Myozap, a Novel Intercalated Disc Protein, Activates Serum Response Factor–Dependent Signaling and Is Required to Maintain Cardiac Function In Vivo

Thalia S. Seeger,* Derk Frank,* Claudia Rohr, Rainer Will, Steffen Just, Christine Grund, Robert Lyon, Mark Luedde, Manfred Koegl, Farah Sheikh, Wolfgang Rottbauer, Werner W. Franke, Hugo A. Katus, Eric N. Olson, Norbert Frey

Rationale: The intercalated disc (ID) is a highly specialized cell-cell contact structure that ensures mechanical and electric coupling of contracting cardiomyocytes. Recently, the ID has been recognized to be a hot spot of cardiac disease, in particular inherited cardiomyopathy.

Objective: Given its complex structure and function we hypothesized that important molecular constituents of the ID still remain unknown.

Methods and Results: Using a bioinformatics screen, we discovered and cloned a previously uncharacterized 54 kDa cardiac protein which we termed Myozap (Myocardium-enriched zona occludens-1-associated protein). Myozap is strongly expressed in the heart and lung. In cardiac tissue it localized to the ID and directly binds to desmoplakin and zona occludens-1. In a yeast 2-hybrid screen for additional binding partners of Myozap we identified myosin phosphatase–RhoA interacting protein (MRIP), a negative regulator of Rho activity. Myozap, in turn, strongly activates SRF-dependent transcription through its ERM (Ezrin/radixin/moesin)-like domain in a Rho-dependent fashion. Finally, in vivo knockdown of the Myozap ortholog in zebrafish led to severe contractile dysfunction and cardiomyopathy.

Conclusions: Taken together, these findings reveal Myozap as a previously unrecognized component of a Rho-dependent signaling pathway that links the intercalated disc to cardiac gene regulation. Moreover, its subcellular localization and the observation of a severe cardiac phenotype in zebrafish, implicate Myozap in the pathogenesis of cardiomyopathy. (Circ Res. 2010;106:880-890.)

Key Words: myocytes ■ cardiac ■ cardiomyopathies ■ serum response factor

A unique morphological feature of cardiac muscle is the intercalated disc (ID), a highly specialized cell-cell contact structure that connects individual cardiomyocytes. Several independent substructures of the ID have been distinguished, including (1) desmosomes, which link the intermediate filament apparatus of the cell, (2) fasciae adhaerentes, to which the actomyosin filament bundles are attached, and (3) gap junctions, which allow free movement of ions and small molecules between adjacent cardiomyocytes. Together, these structures form a network of proteins which ensure mechanical and electric coupling of contracting cardiomyocytes (“functional syncytium”) (reviewed elsewhere). Recently, immunoelectron microscopy and biochemical studies have revealed that major constituents of the desmosomes such as desmoplakin, plakophilin-2 and the cadherins can also be detected in adherens junctions. Correspondingly, typical components of adherens and gap junctions were found to colocalize with desmosomal molecules. Thus, it has been proposed that in the heart these specialized cell-cell contact structures are merged in an “area composita.”

Given the critical importance of the intercalated disc for cardiac integrity and function, it is perhaps not surprising that the ID has become a hot spot of inherited cardiac disease. In particular, arrhythmogenic right ventricular cardiomyopathy (ARVC), has been coined a “desmosome cardiomyopathy,”

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since the majority of the ARVC-associated genes encode for desmosomal ID components. Yet, the complex interplay of the multiprotein network of the ID still remains poorly understood and it is likely that many critical ID components have still not been identified to date.

In an effort to identify such novel cardiac ID components, we explored expressed sequence tag (EST) databases for previously uncharacterized sequences with high abundance in cardiac cDNA libraries. With this approach, we discovered a novel open reading frame encoding for a 466 amino acid protein which we termed Myozap (Myocardium-enriched zonula occludens-1–interacting protein). Here we show that this cardiac-enriched protein colocalizes with β-catenin, N-cadherin as well as plakophilin-2 at the intercalated disc and directly binds to ID components such as desmoplakin and zonula occludens (ZO)-1. In addition, Myozap promotes serum response factor (SRF) signaling in a Rho-dependent fashion, while a newly identified Myozap binding partner, MRIP (myosin phosphatase-Rho interacting protein), inhibits this pathway. Finally, knockdown of the ortholog of myozap in zebrafish results in cardiomypathy with severe contractile dysfunction.

Methods

Experimental procedures for cloning and the subsequent bioinformatics, Northern blot analysis and radioactive in situ hybridization, the generation of a Myozap-specific antiserum and western blot analyses, immunofluorescence and immunoelectronic microscopy, yeast 2-hybrid assays, tissue culture, immunoprecipitations, reporter gene assays as well as zebrafish injection procedures, and fractional shortening measurement are provided in the expanded Methods section in the Online Data Supplement, available at http://circres.ahajournals.org.

Statistical analyses of the data were carried out using ANOVA in several other proteins that link the cell membrane and the cytoskeleton.9 Myozap Is a Highly Conserved Cardiac Protein

In order to identify novel cardiac-enriched genes, we searched the EST database for uncharacterized sequences predominantly found in cardiac cDNA libraries. Several of these ESTs corresponded to the UNIGENE cluster Mm.27585, which we used to construct an open reading frame (GenBank accession no. FJ970029) encoding for a 466 amino acid polypeptide with a calculated molecular weight of 54.2 kDa (Figure 1A). A protein sequence alignment between mouse, rat, Xenopus, and zebrafish Myozap homologs revealed high evolutionary sequence conservation among mammalian species (Figure 1A). Molecular cloning of human, murine, and rat myozap confirmed the predicted amino acid sequences.

According to the draft of the human genome, myozap maps to chromosome 15q21.3 and encompasses 13 exons, spanning a total of ~125.6 kb. Interestingly, the myozap gene is located in close proximity to a gene termed GRINL1A. In fact, the sequence of Myozap has been previously deposited in the National Center for Biotechnology Information databank under the denotation “Grin1a isoform 7.” Moreover, it has been suggested that a common mRNA (Gcom) of Myozap/Grin1a7 and a downstream part of the GRINL1A locus might exist.8 However, utilizing rT-PCR experiments with several primers, we could only detect 2 distinct transcripts (Online Figure I), suggesting that Myozap and Gcom/Gdown are independent proteins. This notion is further supported by the finding that no overlapping ESTs between Myozap and the downstream gene Grin1A exist in the database (Online Figure II). Taken together, these results make the existence of a common mRNA - at least at significant levels rather unlikely. Nevertheless, in order to acknowledge that the sequence of Myozap has been deposited as “Grin1a7,” we subsequently used the gene name “Myozap/Grin1a7.” Myozap does not contain significant sequence homology to any other known protein. However, between amino acids 181 and 348, myozap contains an ERM (Ezrin, Radixin, and Moesin)-like domain (Figure 1B). The murine Myozap/Grin1a7 gene displayed a similar expression pattern, again with predominant expression in the myocardium. In order to be able to analyze myozap on the protein level, an antiserum was generated in rabbits utilizing a myozap-specific synthetic peptide. Because the ERM-like domain of myozap suggested an association with the cell membrane, we performed Western blot experiments with membrane-rich fractions from various rat tissues. Myozap- and control-transfected HEK 293 cells served as positive and negative controls. These experiments revealed strong synthesis of myozap in the heart and in lung tissue at the predicted size of ~ 55 kDa, other tissues examined were completely negative (Figure 2B). As an additional control, the same tissues and cell culture extracts were subsequently probed with the antiserum in combination with the synthetic

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>ARVC</td>
<td>arrhythmogenic right ventricular cardiomyopathy</td>
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<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin, Radixin, and Moesin</td>
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<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>hpf</td>
<td>hours postfertilization</td>
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<tr>
<td>ID</td>
<td>intercalated disc</td>
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<tr>
<td>MO</td>
<td>morpholio-modified</td>
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<tr>
<td>MRIP</td>
<td>myosin phosphatase Rho interacting protein</td>
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<tr>
<td>Myozap</td>
<td>myocardium-enriched zonula occludens-1–interacting protein</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<td>ZO-1</td>
<td>zonula occludens-1</td>
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Myozap/Grin1a7 Is Predominantly Expressed in the Heart

To determine the expression of myozap in different tissues, we performed multi-tissue Northern blot experiments using mRNA from various human and mouse tissues (Figure 2A). Human Myozap mRNA is predominantly expressed in the heart, and to a much lesser degree in skeletal muscle, placenta and lung. The murine Myozap/Grin1a7 gene displayed a similar expression pattern, again with predominant expression in the myocardium. In order to be able to analyze myozap on the protein level, an antiserum was generated in rabbits utilizing a myozap-specific synthetic peptide. Because the ERM-like domain of myozap suggested an association with the cell membrane, we performed Western blot experiments with membrane-rich fractions from various rat tissues. Myozap- and control-transfected HEK 293 cells served as positive and negative controls. These experiments revealed strong synthesis of myozap in the heart and in lung tissue at the predicted size of ~ 55 kDa, other tissues examined were completely negative (Figure 2B). As an additional control, the same tissues and cell culture extracts were subsequently probed with the antiserum in combination with the synthetic...
peptide the antiserum was generated against. No significant signals could be observed, confirming the specificity of the antiserum. To investigate the subcellular distribution of myozap, we treated the membrane rich fraction with a Triton-containing buffer, which yielded a Triton (detergent)-soluble fraction representing the membrane fraction and a Triton-insoluble fraction rich in cytoskeletal proteins. In particular, the Triton-insoluble fraction yielded significant bands for myozap in heart and lung tissue (Figure 2C).

To further examine the expression of myozap in the heart, in situ hybridizations of adult murine cardiac tissue were performed which revealed an intense and specific signal throughout the myocardium (Figure 2D). The coronary vasculature was spared, consistent with a cardiomyocyte-specific expression pattern.

**Myozap Expression Is Developmentally Regulated**

In order to investigate the developmental expression pattern of Myozap, we performed an additional series of in situ hybridization experiments of staged mouse embryos at embryonic day (E)8.0, E9.0, E11.5, E12.5, and E15.5, as well as postnatal day 1. At E8.0 of mouse embryonic development,
Myozap/Grin1a7 gene expression was confined to the embryonic vasculature (Figure 3A, dorsal aortae). At E9.0, the vasculature still shows a strong signal, including the dorsal aortae and head veins. In addition, the endocardium of the outflow tract and the primitive ventricle were intensely stained, whereas the myocardium was still negative at this time point (Figure 3B). At E11.5, not only the vasculature including the ductus cuvieri (which subsequently develops into the vena cava), but also the trabeculated myocardium of the left ventricle revealed a strong hybridization signal (Figure 3C). One day later, at E12.5 (Figure 3D), an intense signal was observed throughout the entire myocardium (left ventricle and left atrium). Mesenchymal tissue within the lung was also myozap-positive. At late embryonic stages (E15.5) and postnatal day 1, a strong hybridization signal of the heart and lung was maintained (Figure 3E and 3F), whereas expression in the vasculature had become undetectable. Taken together, these findings indicate that Myozap/Grin1a7 expression undergoes developmental regulation with a shift from a vascular and endocardial pattern to a strong and specific myocardial staining.

Myozap Is a Novel Component of the Intercalated Disc

Given the high protein levels of myozap in the myocardium, we next aimed to determine its subcellular localization in isolated adult murine cardiomyocytes. Using the myozap-specific antiserum, we detected a strong signal at the ID, as shown by colocalization with N-cadherin (Figure 4A). In addition, a weak staining of the sarcomeric Z-discs (as determined by colocalization with α-actinin) was observed (data not shown).

In sections of intact mouse heart tissue myozap was again predominantly found at intercalated discs where it colocalized with N-cadherin (Figure 4B, top images). Myozap also colocalized with plakoglobin and plakophilin-2, 2 prototypical area composita proteins (Figure 4B, bottom images). These data were further corroborated by immunoelectron microscopy analyses on cryosections through bovine hearts. Again, the myozap-specific antiserum was used, followed by an incubation step using a nanogold-coupled secondary antibody. We observed an intense signal at the cell–cell junctions between adjacent cardiomyocytes, further supporting the notion that myozap is a novel component of the ID (Figure 4C).

Myozap Interacts and Colocalizes With Desmoplakin

Given the ID localization of myozap, we examined whether it binds to other known ID proteins, such as desmoplakin, plakoglobin and plakophilin-2. Coimmunoprecipitation experiments failed to demonstrate a direct interaction of myozap with plakophilin-2 (data not shown). In contrast, experiments with transfected HEK293T cells revealed an intense coprecipitation of desmoplakin with myozap, while no specific band was observed with empty vector alone (Figure 5A). The positive coimmunoprecipitation could be confirmed with endogenous proteins derived from adult mouse heart lysates (Figure 5B). Moreover, these findings were further supported by the colocalization of both proteins at the ID in immunostainings of adult bovine myocardium (Figure 5C).
Myozap Binds to ZO-1 and Colocalizes With the Actin Cytoskeleton

Next, we performed a yeast 2-hybrid screening experiment to discover additional protein interaction partners of myozap. From this screen, several putative binding partners were identified, including zona occludens-1 (ZO-1) and the Rho inhibitor MRIP (Myosin phosphatase RhoA interacting protein). ZO-1 has been suggested to be a scaffold protein linking the actin cytoskeleton and structures of adherens and tight junctions. In cardiomyocytes, ZO-1 has been shown to be part of the area composita of the intercalated disc. In order to confirm the interaction with Myozap, immunoprecipitation experiments were performed using a ZO-1 antibody and Myozap antiserum. The results reproduced the interaction between the 2 proteins (Figure 6A) and immunostaining of adult mouse heart cryosections revealed a colocalization of both proteins at the ID (Figure 6B).

To further analyze which domains of myozap are sufficient to bind to ZO-1, we generated several deletion clones (Figure 6C) and again performed yeast 2 hybrid experiments to determine a potential interaction. The deletion variants, myozap full length or the empty pDest22 vector (activation domain) were transformed together with ZO-1 (cloned into the pDEST32 DNA-binding domain vector) into yeast. Only the deletion constructs myozap1–348, myozap91–250, myozap91–300 and myozap full length together with ZO-1 were able to grow on histidine lacking medium, suggesting that amino acids 91 to 250 of myozap represent the minimal domain necessary to bind to ZO-1.

Given the ability of ERM proteins to bind to filamentous actin, we next tested whether full length Myozap colocalizes with actin. The use of an actin cosedimentation assay as direct proof of a potential actin-myozap interaction was precluded due to spontaneous sedimentation of myozap during ultracentrifugation (data not shown). Therefore, COS7 cells were transiently transfected with HA-tagged myozap. Subsequent coimmunostaining with TRITC-labeled phalloidin for actin filaments and anti-myozap revealed colocalization of both proteins at the actin cytoskeleton (Figure 6E). To further corroborate these results, the experiment was repeated in isolated adult rat cardiomyocytes. Here, a colocalization of myozap with actin could be seen at ID where actin anchors at high densities to the cell membrane (Figure 6F).

Myozap Induces Rho-Dependent SRF Signaling and Binds to the Rho Inhibitor MRIP

The yeast 2-hybrid screen also revealed a direct interaction between myozap and myosin phosphatase Rho-interacting protein (MRIP, its murine homolog is named p116Rip), which binds to Rho and inhibits Rho-dependent SRF signaling. A coimmunoprecipitation experiment was per-
formed in MDCK (Madin–Darby canine kidney) cells which again confirmed the interaction between the 2 proteins (Figure 7A). Moreover, both proteins colocalized at the cell membrane as well as with cortical actin as shown by immunostaining (Figure 7B). For further interaction domain mapping, we cloned several myozap deletion variants (Figure 7C). A yeast 2 hybrid retransformation interaction assay was performed by cotransforming either one of the deletion variants, myozap full length or empty vector together with MRIP3. The minimal domain of myozap necessary for binding MRIP3 was identified between amino acids 91 and 250 which includes parts of the ERM-like domain (Figure 7D).

Next, we investigated whether Myozap might have an impact on Rho-dependent signaling which has been shown to stimulate the transcriptional activity of SRF,13. Myozap and a luciferase reporter gene driven by the SRF-dependent sm22-promoter,14,15 were overexpressed in HEK293T cells. Myozap strongly activated the sm22-promoter (6.7 fold). Conversely, the addition of C3-transferase from Clostridium botulinum, which inhibits Rho via ADP-ribosylation of the GTPase, markedly attenuated sm22 activity (-76.7%, Figure 7E). In addition, HEK293T cells were cotransfected with myozap, MRIP and the sm22-luc reporter. Again, Myozap activated the sm22-promoter, whereas the addition of MRIP led to significant attenuation of sm22 activation (Figure 7F). Baseline promoter activity was not significantly affected by either C3-transferase or MRIP. Thus, myozap robustly induces SRF-dependent transcription, while its direct binding partner MRIP inhibits this activation.

In order to determine which domain of myozap induces the sm22-promoter, we used the same deletion fragments shown in Figure 7C as well as full length Myozap (MyozapFL).
Myozap or its deletion variants were cotransfected with the sm22-promoter luciferase reporter. All constructs except Myozap1–181 and Myozap348–466 significantly activated the sm22-promoter (Figure 7G). Interestingly, the minimal activation domain localizes to the center of the ERM-like domain. Moreover, Myozap appears to specifically target SRF-dependent signaling, since MEF2, another basic loop helix transcription factor, was not significantly activated (Online Figure II).

Knockdown of Myozap in Zebrafish Causes Cardiomyopathy

To assess the role of Myozap in vivo, we performed morpholino-modified (MO) antisense oligonucleotide-mediated knockdown experiments of the Myozap ortholog in zebrafish. We characterized embryos injected with MO1-control and MO2-myozap, the latter of which leads to an abnormal splice product (exon skipping, 95bp) and thus to premature termination of translation of zebrafish Myozap (zMyozap). MO-control injected embryos displayed normal morphology at 48 hours postfertilization (hpf) (Figure 8A), whereas MO2-Myozap–injected embryos developed significant cardiac pathology (Figure 8B): The morphants showed pericardial edema as a sign of cardiomyopathy as well as blood pooling at the sinus venosus suggestive for low contractile performance. Efficacy of the MO2-myozap morpholino was tested by PCR, which showed that the majority of zMyozap mRNA was incorrectly spliced. By injecting 2.5 ng of MO2-myozap, 92% of the injected embryos (n=370) developed similar phenotypic characteristics including contractile dysfunction. Of note, the MO2-myozap–injected embryos also displayed severe skeletal muscle dysfunction (Online Videos I and II). Repetitive measurements of atrial and ventricular fractional shortening of MO-control– and MO2-Myozap–injected embryos at 36, 48 and 72 hpf revealed a progressive loss of contractility (Figure 8D and 8E; Online Videos III and IV). Despite the functional impairment, Myozap morphant hearts displayed normal morphology. Endocardial and myocardial cell layers as well as the atrioventricular canal appeared unaltered in MO2-myozap–injected embryos after 48 hpf (Figure 8F and 8H). At 48 hpf, the expression of cardiac myosin light chain 2, investigated by whole mount antisense RNA in situ hybridization, was similar in MO-control– and MO2-Myozap–injected embryos, indicating no severe changes.
in cardiac development and differentiation (Figure 8G and 8I).

**Discussion**

We describe a previously uncharacterized cardiac protein, Myozap, which localizes to the ID and binds to several members of the area composita, the predominant cell-cell-contact structure of the mature cardiomyocyte. Since the actin myofilament cytoskeleton anchors at fascia adherens and area composita, the ID is directly involved in the versatile role of actin signaling and actomyosin contractility. The actin cytoskeleton acts in a variety of cellular motility processes, including contractility, mitosis, cytokinesis, secretion, and endocytosis. Moreover, transcription and gene expression is regulated by processes involving actin either directly through an association with nuclear chromatin remodeling proteins or indirectly through changes of actin dynamics. Specifically, monomeric actin serves as an upstream regulator of SRF-dependent signaling (reviewed). SRF is a MADS-box transcription factor that controls the expression of a wide range of muscle-specific genes.

In this context, it is noteworthy that myozap contains an ERM-like domain, since ERM proteins have important roles in adherens junction formation and maintenance via binding and stabilization of the actin cytoskeleton. Of note, ERM proteins are also involved in the regulation of the small GTPase Rho (for review see). The small GTPases of the Rho family, including Rho, Rac, and Cdc42, have been shown to function as major activators of SRF-dependent transcriptional activity via changes in actin dynamics. Rho GTPases thereby serve as molecular switches in a multitude of signaling pathways especially those involving actin polymerization and stress fiber formation. Myozap, which colocalizes with filamentous actin, activates SRF signaling as shown by the robust activation of a sm22-promoter driven reporter. This reporter gene activation could be inhibited by cotransfection of C3-transferase, which irreversibly blocks Rho activation, suggesting that Myozap uses a Rho-dependent mech-
anism of SRF activation. Mechanistically, most ERM proteins positively modulate Rho via binding of RhoGDI, a negative regulator of Rho activity. This in turn releases inactive Rho, allowing its subsequent activation.22 However, ERM domains may also function downstream of Rho, where ERM proteins are activated by Rho-dependent threonine phosphorylation. Both mechanisms together form a positive feedback loop.23

In cardiomyocytes, signaling from the small GTPase Rho and Rac diverges into several downstream targets which include SRF, other transcription factors such as NF-κB, as well as myofilament proteins, and ion channels.24 The direct interaction of Myozap with MRIP, an endogenous inhibitor of Rho-mediated SRF signaling,11 adds another layer of complexity. The addition of MRIP in a SRF-dependent reporter assay significantly blunts myozap’s capability of Rho-dependent SRF activation. Consistently, the murine homolog of MRIP, p116RIP, has been shown to interfere with RhoA-mediated transcription through its ability to disassemble the actomyosin cytoskeleton downstream of RhoA.25

An important hallmark of SRF is its ability to orchestrate the activity of numerous upstream signaling events.26 The tight control of SRF activity is essential for maintenance of cardiac contractility and the heart’s ability to respond to stress and diverse disease stimuli. In this regard, it is worth noting that overexpression of the cardiac SRF activator STARS leads to an increased sensitivity to hemodynamic stress.27 These findings are also in line with data derived from mice with cardiac-specific overexpression of SRF, which develop severe dilated cardiomyopathy.28 Conversely, inactivation of SRF in the adult mouse heart has been reported to lead to impaired left ventricular function and rapid progression to dilated cardiomyopathy mediated by a progressive loss of contractile proteins.29 Further support for the essential role of SRF in cardiac integrity comes from the results of embryonic deletion of SRF, which results in cardiovascular defects and intrauterine death.30,31 Moreover, recently the microRNAs 133a-1 and -2 have been shown to be critical components of an SRF-dependent myogenic transcriptional circuit.32 Our data suggest that myozap might represent another novel regulator of myocardial SRF signaling. Consistently, knockdown of myozap in zebrafish results in severe cardiomyopathy.

In addition to its effects on cardiac signal transduction, the localization of myozap at the ID junctions implies a function in the mechanical and/or electric coupling of cardiomyocytes. The ID is known as a hot spot of inherited cardiac disease, which include ARVC and dilated cardiomyopathy. The disruption of desmosomal integrity appears to be a key factor in mediating the development of ARVC,
resulting in defective mechanics, abnormal localization of cell–cell adhesion proteins and gap junction remodeling. It will thus be interesting to see whether myozap also plays a role in the etiology of ARVC. This notion is supported by data from mouse models for dilated cardiomyopathy including the muscle LIM protein knockout mouse and the tropomodulin–overexpressing mouse, which showed a marked upregulation of adherens junction–associated proteins including N-cadherin, α- and β-catenin, plakoglobin, N-RAP, and vinculin. In our zebrafish knockout model, Myozap deficiency leads to severe contractile dysfunction without disturbing cardiac development. Contractile dysfunction is a hallmark of dilated cardiomyopathy suggesting a potential role for myozap in the pathogenesis of cardiomyopathy.

Moreover, a critical feature in end stage heart failure is the progressive loss and altered distribution of connexin 43. This process, referred to as “gap junction remodeling,” may lead to electrophysiological perturbations and contractile dysfunction, as recently reviewed. In this context, the binding partner of myozap, ZO-1, seems to play an essential role since it directly interacts with connexin 43 at the ID where it controls gap junction size. Recent data suggest that the binding of ZO-1 to connexin 43 is increased in heart failure leading to an altered gap junction architecture (elsewhere and references cited therein). It remains to be seen whether the direct interaction of myozap with ZO-1 modulates this process and thus plays a role in these fundamental remodeling processes.

In summary, we have discovered a previously uncharacterized, cardiac-enriched protein, termed myozap (myocardium-enriched ZO-1 associated protein). Myozap acts as an activator of Rho-dependent SRF signaling, which in turn is controlled by a direct interaction with MRIP, a molecule that can attenuate myozap-dependent SRF activation. Interestingly, myozap is localized at the ID where it interacts with desmoplakin and ZO-1, 2 proteins that have been implicated in the pathogenesis of human heart disease. The knockdown of Myozap in zebrafish leads to severe contractile dysfunction. Further in vivo gain and loss-of-function animal experiments as well as the careful analyses of the Myozap/Grin1A7 gene in ARVC patients, will help to clarify whether myozap also participates in the pathogenesis of cardiomyopathy.

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Disclosures

None.

References


Novelty and Significance

**What Is Known?**

- The ID is an essential cell contact structure ensuring mechanical and electric coupling between cardiomyocytes.
- The importance of the ID is further underscored by the findings that many proteins involved in this complex contribute to the pathogenesis of inherited cardiac disease.

**What New Information Does This Article Contribute?**

- Myozap regulates signaling pathways activated by the serum response factor “knockdown” of Myozap in zebrafish causes severe impairment of cardiac contractility.

Given the complexity and the importance of the intercalated disc, we hypothesized that there may be intercalated disc (ID) components that are still unknown. In bioinformatics screening experiments, we identified an ID protein termed Myozap which colocalizes and interacts with several other ID proteins, including desmoplakin and ZO-1. Moreover, we found that Myozap is a positive regulator of serum response factor signaling. When downregulated in vivo, Myozap deficiency caused severe cardiomyopathy in zebrafish. Myozap may be an important component: molecular pathways that regulate normal cardiac function and may be involved in the pathogenesis of cardiomyopathies.
Myozap, a Novel Intercalated Disc Protein, Activates Serum Response Factor–Dependent Signaling and Is Required to Maintain Cardiac Function In Vivo
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Expanded materials and methods

Cloning of Myozap and bioinformatics

ESTs from cardiac cDNA libraries were “blasted” against the NCBI database to detect novel, yet uncharacterized genes. This screen revealed a bioinformatically constructed open reading frame (ORF) encoding for a novel putative protein of 466 amino acids, which we subsequently termed “myozap” (myocardium-enriched ZO-1-interacting protein). Full-length mouse myozap was cloned from mouse heart cDNA by using myozapF (5’-GCTGGCACCATGCTGCGCTCCACGTCCAC-3’) and myozapR (5’-GCTGGGTCGCCCTAAGTCAGCGTTTTCTTTTGAGGA-3’) for a first PCR and attBFor (5’-GGGGACAAGTTTGTACAAAAAAGCTGGCACC-3’) and attBRev (5’-GGGGACCACTTTGTACAAAAAAGCTGGGCACC-3’) for a second PCR. Similarly, human myozap was cloned from a human cardiac cDNA library. The PCR product was recombined into the pDonR201 plasmid (Invitrogen, Karlsruhe, Germany) using the Gateway technology and was subsequently recombined into expression plasmids to obtain HA- or myc-tagged expression constructs.

Northern blot analysis and radioactive In situ hybridization

Multiple tissue Northern Blots (Clontech, Mountain View, USA) containing mouse and human poly(A) RNA were hybridized overnight at 65°C with 32P-dCTP-labeled (Rediprime II Random Prime labelling System, Amersham Biosciences/GE healthcare, Freiburg, Germany) cDNA probes corresponding to the ORF of mouse and human Myozap, respectively. Serial washes were conducted with 2 x SSC/0.1% SDS and 0.2 x SSC/0.1% SDS at 65°C. Autoradiography was performed at –80°C for 24-48 hours with an intensifying screen. The probes used correspond to the C-terminus (mouse, amino acids 238-466), or N-terminus (human, amino acids 7-194).
For radioactive in situ hybridization, RNA probes corresponding to sense and anti-sense strands of myozap cDNAs were prepared, using T7 and T3 RNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) and $^{35}$S-labeled UTP. Sections of mouse embryos at various time points of development were subjected to in situ hybridization as described previously\(^1\). Sense probes were used as negative controls.

**Generation of a myozap specific antiserum, triton-solubility and Western blot analyses**

A peptide consisting of 15 carboxy-terminal amino acids (NH2- (GC) PYTRVLELSSKKTLT-OH) derived from mouse myozap was synthesized (Biosynthesis, Lewisville, USA) and used to generate antisera in rabbits. IgG was purified from rabbit serum using protein A-sepharose beads (Zymed) and subsequently used for Western blotting as well as immunostaining. In order to determine the specificity of the antibody, several rat tissues were homogenized in a Tris-buffer containing 50 mM Tris-HCl pH 7.4, 5mM EDTA and 2mM EGTA. After centrifugation at 360g for 10 min, the supernatant was centrifuged again at 26,500g for 10 min in order to obtain a membrane rich fraction. The pellet was then subjected to SDS-PAGE and Western blotting. The blots were probed with either the myozap antibodies alone or with the myozap antibodies plus the peptide used to generate the antibody.

In order to determine if myozap can be found in the triton-soluble or –insoluble fraction, we performed further fractionation. Several mouse tissues were homogenized in a Tris-buffer and centrifuged at 360g for 10 min. After centrifuging the supernatant at 26,500g for 10 min, a Tris-soluble-fraction (supernatant) was obtained. The pellet was resuspended in a Triton-buffer\(^2\) containing 20 mM Hepes, pH 7.4, 150 mM NaCl, 0.5 mM CaCl\(_2\) and 1% Triton X-100 and centrifuged at 20000g for 15 min. The supernatant (Triton-soluble-fraction) was removed and the pellet, that corresponds to the Triton-insoluble-fraction, was resuspended in Triton-buffer. The different fractions were subjected to SDS-PAGE and Western blotting. The blots were probed with the myozap antibody.
Immunofluorescence microscopy

The subcellular localization of Myozap was determined in adult mouse and rat cardiomyocytes as well as cryosections of bovine and mouse cardiac tissue using indirect immunofluorescence microscopy. Adult mouse and cardiomyocytes were prepared as described\textsuperscript{2,3}. Cryosections were air-dried, fixed in -20°C acetone for 10 min, permeabilized with 0.1% Triton X-100 (Sigma), and blocked in 2% bovine serum albumin or 5% goat serum for one hour. Next, sections were incubated for one hour (at room temperature) or overnight (at 4°C) with the primary antibodies using the following dilutions: polyclonal rabbit anti-myozap 1:200, monoclonal mouse anti-catenin (Zymed, San Francisco, USA, 1:100), monoclonal mouse anti-N-cadherin (BD Biosciences, Heidelberg, Germany, 1:250), ZO-1 (Zymed, San Francisco, USA, 1:200), monoclonal murine anti-desmoplakin 1:2 (desmoplakin (DP)-mix, containing DP-2.17, DP-2.15 and DP-2.20), and monoclonal mouse anti-plakophilin-2 (clone 86, Progen, Bath, UK, undiluted).

Transfected MDCK (Madin-Darby canine kidney cells) and COS7 cells were fixed with 4% paraformaldehyde for 10 min, permeabilized and blocked with 0.1% Triton X-100 in 2.5% BSA in PBS for one hour at room temperature. Cells were then incubated with primary antibodies (anti-Myozap polyclonal antibody, anti-116RIP (Abnova, Heidelberg, Germany, 1:100) and/or TRITC-labeled phalloidin (Sigma, Munich, Germany, 1:500). Secondary antibodies conjugated to either Alexa 488 (Molecular Probes, via Invitrogen, Karlsruhe, Germany), fluorescein (Vector Labs, Burlingame, USA) or Cy3 (Dianova, Hamburg, Germany) were incubated for 30 min to one hour at a dilution of 1:200. Vectashield medium with DAPI (4',6'-diamidino-2-phenylindole) (Vector Labs, Burlingame, USA) or Fluoromount (Biozol, Eching, Germany) was used for mounting. Fluorescence micrographs were taken with one of the following microscopes: Axioskop, LSM 510 UV and Axioskop 2 Plus (Zeiss, Heidelberg, Germany).

Immunoelectron microscopy
For immunoelectron microscopy, 5 µm thick cryosections of bovine tissue (shock-frozen in liquid nitrogen) were mounted on coverslips and fixed with 2% formaldehyde or methanol-acetone followed by three washes with NH₄Cl. After permeabilization with 0.1% saponin for 5 min, the specimens were incubated with polyclonal anti-Myozap for 2 hours, thoroughly washed with PBS, and incubated for at least 2 hours with secondary antibody coupled to nano-gold particles (Biotrend, Cologne, Germany). Further procedures, including silver enhancement, were conducted as described previously⁴. Electron micrographs were taken with the electron microscope model EM 910 (Zeiss, Heidelberg, Germany).

**Yeast-two hybrid screen**

A full length mouse Myozap cDNA, fused to a GAL4 DNA binding domain (in pDEST32, Invitrogen) was used as bait in a yeast two-hybrid screen of approximately 1 x 10⁶ clones of the ProQuest Three-Frame cDNA human heart library (Invitrogen, Karlsruhe, Germany), as described¹. Briefly, clones displaying differential growth on selective plates lacking histidine, leucine and tryptophan were picked and grown in selective medium lacking leucine and tryptophan, plasmid DNA was isolated and subsequently electroporated into DH10B E. coli (Gibco via Invitrogen, Karlsruhe, Germany). The obtained clones were sequenced and retransformed with the Myozap construct to confirm the interaction.

**Tissue culture, immunoprecipitations, and reporter gene assays**

293T (large “T”-transformed human embryonic kidney cells), MDCK dog cells, and COS7 cells were maintained in DMEM containing 10% FBS, 2 mM L-glutamine and penicillin/streptomycin. 1 x 10⁶ 293T cells were transfected with 4 µg of expression plasmids for full-length HA-tagged mouse Myozap, as well as a flag-tagged ZO-1-construct, a myc-tagged MRIP or a GFP-tagged Desmoplakin (generous gift from K. Green), using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) or jetPEI reagent (PolyPlus Transfection, Illkirch, France). Forty-eight hours after transfection, the cells investigated for the interaction with ZO-1 were harvested in ZO-1-buffer, containing 50mM Tris (pH 7.5),
150 mM sodium chloride, 2 mM EGTA, 0.25 mM sodium deoxycholate, 1% NP40, 0.1 mM sodium orthovanadate, 10 mM sodium fluoride and a protease inhibitor cocktail (Complete; Roche). The interaction with desmoplakin was tested in ELB-buffer containing 50 mM HEPES (pH 7.0), 250 mM sodium chloride, 1% NP40, 5 mM EDTA and a protease inhibitor cocktail (Roche). MDCK cells were harvested in ELB-buffer containing 0.5% SDS and diluted 1:10 with ELB-buffer without SDS. Cells were briefly sonicated and debris was removed by centrifugation. Tagged proteins were incubated overnight at 4°C and then immunoprecipitated for 2 hours using protein G agarose (Zymed) and 1 µg of the appropriate antibody (monoclonal anti-flag M2 (Sigma), monoclonal anti-desmoplakin or polyclonal anti-myc. Subsequently, the pellet was washed with CB-buffer and ELB-buffer, respectively, and subjected to SDS-PAGE, followed by transfer to polyvinylidene membranes and immunoblotting using monoclonal an anti-HA antibody (Sigma, Munich, Germany).

293T cells were transfected with expression plasmids encoding HA-Myozap, myc-RhoQ63L, myc-MRIP3, C3-Transferase and a SM22 promoter-luciferase construct as well as Renilla luciferase under control of a thymidine kinase promoter (pRLTK) to normalize for transfection efficiency. For the MEF2 reporter experiments we used a luciferase construct controlled by 3 MEF2 binding sites. All experiments were conducted in triplicates and repeated at least three times. Luciferase assays from 293T-cell lysates were performed using the Dual-Luciferase Reporter Assay (Promega, Mannheim, Germany). In order to determine the minimal part of Myozap which is sufficient to induce the SM-22 promotor, PCR products using MyozapF, Myozap91F (5’-GCT GGC ACC GGC TGG TCC ACC AAT CAG-3’), Myozap181R (5’-GCT GGG TCT CGC CCT ACA CGT CCA CAA GGG GTT TTC TG-3’), Myozap181F (5’-GCTGGCACCACTTTGGAAAGAGCCAGAGCAAGG-3’), Myozap250R (5’-GCTGGGTCGCCCTACCTACTCATACTGGCTGTACAGCCTT-3’), Myozap300R (5’-GCTGGGTCGCCTACCCCATCTTCTTCTGGRCTTTTCTC-3’), Myozap348R (5’-GCTGGGGTCGCCCTAGCTGGCTGACGCTCTCCTC-3’), Myozap348F
(5'-GCTGGCACCCCTCCGGGAGCGGATCAGA-3') and MyozapR (5'-GCTGGGTCGCCCTAAAGTCAGCGTTTTCTTTGAGGA-3') were cloned and recombined into pcDNA3.1myc-Dest, pDest32 and pDest22 using the Gateway technology. The same deletion mutants were used to determine the minimal necessary domains of Myozap to bind to MRIP3 and ZO-1.

**Coimmunoprecipitation of endogenous protein**

Neonatal mouse hearts were homogenized in co-immunoprecipitation (Co-IP) lysis buffer (10 mM Tris-HCl pH 8, 100 mM NaCl, 0.2% Nonidet P-40 and 1X protease and phosphatase inhibitors; Roche Applied Science). Homogenates (250 µg per IP) were cleared by centrifugation at 12,000 x g for 15 mins and incubated for 6 hours at 4°C with Protein G-sepharose beads (GE Healthcare) pre-incubated with mouse anti-desmoplakin antibody (1:300; AbD Serotec) or control mouse serum (1:300; Sigma) for 2 hours. Beads were washed three times with Co-IP lysis buffer and once with PBS (37 mM NaCl, 10 mM sodium phosphate, 2.7 mM KCl, pH 7.4). Bound proteins were eluted in SDS-PAGE sample buffer, fractionated by 4-15% or 5% SDS-PAGE, and immunoblotted with anti-myozap (1:1000) and anti-desmoplakin (1:2000) antibodies, respectively.

**Zebrafish injection procedures, and fractional shortening measurement**

Care and breeding of zebrafish was performed as described. Morpholino-modified antisense oligonucleotides were directed against two different splice donor sites of zebrafish myozap (MO1-myozap: 5´-TGCTTGCATGATTGAGCACCTGGTG-3´ and MO2-myozap: 5´-AATTAGATGTTACTTACTTGGTG-3´). A standard control oligonucleotide (MO-control) (Genetools, LLC) was injected at the same concentration as a negative control. To inhibit pigmentation, 0.003% 1-phenyl-2-thiourea was added to the embryo medium. To analyze contractile force of the morphants and control morpholino injected embryos, we underwent fractional shortening measurements with help of the zebraFS software application (www.benegfx.de).
Zebrafish histology, transmission electron microscopy, and RNA antisense in situ hybridization

Embryos were fixed in 4% paraformaldehyde and embedded in JB-4 (Polysciences, Inc.). 5 µm sections were cut, dried, and stained with hematoxylin/eosin. Electron micrographs were obtained essentially as described. Whole-mount RNA in situ hybridization was carried out essentially as described using a antisense probe for zebrafish cardiac mlc2. All RNA probes were digoxigenin labeled.

RT PCR for detection of different Gcom isoforms

First Choice Human Brain Reference RNA (Ambion, Austin, USA) was reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen, Karlsruhe, Germany). Amplifications of brain cDNA and the ProQuest Three-Frame cDNA human heart library (Invitrogen, Karlsruhe, Germany) were performed using the GoTaq Flexi DNA Polymerase (Promega, Madison, USA). For amplification the following protocol was used: 1 cycle: 95°C 120sec, 15 cycles: 95°C 45sec, 57°C 45sec (The annealing temperature was reduced 0,5°C per cycle), 68°C 90sec and 20 cycles: 95°C 45sec, 52°C 45sec, 68°C 90sec. Primers used:
P1: 5’-GTCTGGGGAGTTAACTGATTCTG-3’
P2: 5’-CTAAGTCAGAGTTTTCTTCATGGTTAA-3’
P3: 5’-TGTTTCTGGCTTCTGCCTG-3’
P4: 5’-CGCAAGTACTAAGAACTTGACAGG-3’
P5: 5’-TCTCGGTATCTCCCTGAAGAC-3’
References:


Online Figures – legends

Online Figures

Online Figure I: (A) PCR strategy to determine the expression of the Grin1la complex transcript unit in the heart. Gene names and exon nomenclature was derived from Roginski et al. The upstream gene of Grin1la consists of the exons 1 to 10, 12, 13 and 15b and the downstream gene of the exons 20b-e and 21 to 28. The combined genes contain the exons 1 to 14, 15a, 16 to 19, 20a, 20f, 21, 22 and 23a. Five primers (P1 to P5) were designed to amplify prototypical isoforms. P1 is located in exon 9, P2 in exon 15, P3 in exon 19a, P4 in exon 21a and P5 in exon 23. (B) Reverse transcription PCR of human heart and human brain RNA. With the primer pair P1/P2 a 421bp PCR-product could be amplified in the heart and brain. The 421bp-product could correspond to Gup1, Gcom8 and/or Gcom13, whereas a 337bp-product (corresponding to Gup2 and/or Gcom9) or a 853bp-product (Gcom10) could not be amplified. No amplicons could be seen with the primer pairs P1/P5 and P1/P3 in the heart and likewise in the brain. A strong 429bp product could be amplified in the heart and in the brain with the primer pair P4/P5, which could correspond to Gcom9, Gcom12, Gdown1, Gdown3, Gdown4 and/or Gdown6. As the primer pairs P1/P5 and P1/P3 were designed to only amplify combined genes, we could not find the combined genes in the heart and in the brain.

Online Figure II: EST blast analyses of the transcripts Gcom1, Gcom2, Myozap (Gup1), and Gup2

A&B, when blasting the Gcom sequences against NCBI’s EST database, both combined transcripts lack overlapping ESTs (101 blast hits each), even in other tissues than heart (e.g., brain). C, in contrast to that, Myozap (Gup1, 100 blast hits) shows an evenly distributed pattern of EST over the whole range of its transcript. D shows the blast results of the Gup2 isoform. Only one EST (out of 103) lacking the exon which defines the Gup2 compared to Myozap is present.
Online Figure III: A MEF2 luciferase reporter gene is not activated by Myozap.

No substantial activation of the MEF2 reporter by Myozap could be observed. MRIP3 did not show strong inhibitory effects on the reporter gene activity.

Online video I:
Normal skeletal muscle function in control morpholino injected zebrafish embryos.

Online video II:
Severe skeletal muscle dysfunction in MO2-Myozap injected embryos.

Online video III:
Normal cardiac performance in control morpholino injected zebrafish embryos.

Online video IV:
Progressive loss of cardiac contractility in MO2-Myozap injected embryos.
# Online Figure I

### A

| Exons      | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25-28 |
|------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|     |
| Gup1       |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gup2       |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom1      |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom2      |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom3      |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom4      |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom5      |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom6      |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom7      |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom8      |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom9      |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom10     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom11     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom12     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gcom13     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gdown1     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gdown2     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gdown3     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gdown4     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gdown5     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gdown6     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| Gdown7     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |

### B

Heart

Brain
Online Figure III

Mef2A-luc + + + + + + +
Myozap - ++
MRIP3 - - -