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Raver1 is an integral component of muscle contractile elements

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Abstract

Raver1, a ubiquitously expressed protein was originally identified as a ligand for metavinculin, the muscle specific isoform of the microfilament-associated protein vinculin. The protein resides primarily in the nucleus, where it colocalizes and may interact with the polypyrimidine-tract binding protein PTB, which is involved in alternative splicing processes. During skeletal muscle differentiation, raver1 translocates to the cytoplasm and eventually targets to the Z-line of sarcomeres. Here, it colocalizes with metavinculin, vinculin and alpha-actinin, all of which have biochemically been identified as raver1 ligands. To obtain more information on the potential role of raver1 in muscle structure and function, we investigated its distribution and fine localization in striated and smooth muscle of mouse. Three monoclonal antibodies that recognize epitopes in different regions of the raver1 protein were employed in immunofluorescence and immuno electron microscopy. Our results show that cytoplasmic accumulation of raver1 is not confined to skeletal muscle, but occurs also in heart and smooth muscle. Unlike vinculin and metavinculin, cytoplasmic raver1 is not restricted to costameres, but also represents an integral part of the sarcomere. In isolated myofibrils and in ultrathin sections of skeletal muscle, raver1 was found concentrated at the I-Z-I band. In addition, a minor fraction of raver1 was present in the nuclei of all three types of muscle. These data indicate that during muscle differentiation raver1 might link gene expression with structural functions of the contractile machinery of muscle.

Introduction

Muscle contraction critically depends on the precise interplay of myofibrils anchored to and associated with structural as well as regulatory proteins. This is best demonstrated for striated muscle, where the contractile apparatus is organized into regular repeating units, the sarcomeres, whose integrity is essential for proper muscle function. Metabolic labelling and transfection studies have shown that fully differentiated muscle, in spite of its rigid appearance, is a highly dynamic structure with a constant turnover of individual components albeit at different rates (Zak et al. 1977; Martin 1981; Michele et al. 1999; Littlefield et al. 2001). Even proteins previously perceived as static components of the Z-disc such as alpha-actinin or myotilin are in a continuous exchange with a cytoplasmic pool (Wang et al. 2005). Furthermore, muscle is able to respond to changes in mechanical strain by altering the equilibrium between protein synthesis and degradation (Janmey 1998; Ruwhof and van der Laarse 2000; Russell et al. 2000). However, it is still poorly understood how the turnover of individual components is achieved while muscle function is being maintained.

Some of the muscle proteins exhibit a dual residency, at the sarcomere and in the nucleus. They have been suggested to function as messengers that link gene expression to myofibril signalling pathways. Indeed, there is increasing evidence that sarcomeric constituents may appear at different subcellular locations in response to different stimuli, both within the sarcomere as well as in other cellular compartments, such as the nucleus [reviewed in (Clark et al. 2002; Lange et al. 2006)]. These findings imply that the sarcomere not only provides the structural basis for muscle contraction, but also serves as a communicative platform where signals are both, perceived and transmitted, allowing for functional plasticity of muscle tissue. A candidate messenger protein in muscle differentiation or maintenance is raver1 (Fig. 1A), a ubiquitously expressed 80 kDa protein that contains three RNA-recognition motifs (RRMs) with a high sequence homology to members of heterogeneous ribonucleoprotein family (Huttelmaier et al. 2001; Jockusch et al. 2003). In most cell types, raver1 is found in the

nucleus, but displays nucleocytoplasmic shuttling presumably mediated by two nuclear localization signals at the N- and C-termini and a putative export sequence located within the central leucine-rich region. In the nucleus, raver1 may associate with another hnRNP, the polypyrimidine tract binding protein (PTB), a potent regulator of alternative splicing of several genes (Valcarcel and Gebauer 1997). Direct binding of raver1 to PTB has been demonstrated *in vitro* and both proteins colocalize in the perinucleolar compartment (PNC), an RNA-dependent structure that is characterized by the accumulation of PTB (Ghetti et al. 1992). Furthermore, raver1-PTB complexes can be immunoprecipitated from vertebrate cells (Huttelmaier et al. 2001). While direct RNA binding of raver1 has yet to be demonstrated, the protein may assist in PTB-mediated splicing events in smooth muscle (Gromak et al. 2003; Spellman et al. 2005). Apart from its association with PTB in the nucleus, raver1 interacts with the microfilament-associated proteins vinculin, its muscle-specific splice variant metavinculin and alpha-actinin (Huttelmaier et al. 2001). In fact, raver1 was originally identified through its interaction with metavinculin in a yeast two hybrid screen. During myotube formation of C2C12 cells, nuclear raver1 was shown to translocate to the cytoplasm and eventually associate with myofibrils at the position of the Z-line. In differentiated skeletal muscle, raver1 colocalized with its cytoplasmic ligands (meta)vinculin and alpha actinin at the region of sarcomeric Z-lines and costameres (Huttelmaier et al. 2001).

To gain further information on raver1 and its role in muscle differentiation, we embarked on a detailed analysis of the localization of raver1 in skeletal, heart and smooth muscle of the mouse. To this end, we generated and characterized three monoclonal antibodies against different regions of the raver1 protein and employed them in immunofluorescence and immuno electron microscopy. Our data reveal that raver1 is associated with cytoplasmic contractile elements in all three muscle types. Furthermore, raver1 is not restricted to costameres, but the protein is a prominent component of the I-Z-I region in striated muscle

and the intercalated disc in heart. In addition, nuclear localization of raver1 was noted for all tissues examined.

Materials and methods

Raver1 Antibodies

Three monoclonal antibodies of the IgG type were generated. The antibody 5G6 was raised against an N-terminal fragment of raver1 (Huttelmaier et al. 2001). In the present study, the epitope of this antibody was mapped by pepscan analysis. Peptides (15-mers) of the raver1 sequence with an overlap of twelve amino acids between consecutive peptides were spot-synthesized on a cellulose membrane (Frank and Overwin 1996) and analyzed for antibody binding as described (Mayboroda et al. 1997). Two additional antibodies (5D5 and 7D3) were raised against a C-terminal peptide (EPSPEGSY, corresponding to amino acids 718-727) of the protein, following standard protocols. The antibodies obtained were tested in solid phase binding assays (enzyme-linked immunosorbent assay) on recombinant protein and in immunoblots using total extracts of HeLa and C2C12 cells subjected to SDS-PAGE. HRP-coupled goat anti-mouse antibodies (Sigma) were used as secondary antibodies in both assays. The raver1 antibodies were purified from hybridoma culture supernatants by affinity chromatography using anti-mouse IgG agarose (Sigma) columns. Binding and elution of antibodies was performed according to the supplier's protocol. 500 µl fractions were collected and immediately neutralized to pH 7.0 with 1M Tris pH 9.5. Aliquots were analyzed by SDS-PAGE. Fractions containing high antibody concentrations were pooled and dialyzed against PBS overnight. The concentration of purified antibody was calculated from the absorption at OD280 nm using an extinction coefficient of 1.35.

Cryosectioning and immunofluorescence labelling

Freshly isolated murine tissues (*extensor digitorum longus*, heart, uterus, urinary bladder) were mounted on egg white and cork (Jockusch and Voigt 2003) with Polyfreeze tissue-freezing medium (Polysciences Inc.). To prevent contraction, muscles were fixed with minuten pins. Specimens were frozen in melting isopentane, prechilled in liquid nitrogen and stored at -80°C. They were sectioned (5-10 µm) in a cryostat microtome at -20°C, mounted on glass slides and dried for at least 30 min. After washing with PBS, the sections were briefly (10-20 s) incubated with 1% Triton X-100 and then fixed with 4% formaldehyde for 20 min. After additional washing steps, they were permeabilised with 0.2% Triton X-100 at room temperature (RT) for 30 min. For indirect immunofluorescence, unspecific background labelling was first quenched by incubation with 5% bovine serum albumin and 0.25 mg/ml Fab fragments (goat anti-mouse IgG, Dianova) in PBS at RT for 1 h. The sections were then washed in PBS and incubated with the monoclonal raver1 antibodies (cell culture supernatants) at RT for 1 h, washed three times in PBS and then incubated with goat anti-mouse Cy3 (diluted 1:200, Jackson Immuno Research). Samples were counterstained for F-actin and DNA with FITC-conjugated phalloidin (Sigma) and DAPI, respectively. Finally, samples were extensively washed in PBS, mounted in Mowiol, and examined with a Zeiss Axiophot microscope equipped with epifluorescence optics.

Preparation and staining of myofibrils

Skeletal muscles were dissected from adult mice and incubated in Ringer's solution (100 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, 10 mM NaH₂PO₄, 0.1% glucose, 20 µM leupeptin, pH7.4) on ice for 30 min. The tissue was transferred to rigor buffer (100 mM KCl, 2 mM MgCl₂, 2 mM EGTA, 0.3 mM DTT, 20 µM leupeptin pH 7) using 5 ml per 0.5 g of tissue. After homogenisation using a dounce homogeniser, samples were incubated for 10 min on ice under constant agitation, centrifuged at 1,500 xg for 30 min and resuspended in 10 volumes of ice-

cold rigor buffer. These steps were repeated three times. Isolated myofibrils were stored in rigor buffer containing 50% glycerol at -20°C until use. For immunofluorescence, the myofibrils were allowed to adhere to glass slides for 1 min, rinsed with PBS and fixed with 4% formaldehyde for 10 min. After washing the samples were blocked with 5% bovine serum albumin in PBS at RT for 2 hours, to reduce background labelling and stained for immunofluorescence as described above. Sarcomeric alpha-actinin was stained using a monoclonal antibody (Sigma).

Immuno electron microscopy

For immunogold labelling, ultrathin sections were mounted on 200 mesh carbon/parlodion-coated copper grids. After blocking unspecific binding sites by two 5 minute incubations in 2% BSA/PBS, grids were incubated on droplets of raver1 antibody solutions in a moist chamber at RT for two hours. The optimal antibody concentrations were determined by titration experiments. For direct labelling, purified raver1 antibodies were conjugated to colloidal gold (Slot and Geuze 1985). For indirect labelling, grids were rinsed with PBS after primary antibody incubation, blocked with 2% BSA in PBS for two times 5 min, and then incubated with a secondary 10 nm gold-conjugated goat anti mouse IgG (BBInternational) for one hour at room temperature. After washing in PBS and dH₂O, grids were stained with 6% uranyl acetate for 1 hour, rinsed with dH₂O and then counterstained with lead-citrate for two minutes. For control experiments the primary antibody was omitted (data not shown). Stained specimens were examined in a Hitachi H7000 transmission electron microscope operated at 100 kV at 20.000x magnification. Micrographs were recorded on Kodak® electron image film SO-163.

Gel electrophoresis and immunoblotting.

Total protein extracts from C2C12 and HeLa cells were obtained as described (Huttelmaier et al. 2001). Equal amounts of total protein were loaded onto 10% polyacrylamide gels. After electrophoresis, samples were transferred onto nitrocellulose membranes and analyzed for raver1 proteins following standard procedures as described (Huttelmaier et al. 2001).

Recombinant protein expression and purification

Recombinant raver1 proteins were expressed using the Bac-to-Bac™-system according to the manufacturer's (Invitrogen) protocol. Briefly, cDNAs encoding full-length raver1, raver1ΔC (amino acids 1-441) and raver1ΔN (amino acids 442-748) were cloned into a modified pFastBac™ donor plasmid harbouring an additional FLAG tag sequence downstream of the histidine tag and transformed into *E.coli* DH10Bac cells. Recombinant bacmid DNA was isolated and transfected into sf9 (*Spodoptera frugiperda*) cells. The virus-containing supernatant was harvested and used for the infection of High Five™ insect cells. Cells were collected by centrifugation at 500 xg for 5 min, washed with PBS containing leupeptin (20 μM) and stored at -70°C. His-tagged proteins were purified from insect cells according to the manufacturer's instructions (Qiagen) with slight modifications as described (Witt et al. 2004). Fractions containing the protein of interest were dialyzed against storage buffer (50 mM sodium phosphate buffer, pH 7.2, containing 0.2mM EGTA, 4 μM pepstatin A, 40 μM leupeptin, Trasylol® (2.8mg/ml in H₂O) and 14.3 mM β-mercaptoethanol) and stored on ice until use.

Results

Characterization of raver1 antibodies

In the present study three monoclonal antibodies, directed against different regions in the raver1 sequence (Fig. 1A) were employed to analyze the subcellular distribution of raver1 protein in muscle. The 5G6 antibody was raised against a recombinant MBP-tagged N-terminal raver1 fragment (amino acids 1-625) for the initial characterization of raver1 (Huttelmaier et al. 2001). We mapped the epitope of this antibody by pepscan analysis (Frank and Overwin 1996) and found that it recognizes a series of four 15mer peptides (Fig. 1B). From the minimal sequence overlap the epitope ETVDRR was deduced, which corresponds to amino acids 25-30 of raver1. The antibodies 5D5 and 7D3 were raised against a C-terminal synthetic peptide (EPSPEGSY, comprising amino acids 718-727) and specifically recognized this peptide in ELISA and immunoblot assays (data not shown).

All three antibodies were tested for their reactivity with recombinant raver1 proteins in immunoblot assays (Fig. 1C and data not shown). Full length raver1 and the two complementary deletion constructs raver1 Δ C (amino acids 1-441) and raver1 Δ N (amino acids 442-748) were purified from insect cells and subjected to SDS-PAGE (left panel), revealing major protein bands of 80 kDa for full-length raver1, approximately 51 kDa for raver1 Δ C and approximately 35 kDa for raver1 Δ N. Degradation was only noted for the full-length protein. In immunoblots, antibody 5G6 recognized full-length raver1 and its proteolytic fragments as well as the N-terminal fragment raver1 Δ C (centre panel). In contrast, antibodies 5D5 and 7D3 bound to the C-terminal construct raver1 Δ N in addition to the full length protein, but failed to detect any degradation products (right panel and data not shown). This indicates that the full-length protein is degraded from its C-terminus.

When tested on cell extracts (Fig. 1D), all three antibodies recognized a protein band of approximately 80 kDa in murine C2C12 cells. In (human) HeLa cells, however, endogenous

raver1 protein was only detected by the C-terminal antibodies (right panel and data not shown). Consistent with this finding, a comparison of known raver1 sequences reveals that the 5G6 epitope is not highly conserved among vertebrate species, whereas the epitope recognized by 5D5 and 7D3 is present in man, mouse, rat and *Xenopus* raver1 (Fig. 1E).

Identification of raver1 as a myofibril component

In striated muscle, the raver1 ligand vinculin is restricted to attachment sites of the contractile apparatus to the sarcolemma, such as costameres and intercalated disks (Tokuyasu et al. 1981; Koteliensky and Gneushev 1983; Pardo et al. 1983; Witt et al. 2004), whereas alpha-actinin, another raver1 binding partner, is additionally present in the Z-disc (Tokuyasu et al. 1981; McKenna et al. 1986). Thus, we wanted to determine whether raver1 is confined to the costamere or constitutes an integral part of sarcomeric structures. Frozen longitudinal sections of skeletal muscle from adult mice (*Extensor digitorum longus*) yielded an identical staining pattern with all three raver1 antibodies (Fig. 2 and data not shown). Raver1 was present as a doublet in the I-Z-I region, which was identified in the corresponding phase contrast image. Notably, these doublets could only be seen in well relaxed or stretched muscle. In contracted muscle, raver1 appeared as a single band colocalizing with the Z-line, which may account for the staining observed in our previous investigation (Huttelmaier et al. 2001). To further ascertain the localization of raver1 in muscle, myofibrils were isolated from murine skeletal muscle and labelled with raver1 antibodies yielding identical staining pattern (Fig. 3A and data not shown). Again, raver1 was present as a doublet flanking the Z-disc on both sides as was evident from comparison with the corresponding phase contrast images (left panels) and staining of myofibrils for alpha-actinin (Fig. 3B) or actin (data not shown).

The sarcomeric localization of raver1 was further examined by immuno electron microscopy (Figs. 3C and D). Gold particles were mainly distributed along the Z-disc in the I-Z-I region,

but were occasionally also found at the M-line (Fig. 3D). Additionally, sections including the nucleus revealed raver1 also as an intranuclear component (Fig. 3C).

Localization of raver1 in cardiac muscle

To study the distribution of raver1 in cardiac muscle, immunolabelling experiments were performed on murine ventricular heart tissue (Fig. 4). Frozen sections were stained with 5G6 (Figs. 4A) and 7D3 (Fig. 4B). In longitudinal sections, a distinct sarcomeric banding pattern was observed that colocalized with the Z-discs of cardiomyocytes (Fig. 4A). Distinct doublets were not as well resolved as in skeletal muscle (compare inset in Fig. 4A with Fig. 2). In addition, a strong raver1 signal was also present at the intercalated discs connecting cardiomyocytes (arrowheads in Fig. 4A). Raver1 staining in cardiomyocyte nuclei was fairly weak (asterisks in Fig. 4B) and often obscured by the sarcomeric signals. A similar reduction of nuclear signal intensity has already been described during the differentiation process of skeletal muscle (Huttelmaier et al. 2001). Ultrastructural analyses by immuno electron microscopy with 7D3 confirmed the dual residency of raver1 (Figs. 4C, D). Immunogold labelling was prominent at the I-Z-I region (Fig. 4C) and in the nucleus (Fig. 4D). These data reveal that the localization of raver1 at the sarcomeric boundary and in the nucleus is a common feature of striated muscle.

Analysis of raver1 distribution in smooth muscle

We also investigated the subcellular distribution of raver1 in smooth muscle, where the contractile apparatus is not organized in sarcomeres. Sections of murine urinary bladder (Fig. 5A) and uterus (Fig. 5B) were analyzed with 7D3 or 5D5, respectively. The smooth muscle cells of the urinary bladder (Fig. 5A, upper left panel) were readily distinguished from adjacent connective tissue by strong cytoplasmic staining for raver1 (upper right panel), and by the bright staining of filamentous actin by phalloidin (lower left panel). Comparison of

these images with DAPI staining (lower right panel) clearly showed that raver1 was also present smooth muscle nuclei (arrows). Increased raver1 staining was also noted for the smooth muscle layer of blood vessels (boxed areas). Sections of the uterine smooth muscle (Fig. 5B, upper left panel) confirmed the presence of raver1 in myofibrils (upper right panel) which were identified by phalloidin staining (lower left panel) and the characteristic elongated shape of their nuclei (lower right panel). The presence of raver1 in smooth muscle myofibrils is evident in Fig. 5B, but the resolution of the immunofluorescence images was not good enough to claim unambiguously its presence in smooth muscle nuclei.

At the ultrastructural level, the distribution of raver1 was examined in ultrathin sections of urinary bladder labelled with 5D5 (Fig.6). Indirect immunogold labelling revealed that raver1 was present in both, nuclei and cytoplasm. In some instances, an association of raver1 with cytoplasmic filamentous structures that could represent microfilaments was observed.

Discussion

We have previously observed that during skeletal muscle differentiation the hnRNP-like protein raver1 translocates from the nucleus to microfilament attachment sites in differentiated skeletal muscle at the sarcolemma (Huttelmaier et al. 2001). Based on these findings, we analyzed the subcellular distribution of raver1 in skeletal muscle in more detail and expanded our investigation to heart and smooth muscle. We demonstrate that the cytoplasmic accumulation of raver1 and its association with contractile elements is a common feature of all three types of muscle. In addition, a minor fraction of the protein was always present in the nucleus. A detailed analysis of stretched myofibrils and ultrathin sections of skeletal muscle showed that raver1, like alpha-actinin but unlike vinculin, is not restricted to the costameres. Instead, it is also an integral sarcomeric component, mainly localized in the I-Z-I region. In heart muscle, raver1 was also found as a prominent component of intercalated

discs, which are also rich in vinculin, metavinculin and alpha actinin (Tokuyasu et al. 1981; Witt et al. 2004).

Immunolabelling of myofibrils (Fig. 3) revealed that the distribution of raver1 does not exactly correspond to that of its ligand alpha-actinin. As expected, the latter is strictly confined to the Z-disc (Tokuyasu et al. 1981; McKenna et al. 1986), whereas raver1 flanks this structure being thus mainly located in the I-regions. This partial overlap *in situ* may either reflect a true differential localization of the two proteins, or, alternatively, it could be the consequence of steric hindrance of antibody binding caused by additional proteins. *In vitro*, raver1 directly interacts with vinculin and alpha-actinin and complexes containing all three proteins can be recovered from cell extracts by immunoprecipitation (Huttelmaier et al. 2001). Based on our immunolabelling analyses, a direct interaction between raver1 and alpha actinin *in situ* might be possible, but cannot be proven.

As revealed by immuno electron microscopy, raver1 was occasionally also found at the M-band (Figs. 3 and 4). Different locations within the sarcomere have also been described for other proteins such as obscurin (Young et al. 2001) and FHL2/DRAL (Lange et al. 2002), but at present it is not clear whether translocations of such proteins occur within the sarcomere, or whether there are distinct subpopulations of these components present at different sites (Lange et al. 2006).

The dual localization of raver1 in the nuclear and sarcomeric compartment of muscle, together with its structural organization, based on which it was identified as a member of a protein family engaged in RNA modification, suggests that raver1 may also have a dual function. Since raver1 harbours three RRM and affects PTB-mediated RNA splicing *in vitro* (Gromak et al. 2003) it has been speculated that raver1 could be involved in processing and delivery of specific mRNAs to the sarcomere and thus mediate site-specific synthesis of sarcomeric proteins (Jockusch et al. 2003). Such sarcomeric localization has been reported for the mRNAs of vimentin, desmin and vinculin (Morris and Fulton 1994). However, direct

RNA binding of raver1 remains to be shown. Alternatively, the RRM s may act as binding modules mediating protein-protein interactions and could be involved in vinculin binding (Huttelmaier et al. 2001).

However, other possibilities need to be considered. It is commonly accepted that the functional plasticity of muscle critically depends on intracellular communicators which translate mechanical signals into altered protein activities or gene expression profiles. For example, a signalling complex involving the kinase domain (TK) of the giant sarcomeric protein titin regulates nuclear translocation of MURF-2. Nuclear MURF-2 interacts with the transactivation domain of serum response factor (SRF), which indirectly leads to suppression of SRF-dependent muscle gene expression (Lange et al. 2005). MURF-2 thus represents a member of a constantly growing, heterogeneous group of proteins that may translocate from the sarcomere to the nucleus to perform a sensory or messenger function. Other examples of such proteins are for example myopodin (Weins et al. 2001; Sanchez-Carbayo et al. 2003), myopalladin (Bang et al. 2001) and CARP (Jeyaseelan et al. 1997; Bang et al. 2001; Miller et al. 2003). However, for most proteins the physiological consequences of these translocations remain to be shown (Lange et al. 2006).

At present, we cannot determine whether raver1 exerts primarily structural, transport, signalling or regulatory functions in muscle. Raver1 knock-out mice are viable and fertile and myofibrils isolated from these animals are morphologically indistinguishable from their wild type counterparts (B. Kleinhenz and H.H. Arnold, personal communication). Even though we recently identified a raver1-related protein (raver2, Kleinhenz et al. 2005) the possibility of mere functional redundancy is rather unlikely, since raver2 is not expressed in muscle tissue. Furthermore, genetic analyses do not indicate any other raver isotypes. In summary, these facts argue against a purely structural function of raver1. Apparently, the protein is not required for initial sarcomere formation and maintenance. However, the possibility remains that raver1 in costameres and intercalated disks contributes to proper muscle function by

modulating either the molecular architecture of microfilament attachment sites or signalling pathways that target these attachment sites. Further studies, involving physiological analyses of animals, muscle and isolated myofibrils on raver1-depleted mice are required to clarify these issues.

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Figure legends

Figure 1: Characterization of monoclonal antibodies against different raver1 epitopes.

(A) Schematic representations of full-length raver1 and two complementary deletion constructs used in this study. The first and last amino acids of each construct are given. RNA recognition motifs are shown as black boxes, nuclear localization signals are indicated in grey. A central leucine-rich region (putative NES) is depicted as a hatched box. Arrows indicate the binding positions of the monoclonal antibodies 5G6, 5D5 and 7D3. (B) The epitope recognized by 5G6 comprises amino acids 25-30 of raver1 as revealed by pepscan analysis. (C) Reactivity of the raver1 antibodies with recombinant raver1 and its deletion fragments (SDS-PAGE, left panel) in immunoblots. 5G6 (centre panel) recognizes full-length raver1, a bouquet of its degradation products and raver1 Δ C, while 7D3 (right panel) and 5D5 (not shown) react with full length raver1 and raver1 Δ N. (D) Reactivity of the raver1 antibodies with cell extracts. Equal amounts of proteins from murine C2C12 and human HeLa were subjected to SDS-PAGE, blotted and incubated with 5G6 (left), 5D5 (right) and 7D3 (not shown). While the C-terminal antibodies recognize raver1 protein in samples of both cell types, 5G6 detects only murine raver1. (E) Part of the amino acid sequence of raver1 comprising the epitopes for 5G6 (left) and 7D3/5D5 (right) from different species. Note that the C-terminal sequence is highly conserved among vertebrate raver1 proteins, whereas the 5G6 epitope is only conserved in mouse and rat.

Figure 2: Localization of raver1 in skeletal muscle of the mouse (*Extensor digitorum longus*).

Immunofluorescence analysis of frozen sections (A, B left panels) stained for raver1 with 5G6 (A) 5D5 (B) and 7D3 (not shown) revealed a striated pattern for each antibody. Higher magnifications (insets) of well stretched muscle tissue (boxed regions) suggest that raver1 is localised as a doublet embracing the Z-lines. Bars: 10 μ m.

Figure 3: Identification of raver1 as an integral component of skeletal muscle myofibrils.

In phase contrast images of myofibrils isolated from mouse skeletal muscle (A and B, left panels), I-bands (arrows) and Z-lines (arrowheads) are well discernible. Immunostaining of myofibrils for raver1 (5G6, A) and alpha-actinin (B) reveals that raver1 concentrates as a doublet in the I-Z-I region whereas alpha-actinin clearly marks the Z-line. Bars: 5 μ m. (C, D). Electron micrographs of ultrathin sections of mouse skeletal muscle directly labelled with 10 nm gold-conjugated raver1 antibody 5G6 (C) and 7D3 (D) show prominent labelling of the I-band with some accumulation in the vicinity of the Z-line. Fewer gold particles are also found at the M-band (M in D) and within the nucleus (C). In both pictures, gold is enlarged and pseudocoloured red. Bars: 200 nm.

Figure 4: Subcellular localization of raver1 in cardiomyocytes. Frozen longitudinal sections (A, B) of mouse heart muscle were analysed for raver1 by 5G6 (A) and 7D3 (B) and counterstained with either FITC-conjugated phalloidin (A) or DAPI (B) to visualize the filamentous actin cytoskeleton or nuclei, respectively. Left panels (A, B) represent the corresponding phase contrast images. (A, B centre panel): Raver1 is present in a striated pattern in cardiac sarcomeres (inset in A represents a higher magnification of the boxed area) and in intercalated discs (arrowheads in A). A comparably weak raver1 signal which is mainly obscured by sarcomeric striations is detected in the centrally located nuclei of cardiac cells (asterisks in B, placed immediately right to nuclei) which were counterstained by DAPI (right panel). Bars: 10 μ m. (C, D) Ultrathin sections of neonatal rat heart muscle, indirectly labelled with 7D3 and 10 nm gold (enlarged and pseudocoloured red). The sarcomeric (C, D) and nuclear (D) localization of raver1 seen at the light microscopic level is confirmed. M: M-band, N: nucleus, Z: Z-line, Bars: 200 nm.

Figure 5: Immunolocalization of raver1 in smooth muscle. (A) Urinary bladder. The phase contrast image (upper left) shows smooth muscle (left), identified by the intense staining of filamentous actin by phalloidin (left lower panel) and connective tissue (right), separated by the dotted line. The position of a blood vessel is indicated (boxed area). Immunostaining with 7D3 (upper right) reveals a cytoplasmic concentration of raver1 in the smooth muscle layers of the urinary bladder and the blood vessel. Less prominent raver1 staining was observed in smooth muscle nuclei (arrows) identified by DAPI staining (lower right). Cells of the connective tissue show primarily a nuclear localization of raver1. (B) Mouse uterus. The smooth muscle fibres seen in the phase contrast image (upper left) and recognized by their fibrillar actin staining with phalloidin (lower left) are intensely stained for raver1 with 5D5. Nuclei were identified by DAPI staining (lower right), but a nuclear localization of raver1 could not be determined at the resolution of such images. Bars: 20 μ m.

Figure 6: Immuno electron microscopic analysis of raver1 in smooth muscle tissue. (A and B) Ultrathin sections of smooth muscle from mouse urinary bladder (A) and mouse aorta (B) were labelled with 5D5 and a 10 nm gold-conjugated secondary antibody (enlarged, pseudocoloured red). Raver1 is seen present in the nuclei (N) and the cytoplasm (C) of both types of smooth muscle cells. Bars: 200 nm

A

B

AS 16 – 30: E A E A E A E T P E T V D R R

AS 19 – 33: A E A E T P E T V D R R A P E

AS 22 – 36: E T P E T V D R R A P E Q E L

AS 25 – 39: E T V D R R A P E Q E L P P L

C

D

E

5G6 5D5/7D3

H. sapiens VEAGDAAE**RR**AP**EE** LPSP**EP**SP**EG**SY**VG**QH**SQ**

M. musculus AET**PET**VDR**RR**AP**EG** LPSP**EP**SP**EG**SY**VG**GH**SG**

R. norvegicus AET**PET**VDR**RR**T**PEQ** LPSP**EP**SP**EG**SY**VG**GH**SQ**

X. laevis ESRGWRSE**RE**AV**ES** IPS**P**EP**SP**EP**GV**Y**IG**QH**SQ**

A

B

Fig. 2 Zieseniss et al.

A

B

C

D

Fig.3 Zieseniss et al.

A

B

C

D

Fig. 4 Zieseniss et al.

A

B

Fig. 5 Zieseniss et al.

A

B

Fig. 6 Zieseniss et al.