteins with anti-CSF114(Glc).

Figure 3. MALDI-TOF MS analysis of the four trypsin-digested bands excised by SDS-PAGE. For each spectrum signals corresponding to the peptides used for MS/MS analysis are highlighted. Spectra for the 130 kDa (A), 98 kDa (B), and the two 47 kDa bands analyzed (C and D).

Figure 4. Western blot of authentic proteins with CSF114(Glc) specific IgGs from MuS patients or commercially available mAbs. (A) WB of rat brain proteins detected with commercial monoclonal IgGs; lane 1: Mr Markers, lane 2: Rat brain proteins detected by: anti-alpha fodrin (1:400), anti-CNPase (1:500), anti-alpha actinin (1:100); lane 3: Rat brain proteins incubated with: anti-CKBB (1:400). (B) WB of authentic proteins incubated with commercial monoclonal IgGs; lane 1: alpha fodrin; lane 2: alpha actinin; lane 3: CKBB; lane 4: CNPase. (C) WB of authentic proteins detected by anti-CSF114(Glc) IgGs purified from individual MuS patients’ sera (six patients).
Table I. Identification of putative autoAgs following peptide mass fingerprint search on Mascot engine and MS/MS analysis of selected signals for each protein

| SDS band | Identified protein | Accession number | Mascot search Score | Matched / Searched | Sequence coverage | MSMS Sequence confirmation | Mass | Position |
|----------|-------------------|------------------|---------------------|--------------------|-------------------|---------------------------|------|----------|
| 130 kDa  | alpha fodrin      | P16086           | 94                  | 51 / 87            | 25.9%             | SADESGQALLAAGHYASDEVR     | 2147 | 1587     |
|          |                   |                  |                     |                    |                   | REELITNWEQIR              |      |          |
| 98 kDa   | alpha actinin     | Q6T487_rat       | 90                  | 29 / 101           | 38.5%             | LASDLLEWIR                | 1215 | 282-291  |
| 47 kDa   | creatine kinase   | P07335           | 133                 | 13 / 58            | 35.2%             | TFLVWINEEDHLR              | 1672 | 1965     |
|          |                   |                  |                     |                    |                   | GTGGVDTAAVGGVFDVSNADR      | 1672 | 224-236  |
| 47 kDa   | 2',3'-CNPase      | P13233           | 83                  | 16 / 96            | 35.2%             | ATGAEEYAQVDVVR             | 1536 | 261-274  |

Any score greater than 63 is considered significant. Identification was confirmed by manual sequencing of prominent peptides for each protein (reported as Supplemental Figures), of which amino acid sequence, mass of the precursor and position within the protein is reported.
FIGURES

Figure 1

Figure 2
