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Autoantibodies in Canine Masticatory Muscle Myositis Recognize a Novel Myosin Binding Protein-C Family Member

Xiaohua Wu,*† Zhi-fang Li,‡ Randolph Brooks,* Elizabeth A. Komives,§ Justin W. Torpey,§ Eva Engvall,‡ Steven L. Gonias,* and G. Diane Shelton1*

Inflammatory myopathies are a group of autoimmune diseases that affect muscles. In humans, the most common inflammatory myopathies are polymyositis, dermatomyositis, and inclusion body myositis. Autoantibodies may be found in humans with inflammatory myopathies, and these play an important role in diagnosis and disease classification. However, these Abs are typically not muscle-specific. Spontaneously occurring canine inflammatory myopathies may be good parallels to human myositis. In dogs with inflammatory myopathy, muscle-specific autoantibodies have been found, especially in masticatory muscle myositis. We have identified the major Ag recognized by the autoantibodies in canine masticatory muscle myositis. This Ag is a novel member of the myosin binding protein-C family, which we call masticatory myosin binding protein-C (mMyBP-C). mMyBP-C is localized not only within the masticatory muscle fibers, but also at or near their cell surface, perhaps making it accessible as an immunogen. The gene for mMyBP-C also exists in humans, and mMyBP-C could potentially play a role in certain human inflammatory myopathies. Understanding the role of mMyBP-C in this canine inflammatory myopathy may advance our knowledge of mechanisms of autoimmune inflammatory muscle diseases, not only in dogs, but also in humans. The Journal of Immunology, 2007, 179: 4939–4944.

The idiopathic inflammatory myopathies (IM)2 are characterized by acquired muscle weakness and inflammatory cell infiltrates within skeletal muscle and include a number of different autoimmune diseases. The most common IMs in humans are polymyositis (PM), dermatomyositis, and inclusion body myositis. Myositis-specific or myositis-associated autoantibodies are found in approximately half of the patients with idiopathic IM and are useful in predicting clinical presentation, response to therapy, clinical outcome, and in immunogenetics (1, 2). Although these autoantibodies are myositis-specific and characteristic of IM, they do not yet fully explain the disease manifestations, as the targeted Ags are not specific for muscle tissue (1–5). Instead, the myositis-specific Abs target more ubiquitously expressed components of the translational machinery, including tRNA synthetase and signal recognition particles, or components of the nucleosome remodeling complex, such as MI-2 (1–5). It is not known whether these autoantibodies play a role in the pathophysiology of IM or whether they are merely an epiphenomenon. Therefore, the triggers for the aberrant immune response and the main targets of autoimmunity in human myositis remain to be discovered (6).

Spontaneous IMs occur in dogs (7–16), and canine IMs may provide good parallels and insights into human IMs (17). In a clinicopathologic review of 200 cases of canine IM (8), a spectrum of clinical forms was identified, including polymyositis, dermatomyositis, and masticatory muscle myositis. To date, a canine homologue of inclusion body myositis has not been identified. The most common generalized IMs are idiopathic immune-mediated polymyositis and myositis caused by infectious agents such as the protozoan parasite Neospora caninum (8). A search for the myositis-associated autoantibodies found in human patients has not yet been performed in dogs with IM. Instead, a general screening of muscle biopsy specimens and serum for muscle-specific Abs resulted in the identification of an as-yet-uncharacterized sarcolemmal Ag(s) in canine polymyositis (9). In some breeds with a high incidence of PM, particularly in Boxers and Newfoundlands (8, 9), autoantibodies were shown to be directed against cell surface Ags, which are muscle specific, binding to skeletal and heart muscle, but not to liver, spleen, lung, or stomach (9). Furthermore, the Abs are directed against conserved muscle Ags, as in addition to binding to normal dog muscle, they also bound to muscle from normal cat and mouse, although with lower titers (9).

The most extensively studied inflammatory myopathy in dogs is canine masticatory muscle myositis (CMMM; Ref. 10–16), which affects only muscles of the masticatory muscle group. Autoantibodies specifically target type 2M fibers (11), and these Abs are currently used to diagnose the disease in dogs (18). In previous studies, it was shown that autoantibodies in CMMM recognize masticatory muscle-specific myosin heavy and light chains plus an unknown protein (11, 12). Such Abs are not present in dogs with PM, even though cellular infiltration and myofiber damage is clearly present in the masticatory muscles of such dogs (8, 11, 18). Thus, the autoantibodies in CMMM are disease specific and not just an epiphenomena secondary to myofiber damage.

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Abbreviations used in this paper: IM, inflammatory myopathy; PM, polymyositis; CMMM, canine masticatory muscle myositis; MyBP-C, myosin binding protein-C; mMyBP-C, masticatory muscle myosin binding protein-C; TTBS, Tween 20 Tris buffered saline; MS, mass spectrometry.

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In the present study, we have identified and characterized the unknown protein and show here that it is a novel myofibril-associated protein, which is a member of the myosin binding protein-C family (MyBP-C), and which is specifically expressed in masticatory muscle type 2M fibers.

**Materials and Methods**

**Canine sera and tissue specimens**

Canine sera and frozen tissue samples used in this study were from archived specimens of the Comparative Neuromuscular Laboratory, University of California, San Diego. All tissue and serum samples were stored at −70°C until used. Specimens were from clinical cases of naturally occurring CMMM that were submitted for diagnostic testing. A diagnosis of CMMM was based on appropriate clinical signs, cellular infiltrates within fresh frozen masticatory muscle biopsy specimens, and the documentation of autoantibodies against type 2M fibers.

**Affinity chromatography purification of IgG**

IgG was purified from the pooled serum of 10 dogs with confirmed CMMM, using protein A Sepharose affinity chromatography (Zymed Laboratories). Five ml of protein A Sepharose (Zymed Laboratories) was incubated with 10 ml of pooled serum on a rocking platform for 2 h at 22°C. The protein A Sepharose and adsorbed proteins were then loaded into a column and washed with 10 mM sodium phosphate and 150 mM NaCl (pH 7.4). IgG was eluted with 0.1 M glycine (pH 2.7) into collection tubes that contained 2 M Tris buffer (pH 8.0) so that the final pH was adjusted to pH 7.0. Protein concentrations were determined by bicinchoninic acid assay (Sigma-Aldrich).

**Immunoblot analysis**

Normal canine tissues, including masticatory (temporalis) and limb muscles, heart, kidney, liver, brain, stomach, and testes, were cut into small pieces and homogenized in 2% Triton X-100 in 0.1 M sodium phosphate (pH 7.4). Cell debris was removed by centrifugation at 15,000 × g for 5 min at 4°C. Extracts were separated by 4–15% gradient SDS-PAGE (Bio-Rad) and proteins were electrotransferred to polyvinylidene difluoride membranes. Proteins on the membranes were stained with Ponceau S. After destaining with 200 μM NaOH, membranes were blocked with Tween 20 Tris buffered saline (TTBS) containing 5% nonfat dry milk for 1 h at 22°C, incubated with 2 μg/ml affinity-purified CMMM-specific dog IgG, control serum, autoimmune serum, or monoclonal anti-β-tubulin Ab (1/100; Sigma-Aldrich) in TTBS, 5% milk for 16 h at 4°C, washed, and incubated with peroxidase-conjugated rabbit anti-dog IgG (1/25,000; Jackson ImmunoResearch Laboratories) or goat anti-mouse IgG (1/200; Sigma-Aldrich) in TTBS 5% milk for 1.5 h at 22°C. Peroxidase was detected by chemiluminescence (Bio-Rad).

**Immunofluorescence staining of tissue sections**

Fresh frozen temporalis muscle sections (8 μm) were dried at room temperature, blocked with 10% FBS in PBS for 1 h at 37°C, and washed for 10 min with PBS. Sections were incubated for 1 h at 37°C with mouse mAbs diluted in PBS against actin (1/200, Clone AC-40; Sigma-Aldrich) or the rod domain of dystrophin (1/50, Clone Dy4/6D3; Vector Laboratories), and 5 μg/ml affinity purified CMMM IgG. CMMM IgG was titrated and used at the highest dilution that would give positive staining. Following further washes, sections were incubated for 45 min at 37°C with affinity purified rabbit anti-dog IgG conjugated with Texas Red (1/200; Jackson ImmunoResearch Laboratories) and Alexa Fluor 488 goat anti-mouse IgG (1/100; Molecular Probes). The slides were again washed in PBS and images taken with an inverted fluorescence microscope (Olympus IX71).

**FIGURE 1.** Autoantibodies in CMMM bind to a 150 kDa (p150) protein present in normal dog temporalis muscle (Au) but not in limb muscle (Ab). Affinity purified IgG from dogs with CMMM and were used in immunoblotting. p150 is not detected by control dog sera (Ac). Sera from an additional 10 dogs (S1–10) were individually tested and compared with CMMM IgG. In all cases, Abs recognize the p150 protein (B).

**FIGURE 2.** Autoantibodies in CMMM are masticatory muscle specific. Affinity purified IgG from dogs with CMMM were used in immunoblotting to show that CMMM autoantibodies do not bind to proteins in detergent extracts of canine limb muscle, stomach (smooth muscle), heart muscle, or other tissues. An Ab against tubulin was used as a control for protein loading.
Immunoprecipitation

Purified IgG was incubated with protein A Sepharose, and unbound proteins were removed by centrifugation and washing. An extract of canine temporalis muscle was preincubated with protein A Sepharose and centrifuged. The precleared muscle extract was added to the IgG protein A Sepharose and incubated for 16 h at 4°C. The Sepharose was washed with PBS, and resuspended in Laemmli sample buffer containing 100 mM DTT. Samples were denatured 5 min at 95°C and separated by 8% resolving SDS-PAGE. Gels were stained with Spyro Ruby (Bio-Rad) protein stain and imaged under UV illumination.

Mass spectrometry (MS)

Protein containing gel pieces were transferred to a siliconized tube and washed and destained in 200 μl 50% methanol overnight. The gel pieces were dehydrated in acetonitrile, rehydrated in 30 μl of 10 mM DTT in 0.1 M ammonium bicarbonate and dried by vacuum centrifugation. The gel pieces were rehydrated with 20 μg/ml trypsin in 50 mM ammonium bicarbonate on ice for 10 min. Any excess trypsin solution was removed and 20 μl 50 mM ammonium bicarbonate added. The sample was digested overnight at 37°C, and the resulting peptides were extracted from the gel pieces in two 30 μl aliquots of 50% acetonitrile and 5% formic acid. The extracts were combined and evaporated to 25 μl for MS analysis.

The liquid chromatography-MS system consisted of a Finnigan LTQ (Fisher Scientific) ion trap mass spectrometer system with a Protana nanospray ion source interfaced with Phenomenex Jupiter 8 cm X 75 μm C18 reversed-phase capillary column. Samples (0.5–5 μl) of the digest were injected, and the peptides were eluted from the column with an acetonitrile and 0.1M acetic acid gradient at a flow rate of 0.25 μl/min. The data were analyzed by database searching using the Sequest search algorithm against the NR database (19, 20). MS was performed and the data analyzed at the Biomolecular Mass Spectrometry Facility, University of California at San Diego, and confirmed at the W.M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia.

Peptide Ab generation

The predicted canine masticatory MyBP-C (mMyBP-C) sequence, which was based on mass spectrometry and bioinformatics annotation, was compared with that of expressed sequence tags and genomic DNA from the canine and other species in the National Center for Biotechnology Information (NCBI) public database. Four peptide sequences were selected based on conservation between species, hydrophilicity, immunogenicity, and predicted secondary structure. Synthetic peptides and rabbit polyclonal Abs against them were generated by Pacific Immunology: CKELKRDEKYEITVS (rabbits 697,698), CARNEIGDSPLDSK (rabbits 699,700), CSVLVENELGKDRS (rabbits 701,702), and CDKDDKSILASVTES (rabbits 703,704).

Results

Abs in canine masticatory muscle myositis recognize a 150 kDa (p150) masticatory muscle protein

Purified IgG from pooled CMMM sera was used to identify autoantigens in canine masticatory muscle. Immunoblotting showed that the pooled IgG bound to a single 150 kDa (p150) protein (Fig. 1Aa). This p150 was not detected in limb muscle extracts using the same purified IgG (Fig. 1Ab). Furthermore, pooled serum isolated from control dogs failed to detect the p150 band (Fig. 1Ac). Serum samples from an additional 10 dogs with CMMM were individually used to identify targets in canine masticatory muscle. All ten identified protein with an apparent molecular mass of 150 kDa (Fig. 1B). Although other proteins with an apparent molecular mass of >150 kDa were also identified, these proteins were not identified consistently by all sera.

FIGURE 3. p150 is a myofibril-associated protein and partially colocalizes with actin and dystrophin. Immunofluorescence staining of fresh frozen normal canine temporalis muscle with purified CMMM IgG and mAbs against dystrophin or actin showed that p150 is present in the myofibrils in type 2M fibers but not type 1 fibers, and is also present at the sarcolemma, partially colocalizing with dystrophin. Bar = 50 μm for all figures.

FIGURE 4. p150 protein was isolated by immunoprecipitation and SDS-PAGE. The 150 kDa protein band recognized by CMMM IgG (auto IgG) was cut from the gel (band enclosed in box) and processed for MS. The p150 protein was not detected by Protein A alone (C-ProtA) or by pooled normal control dog serum (C-serum).
FIGURE 5. Protein alignment revealed that the 35 fragments from MS perfectly matched the deduced product of canine cDNA. A cDNA clone of human p150 was 92% identical to canine p150 (A). The fragments generated by MS are highlighted in red, and peptide sequences used for generation of Abs are underlined. The deduced protein from this cDNA contained six Ig-like domains and four fibronectin type 3 domains, very similar to the molecular domain organization of myosin binding protein-C (B).
Autoantibodies in canine masticatory muscle myositis are muscle specific

To determine the expression of p150, we tested extracts of different canine organs, including liver, testes, brain, kidney, heart, and stomach with purified IgG from pooled CMMM sera. p150 was detected only in masticatory muscle (Fig. 2) and not in any other organ. An Ab against tubulin was used as a control for protein loading.

p150 is a myofibril associated protein and partially colocalizes with actin and dystrophin

To localize p150, we performed immunofluorescence microscopy, analyzing sections of fresh frozen normal canine temporalis muscle. Muscle sections were incubated with purified canine CMMM IgG and with mAbs specific for actin or dystrophin. CMMM IgG stained the myofibrils in type 2M fibers, and the staining partially colocalized with that for actin (Fig. 3). CMMM sera IgG also stained the sarcolemma, partially colocalizing with staining for dystrophin (Fig. 3).

Isolation and characterization of p150

Using purified IgG from a pool of sera from dogs with CMMM, autoantigens were purified from masticatory muscle by immunoprecipitation and by affinity chromatography. The same major protein, with an apparent molecular mass of 150 kDa, was obtained by both approaches (Fig. 4). The p150 gel band was trypsinized and subjected to MS. The 35 peptide fragments from the mass spectrometer were found to perfectly match a canine hypothetical protein (Accession NM_173588) was sequenced and shown to be very similar to myosin binding protein-C (21, 22). A basic local alignment search tool search against the Swiss-prot database revealed that p150 has significant overall homology (~30%) to slow- and fast-type myosin binding protein-C as well as to a number of characterized muscle-related proteins such as titin (Fig. 5).

Abs generated to p150 peptides recognize p150

Four peptide sequences were synthesized based on the known sequence of canine p150 (underlined in Fig. 5); they were selected based on conservation between species, hydrophilicity, immunogenicity, and predicted secondary structure. Peptide Abs were generated in rabbits and tested in immunoblotting (Fig. 6). Abs to peptides CSVLVENELGKDRS (rabbits 701,702) and CDKDDK SILASVTES (rabbits 703,704) bound strongly to p150 protein (Fig. 6).

Discussion

In this study, we identified a major target for muscle-specific autoantibodies in canine masticatory muscle myositis, and show that this 150 kDa protein is a member of the myosin binding protein-C family. Autoantibodies against the 150 kDa protein bind to type 2M fibers in the masticatory muscle group but not to type 1 fibers (11, 12), or to limb, smooth, or cardiac muscle, or other tissues (Fig. 2). In previous studies, we showed that other proteins including masticatory muscle myosin heavy and light chains are autoantigens in CMMM (10, 11). Indeed, proteins with a molecular mass less than and greater than 150 kDa were identified in immunoblotting with individual dog sera consistent with this observation (Fig. 1B). However, the significance of autoantibodies to these other Ags is not known, because they are not present in all cases. Results of immunoblotting with purified IgG from dogs with MMM showed that the IgG Abs did not bind to myosin or other proteins at the concentrations used (Fig. 1A), demonstrating that the 150 kDa protein is the major autoantigen. Because the identity and function of the 150 kDa protein has not been previously described, and because it is a member of the MyBP-C family, we propose to name this protein masticatory myosin binding protein-C.

It is unlikely that autoantibodies against mMyBP-C are just an epiphenomenon. Autoantibodies against mMyBP-C are consistently found in dogs with CMMM, but are not present in generalized canine IMs where cellular infiltrates are present in both masticatory and limb muscle groups, including IMs caused by infectious agents and in immune-mediated PM (8, 11). Autoantibodies should be detected in all inflammatory muscle diseases that damage the masticatory muscle group, if the presence of autoantibodies was just a secondary effect of myofiber damage and exposure of the immune system to sequestered proteins. In a previous study of a large group of dogs with IMs (8), Abs were not detected against type 2M fibers in 140 cases of generalized inflammatory myopathy of both immune-mediated or infectious origin.

According to the sequence and m.w., expression pattern and localization, MyBP-C is a novel member of the skeletal muscle myosin binding protein-C family. MyBP-C proteins belong to the intracellular Ig superfamily (21, 22). Four different isoforms of MyBP-C are expressed in cardiac muscle, and in skeletal muscle fast and slow fibers (23). Similar to the other MyBP-C family members, mMyBP-C is composed of six Ig-like domains and four fibronectin-3-like domains (Fig. 5B) (23). In addition to being common building blocks of many extracellular proteins involved in ligand recognition and cell adhesion, Ig and fibronectin-3 domains are also the main domains of other intracellular proteins associated with the contractile apparatus of muscle and a physiological substrate of cAMP-dependent protein kinase (24). MyBP-C proteins are thick filament-associated proteins localized to the crossbridge-containing C zones of striated muscle sarcomeres (25). As such, they control the attachment of myosin heads by the interaction with myosin S2 (25).

The role of MyBP-Cs, and by extension mMyBP-C, in the regulation of muscle contraction may be of particular relevance to CMMM. The binding of the N terminus of MyBP-C to the subfragment-2 portion of myosin reduces actomyosin ATPase activity; phosphorylation abolishes this interaction, resulting in release of the “brake” on crossbridge cycling (26). A clinical characteristic of CMMM is the inability to open the jaw, even under general anesthesia (11). In the acute phase of the disease, there is swelling.
of the masticatory muscles with restricted jaw movement (trismus). Variable degrees of cellular infiltration are present within the muscle, but fibrosis is not usually observed. Thus, fibrosis is not the cause of restricted jaw movement in the acute phase. Following immunosuppressive corticosteroid therapy, jaw mobility gradually returns to normal. We hypothesize that an interaction of the autoantibodies with mMyBP-C may interrupt the muscle contractile mechanism by maintenance of a contracted state or interfering with normal relaxation. Resolution of restricted jaw mobility follows corticosteroid therapy; removal of autoantibodies by the immunosuppressive action of corticosteroids may result in effective relaxation of muscle. When the disease is in remission, and the dog is off corticosteroid therapy, autoantibodies against type 2M fiber proteins are no longer detectable either by ELISA or by immunocytochemistry (G. D. Shelton, unpublished observations).

The partial colocalization of mMyBP-C with dystrophin suggests that mMyBP-C may be expressed at or close to the sarcolemma in addition to inside the cell. A sarcolemmal location would expose it to the immune system and perhaps even be a trigger for an autoimmune reaction. As there are multiple alternative splice forms of mMyBP-C, one splice form could be surface associated and other splice forms intracellular. mMyBP-C is highly conserved among mammalian species and also present in humans, so theoretically, mMyBP-C could play a role in the initiation or maintenance of human inflammatory myopathies.

In conclusion, we have identified and molecularly characterized the first myositis- and muscle-specific protein that is localized not only within the muscle fiber but also associated with the sarcolemma. Further investigations on this novel protein may shed some light on possible mechanisms of the development of autoimmunity in inflammatory muscle disease not only in dogs but also in humans.

Disclosures

The authors have no financial conflict of interest.

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