Molecular determinants for the recruitment of the ubiquitin-ligase MuRF-1 onto M-line titin

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ABSTRACT Titin forms an intrasarcromeric filament system in vertebrate striated muscle, which has elastic and signaling properties and is thereby central to mechanotransduction. Near its C-terminus and directly preceding a kinase domain, titin contains a conserved pattern of Ig and FnIII modules (IgA168-IgA169-FnIIIA170, hereby A168-A170) that recruits the E3 ubiquitin-ligase MuRF-1 to the filament. This interaction is thought to regulate myofibril turnover and the trophic state of muscle. We have elucidated the crystal structure of A168-A170, characterized MuRF-1 variants by circular dichroism (CD) and SEC-MALS, and studied the interaction of both components by isothermal calorimetry, SPOTS blots, and pull-down assays. This has led to the identification of the molecular determinants of the binding. A168-A170 shows an extended, rigid architecture, which is characterized by a shallow surface groove that spans its full length and a distinct loop protrusion in its middle point. In MuRF-1, a C-terminal helical domain is sufficient to bind A168-A170 with high affinity. This helical region predictably docks into the surface groove of A168-A170. Furthermore, pull-down assays demonstrate that the loop protrusion in A168-A170 is a key mediator of MuRF-1 recognition. Our findings indicate that this region of titin could serve as a target to attempt therapeutic inhibition of MuRF-1-mediated muscle turnover, where binding of small molecules to its distinctive structural features could block MuRF-1 access.—Mrosek, M., Labeit, D., Witt, S., Heerklotz, H., von Castelmur, E., Labeit, S., Mayans, O. Molecular determinants for the recruitment of the ubiquitin-ligase MuRF-1 onto M-line titin


Key Words: elastic filament titin • muscle atrophy • X-ray crystallography • binding studies

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proteins could form stable assemblies on the titin filament. MuRFs are muscle-specific proteins with a RING-finger/B-box/coiled-coil tripartite fold known as TRIM (Fig. 1B) (10). Although three MuRF isoforms (MuRF-1, -2, -3; encoded by different genes; 9) have been identified to date, only MuRF-1 has been shown to interact directly with titin. MuRF-1 is an E3 ubiquitin-ligase that becomes up-regulated during muscle loss and has been proposed as a molecular mediator of muscle atrophy (11). Muscle atrophy is characterized by an accelerated protein degradation via the ubiquitin proteasome system and it is an associated condition to numerous diseases, including cancer, diabetes and AIDS, as well as physiological states such as long-term immobilization and microgravity. A role of MuRF-1 in muscle turnover has been confirmed by a knockout mouse model that showed increased resistance to muscle atrophy (11), which suggests the potential pharmacological relevance of this protein. Yet, the cellular function of MuRF-1 might be complex to modulate, since it interacts with a broad range of cellular factors, including the ubiquitin-like modifier SUMO-3 (12), isopeptidase T-3, and the SUMO E2 ligase Ubc9 (8), which thereby might exert their influence on the titin cytoskeleton. Furthermore, nuclear forms of MuRF-1 in muscle turnover has been confirmed by a knockout mouse model that showed increased resistance to muscle atrophy (11), which suggests the potential pharmacological relevance of this protein. Yet, the cellular function of MuRF-1 might be complex to modulate, since it interacts with a broad range of cellular factors, including the ubiquitin-like modifier SUMO-3 (12), isopeptidase T-3, and the SUMO E2 ligase Ubc9 (8), which thereby might exert their influence on the titin cytoskeleton. Furthermore, nuclear forms of MuRF-1 interact with the transcription regulator GMEB-1 (8). Taken together, current functional data on MuRF-1 suggest its involvement in turnover and muscle gene expression, possibly forming part of mechanotransduction pathways in control of the titin composition of the sarcomere which, in turn, determines mechanical stiffness. Thus, in order to gain an insight into the structure of M-line titin and its recruitment of MuRF-1, we have elucidated the crystal structure of A168-A170 and interpreted it in the context of its binding to MuRF-1. Our findings aid the understanding of the molecular determinants of the interaction and point to a therapeutic interest of this region of titin.

**Materials and Methods**

**Cloning**

For crystallographic studies, A168-A170 from human titin (residues 24431–24731, EMBL X90568) was cloned into the pETM-11 vector (EMBL collection) at restriction sites NcoI and MluI. pETM-11 is a variant of pET-24d (Novagen, Madison, WI, USA), which includes an N-terminal His6-tag and a TEV (tobacco etch virus) protease cleavage site prior to the inserted gene. Pull-down assays used A168-A170 cloned in pETM-44 (EMBL collection), which provided the N-terminal fusion of maltose binding protein (MBP) to the titin fragment. A mutated version of this construct, where the 102KTLE105 motif had been replaced by AAAA, was obtained using the QuikChange protocol (Stratagen, La Jolla, CA, USA). Fragments from MuRF-1 (Swiss-Prot Q969Q1), MuRF-1(166–341) and MuRF-1(166–315), were inserted in pETM-11 via NcoI and KpnI restriction sites. Both constructs correspond to a C280S mutated variant designed to prevent unspecific aggregation of the samples due to oxidation during storage.

**Protein Production**

Overexpression of A168-A170 for structural analysis was carried out in *E. coli* BL21 (DE3) Rosetta (Novagen). Cultures were grown at 37°C up to an OD600 of 0.6 in Luria Bertani medium supplemented with 25 μg/ml kanamycin and 34 μg/ml chloramphenicol. Expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cultures were further grown at 25°C for 18 additional hours. Cells were harvested by centrifugation at 2800 × g and 4°C. Bacterial pellet was resuspended in 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and DNase I. Lysis was by addition of lysozyme and sonication. The homogenate was clarified by centrifugation at 15000 × g and 4°C. The supernatant were loaded onto a Ni2+-chelating HisTrap column (GE Healthcare) equilibrated in lysis buffer and eluted using 250 mM imidazole. The eluent was dialyzed against 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and DNase I. Lysis was by addition of lysozyme and sonication. The homogenate was clarified by centrifugation at 15000 × g and 4°C. The supernatant was dialyzed against 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; and 2 mM DTT in the presence of TEV protease. Given that A168-A170 still interacted with the Ni2+-chelating resin after tag removal, the digested mixture was further purified by gel filtration on...
a Superdex 200 Hiload 16/60 PG column (GE Healthcare) equilibrated in dialysis buffer. The samples were finally dialyzed against 25 mM MOPS, pH 8.0; 150 mM KCl; 1 mM DTT; 1 mM EGTA; and stored at 4°C for further use.

MuRF-1 fragments were prepared as above, but purification of the protease digest used subtractive affinity chromatography prior to gel filtration.

Structure elucidation

Crystals grew in hanging drops at 20°C from solutions containing 2.1 M NaCl; 0.1 M MES, pH 5.5; and 0.6 M Li₂SO₄, mixed in a 1:1 ratio with a 12 mg/ml protein solution. Crystals were in the habit of long rods, hexagonal in cross section with approximate dimensions of 600 × 100 × 100 μm³ and reached their final size over a 2 month period.

For X-ray data collection, crystals were frozen at 100 K in mother liquor solutions supplemented with 20% [v/v] glycerol as cryoprotectant. X-ray data processing was in XDS (13), and data statistics and crystal parameters are given in Table 1. Crystals contained two copies of A168-A170 per asymmetric unit, which were related by an imperfect two-fold axis approximately colocalized with the crystallographic z-axis. Since attempts at phasing by molecular replacement were unsuccessful, experimental phases were obtained by SIR combined with a 3λ MAD experiment on a crystal derivatized with [Ta₆Br₁₂]²⁺ by soaking for 30 min at saturation. Derivative data statistics are given in Table 3. Anomalous scatterers (2 sites) were located using CNS (14) and phases calculated and refined in SHARP (15) (Table 3). Density modification used solvent flattening, histogram matching, and NCS domain averaging and a test set using FREERFLAG (16). Solvent molecules were built using the water-pick routine in CNS and validated visually in electron density maps. The final model includes all protein residues of A168-A170, at exception of the 12 C-terminal residues that were disordered in both NCS copies. Two N-terminal residues in positions –1 and –2, remnants of the TEV cleavage site, could be observed and are part of the final model.

Model coordinates and experimental X-ray data have been deposited at the Protein Data Bank with accession code 2NZI.

Circular dichroism

MuRF-1(166–315) was extensively dialyzed against 10 mM sodium tetraborate, pH 8.5; 20 mM NaCl; and concentrated up to 0.1 mg/ml as determined by BCA (Pierce, Rockford, IL, USA) assay. Data were acquired at 25°C on a 62A DS circular dichroism spectropolarimeter (AVIV) equipped with a temperature-controlled quartz cell of 1 mm optical path. The spectrum was recorded at a scan speed of 50 nm/min with a response time of 5 s and averaged over 8 scans. Protein conformation was calculated by fitting the experimental data with a combination of reference spectra for known secondary structure elements (18).

Size exclusion chromatography combined with multiangle light scattering (SEC-MALS)

The oligomeric state of MuRF-1(166–315) in solution was determined via SEC-MALS measurements performed on an AKTA basic system (GE Healthcare) connected to a triangle static light-scattering detector and a differential refractometer (miniDAWN Tristar and Optilab, respectively). A Superdex 200 HR 10/300 GL column (GE Healthcare) was used in 50 mM Tris, pH 8.0, and 100 mM NaCl at a flow rate of 0.7 ml/min.

### Table 1. Native X-ray data and model refinement statistics

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<td>Ramachandran plot (%)</td>
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*Calculated using PROCHECK (32). The one residue in disallowed conformation is His146B.
ml/min. Sample volumes of 100 μl were injected at a concentration of 2 mg/ml. A specific refractive index increment (dn/dc) of 0.185 ml/g was used. The data were recorded and processed using ASTRA software (Wyatt Technology, Santa Barbara, CA, USA). To determine the detector delay volumes and normalization coefficients for the light scattering detector, a BSA sample (Pierce) was used as reference.

**Isothermal titration calorimetry**

Data were recorded with a VP-ITC calorimeter (Microcal, Beverly, MA, USA). All samples were previously dialyzed against 50 mM Tris-HCl, pH 8.0, and 20 mM NaCl. Titrations consisted of 5–7 μl injections of A168-A170 concentrated to 1.4 mg/ml into MuRF-1(166–341) or MuRF-1(166–315) solutions at 1 mg/ml and 0.7 mg/ml, respectively (as determined by BCA diagnostics) used as indicated by the manufacturer.

**Binding assays**

Titin residues mediating MuRF-1 binding were revealed by using a SPOTS blot membrane (JPT, Berlin, Germany) that displayed Titin residues mediating MuRF-1 binding were revealed by using a SPOTS blot membrane (JPT, Berlin, Germany) that displayed Titin residues mediating MuRF-1 binding were revealed by using a SPOTS blot membrane (JPT, Berlin, Germany) that displayed

| TABLE 3. | Domain arrangement in poly-domain fragments from titin |  |
| --- | --- | --- | --- |
| Opening angle | Torsion | Distance | Buried surface area (Å²) |
| IgA₁₁₀⁻IgA₁₁₀ | 176° | +89° | 43.3Å | 212 (~1.4%) |
| IgA₁₁₀⁻FnA₁₁₀ | 127° | — | 40.2Å | 308 (~2.0%) |
| IgZ₁₂⁻XTAL | 146° | +75° | 49.4Å | 165 (~1.4%) |
| IgZ₁₂⁻RDC/SAXS | 136° | +85° | 44.5Å | 235 (~1.4%) |
| IgZ₁₂⁻.Tele | 167° | +48° | 52.4Å | 160 (~2.0%) |

*Angle defined by the primary axes of inertia (longitudinal) of consecutive domains. b Angle defined by the projection of secondary axes of inertia (cross sectional) of consecutive domains onto a common plane. c Distance between centers of mass of individual domains. d Calculated with AREAIMOL (33). e Crystallographic coordinates for free ZIZZ (IgZ₁₂⁻XTAL) derive from PDB entry 2A38 (3); domain arrangement of ZIZZ in solution was calculated from SAXS data and NMR residual dipolar couplings (IgZ₁₂⁻RDC/SAXS) as reported (3) and crystal coordinates of ZIZZ complexed to telethonin (IgZ₁₂⁻Tele) derive from IAY5 (4).
fectly coaxial but the FnIII domain is bent away from the molecular axis (Fig. 2A). Domain arrangements are described in Table 2. The crystal form used in this study contains two A168-A170 copies in its asymmetric unit. These are essentially identical in conformation (rmsd 0.68 Å for 288 Ca atoms of the whole molecule, calculated with SPDBV; 20), suggesting that this fragment of titin has a well-defined long-range order, and a high degree of stiffness.

Domains IgA168 and IgA169 belong to the I(intermediate)-set of Ig folds (21). They share 20% seq. id. and a high structural similarity (rmsd 0.99 Å for 82 matching Ca atoms, SPDBV). Both belong to the “N-conserved” type of Ig from titin (22), characterized by a conserved N-terminal loop cluster comprising proline residues prior to β-strand A, a PxP motif in the BC loop, and an extended FG-hairpin hosting an NxxG sequence (Fig. 3C). This Ig type predominates in the Z-disc, the skeletal I-band and the A-band fractions of titin, but not in its constitutive cardiac I-band. Domains IgA168 and FnA170 show no significant deviation from other equivalent modules across titin. However, IgA169 shows a unique 9-residue loop protrusion between β-strand A and A’ with sequence PKTLEGMGA, which adopts in part an α-helical conformation (Figs. 2A and Fig. 5A). According to structure and sequence data on titin domains, a loop insertion at this position is not detectable in any other Ig of titin.

Domain interfaces

The domains of A168-A170 form a tight tandem. They are connected through one-residue linkers in extended conformation that effectively result in the continuation of structural elements from one domain into the next. The extended arrangement of the molecule is characterized by domain interfaces with unusually small buried areas (Table 2) and engages a minimal number of residues in each domain. Both Ig-Ig and Ig-FnIII interfaces are void of specific interactions and only involve small hydrophobic clusters.

Domains IgA168-IgA169 interact through hydrophobic groups that surround the linker strand dorsally and ventrally (Fig. 3A). Given their coaxial orientation, the interacting residues originate from the linker region (V92, aliphatic portion of E93, V94) and the C-terminus of β-strand A’ (aliphatic portion of R13, Y14) in IgA168 and the conserved elements of this Ig type in IgA169, namely the FG turn (F180) and the PxP motif of the BC loop (P128, K127) (Fig. 3C). A comparison of IgA168-IgA169 and Z1Z2 (the only other Ig doublet of titin structurally characterized to date) (3, 4) reveals certain similarities. Also Z1Z2 exhibit an extended conformation, lack specific interdomain contacts, and include a small hydrophobic cluster between modules. As that of IgA168-IgA169, the cluster of Z1Z2 comprises residues C-terminal to β-strand A’, the BC loop and linker residues (Fig. 3C). Despite, Z1Z2 displays a different domain orientation (Table 2) and has a longer, three-residue linker that allows certain modular dynamics (3). It can then be concluded that the mere presence of elements characteristic of the “N-conserved” Ig type does not determine domain orientations in these doublets. Since the identified interface residues are not conserved in Ig across titin (23), it

![Figure 2. Crystal structure of A168-A170. A) Ribbon representation. The insertion loop in IgA169 is displayed in cyan. The inset shows the molecular conformation schematically; (B) Surface representation in two related views, where green emphasizes the local curvature. A ridge is present in the concave side of the molecule. The distinct loop of IgA169 is marked; (C) Docking of an α-helical peptide in coiled-coil conformation onto the surface of A168-A170 to satisfy shape complementarity. The helical peptide shown derives from lamin A coil 2B (PDB code 1X8Y).](image-url)
Figure 3. Domain interfaces. (A) Ig168-Ig169 and (B) Ig169-Fn170 interfaces. Domain color code as in Fig. 2A. Hydrophobic contributions are shown in green. Selected van der Waals surfaces are displayed. The Ca atoms of residues in the NxxG motif of the FG β-turns of both Ig169 and Fn170 are shown as spheres. Hydrogen bonds are indicated by dotted lines; (C) Structure-based sequence alignment of titin Ig and FnIII of known structure (PDB accession codes are given). β-Strand composition is shown in yellow; residues at Ig-Ig and Ig-FnIII interfaces are highlighted in red and cyan, respectively. Features characteristic of “N-conserved” Ig (A168, A169, I1, Z1, Z2 and M5) are shown in blue. I91 was formerly I27. Strand nomenclature for Ig as in (21) and for FnIII as in (31). The distinct loop of Ig169 is shown in gray; (D) A-band of titin. Ig and FnIII domains are shown as filled boxes, where Ig domains flanked by FnIII are in brown, FnIII following an N-terminal Ig in gray, other FnIII in white and Ig followed by another C-terminal Ig in blue. Fn171, the only other FnIII of titin with known structure (1BPV), is in cyan. The two residues in variable positions of the NxxG β-turn of FnIII domains are displayed. Domain super-repeats are indicated. The pattern of alternating charges is displayed, where red and blue indicate negative and positive charges, respectively.
cannot be predicted at this point how closely the observed conformations represent those of other tandems. Yet, the overall structural principles of Ig arraying revealed by these studies might be generic to the titin filament as indicated by the fact that all poly-Ig arrays of titin characterized to date by small angle X-ray scattering have revealed comparable extended arrangements independently of their domain or linker composition (22).

The interface of domains Ig\textsubscript{A169}-Fn\textsubscript{A170} exhibits a hydrophobic component as that of Ig\textsubscript{A168}-Ig\textsubscript{A169} (Fig. 3b). Similarly, contacts involve \(\beta\)-strand A' (H111) in the initial domain Ig\textsubscript{A169} and the NxxG motif of \(\beta\)-turn FG in Fn\textsubscript{A170} (K272, F273) (Fig. 3C). However, in contrast to Ig, the FG turns in FnIII of titin are highly conserved in sequence, as revealed by the alignment of 123 FnIII sequences of A-band titin (CLUSTALW; 23). This analysis showed that those FnIII domains following an Ig within the 11-domain super-repeats of the central C-zone region of A-band titin, supposedly part of the myosin-based thick filament, consistently host a conserved residue pair in the variable positions of their NxxG motif (Fig. 3D). These invariably include a charged residue followed by an aromatic group, most often tyrosine or phenylalanine. Since these residues are part of the modular interface, Ig-FnIII pairs from the C-zone of titin can be expected to exhibit a similar interdomain conformation as that of A169-A170. Strikingly, the charges within this motif are distinctly distributed along the C-zone repeats, where every first FnIII hosts a negative charge and the rest a positive group (Fig. 3D). Such segregation is not observed in the 7-domain super-repeats of the preceding D-zone (Fig. 3D). In the crystal structure, the lateral amino group of K272 is not involved in interactions, suggesting its availability for binding to other sarcomeric proteins. Myosin-binding protein C (MyBP-C) binds titin by interacting with the first Ig domain of every super-repeat in the C-zone, so that titin possibly aids thereby the regular polymerization of myosin filaments (25). Given that the FG \(\beta\)-turn of Fn\textsubscript{A170} is located toward the intermodular space, its charge might be speculated to contribute to MyBP-C docking. Interestingly, the FG turns of FnIII domains at other positions within repeats of the D- and C-zones also exhibit certain conservation of their sequence motifs (Fig. 3D), suggesting that these might be generically important for FnIII-FnIII arrangements and/or interactions to other A-band components.

**MuRF-1 binding**

The surface of A168-A170 reveals a shallow groove at the concave side of the molecule that spans its full length (Fig. 2B). This could be expected to accommodate the binding counterpart of MuRF-1. Based on SPOTS blots of MuRF-1 peptides, the latter has been proposed to bind titin through a sequence just prior to its acidic C-terminal tail (Fig. 1B) (26). According to our own analysis (jpred software; 27), the C-terminal half of MuRF-1 (residues 166–315, which include the proposed binding sequence) has high tendency to \(\alpha\)-helical formation, with at least its middle section predictably following a coiled-coil association (COILS; 28) (Fig. 1B). This is supported by CD data on a correspondingly truncated MuRF-1 variant, MuRF-1(166–315), whose spectrum exhibits a maximum at 190 and minima at 208 and 222 nm indicative of a predominant \(\alpha\)-helical composition (Fig. 4A). An estimation of the secondary structure content of this sample based on these data suggested an approximate \(\alpha\)-helical content of 70% and 30% random coil (fitting parameter \(R^2=99.8\%\)). This is in excellent agreement with results from both manual examination and docking prediction software (PatchDock; 29), which indicated that the dimensions and geometry of the surface groove in A168-A170 can well accommodate an \(\alpha\)-helix along its length. In particular, a best docking is obtained if the helix exhibits a long-range bending of its axis as that of components of a dimeric coiled-coil. For example, as seen in human lamin A coil 2B (30) used as a prototypic structural template to probe the surface of A168-A170 in this study (Fig. 2C). To explore further the interaction area between titin and MuRF-1, we investi-
gated their binding by SPOTS blots of peptide fragments of A168-A170 assayed against the central helical fraction of MuRF-1, MuRF-1(166–315). The results confirm that all three modules in A168-A170 are involved in the interaction (Fig. 5A). Furthermore, the mapping of the selected peptides onto the crystal structure of A168-A170 showed that, with the exception of one peptide, all interacting fragments colocalized at the concave face of the molecule (Fig. 5B). Thus, it could be concluded that this is the primary MuRF-1 binding interface.

Further, we have established the interaction between A168-A170 and the helical fraction of MuRF-1 in solution and quantified it using isothermal calorimetry (ITC). This study used two N-terminally truncated MuRF-1 constructs, MuRF-1(166–341) and MuRF-1(166–315) (Fig. 1B), where the latter lacks in addition the acidic C-terminal tail. Binding monitored by ITC showed that both MuRF-1 variants interacted strongly with A168-A170, yielding heat release values of 14 and 28 kcal/mol and Kd affinities of 35 and 37 nM, respectively (Fig. 5C). Since Kd values are similar for both constructs, it can be concluded that the acidic tail of MuRF-1 does not influence binding but that this is determined by its helical fraction. It was also found that saturation occurs at a molar ratio of 1:1, which indicates that ~10 MuRF-1 molecules bind one A168-A170. Such high ratio, however, is unusual and points to either a high oligomerization state of MuRF-1 variants or possible technical problems in the estimation of this parameter by ITC. To gain a further insight into

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**Figure 5.** Binding of MuRF-1 to A168-A170. A) SPOTS blots of peptide fragments from A168-A170 assayed vs. MuRF-1(166–315). Data corresponding to the insertion motif in IgA169 are boxed in red; B) Mapping of interacting SPOTS peptides (orange) onto the molecular surface of A168-A170. They cluster at the concave face (left), while only one peptide (peptide 18) can be mapped to the convex side of the molecule (right), which otherwise remains unmatched. The distinct loop insertion of IgA169 is marked; C) Binding of MuRF-1 to A169-A170 monitored by ITC. The experimental pattern of injections of MuRF-1(166–341) is shown (upper panel). Heats of binding for titrations of A168-A170 into MuRF-1(166–341) (filled circles) and MuRF-1(166–315) (open triangles) recorded in 50 mM Tris-HCl, pH 8.0; 20 mM NaCl at 25°C is displayed (lower panel). Both curves show the same Kd and ΔH and differ only slightly in the active fraction of MuRF-1; D) Superimposition of Ig domains from titin for which a structural model is available, namely IgA169 (cyan), IgA168 (green), I1 (magenta; PDB accession code 1G1C), M5 (yellow; 1NCT), Z1 (salmon; 2A38) and Z2 (gray, 2A38). The unique loop insertion of IgA169 is clearly identifiable. The Ca atoms of its component residues are displayed as solid spheres and the sequence given. I91 (formerly I27), which does not belong to this Ig subgroup and has shorter loop regions, is excluded from the superimposition to ease visualization. As the other Ig, I91 does not comprise loop insertions in its N-terminal β-strand region. The KTLE motif altered by site-site directed mutagenesis is in red; E) SDS-PAGE of a pull-down assay where MBP-A168-A170 was immobilized in affinity resin in the presence of MuRF-1(166–315). Lane 1 shows isolated MBP as control; lane 2 reveals the binding of MuRF-1 to wild-type A168-A170; lane 3 employed tetra-mutated A168-A170, where the KTLE motif had been disrupted, with the resulting loss of MuRF-1 binding.
this issue, we investigated the association state of the helical fraction of MuRF-1 in isolation using size-exclusion chromatography combined with multiangle static light scattering (SEC-MALS). This technique yields an accurate determination of the molecular mass (MM) without being influenced by molecular shape or hydrodynamic parameters. SEC-MALS measurements of MuRF-1(166–315) yielded a MM of 32.3 kDa (protein concentration estimated from refractive index), which is in excellent agreement with the calculated MM of 34.7 kDa for a dimer of this sample (Fig. 4B). This supports predictions based on sequence data that suggest a self-association of MuRF-1 via a dimeric coiled-coil segment (COILS software; 28) (Fig. 1B). Since A168-A170 is monomeric according to structural and chromatographic data, it could be expected that the basic stoichiometry of the titin/MuRF-1 complex is 1:2 (or a multiple of this ratio). Unfortunately, we have not been able to investigate directly the composition of titin/MuRF-1 assemblies by other biophysical techniques during this study. Mixing and storage of complexed samples at concentrations and in conditions compatible with biophysical analysis led to precipitation that rendered the preparations unsuitable for study. Thus, at this time, we cannot discard the possibility that the ratio measured by ITC might represent a more complex scenario in which, on binding, conformational changes take place in the individual components that result in a higher level of assembly. However, the differing ITC value is more likely to be of technical origin. Since ITC curves were consistent over the several repetitions of the recordings and for both constructs, MuRF-1(166–341) and MuRF-1(166–315), the measured stoichiometry cannot be due to random or variable effects, such as unspecific aggregation of MuRF-1 samples. The latter, for which no experimental evidence exists, would have caused drifts in the saturation point of the different ITC measurements depending on sample batch, storage times and conditions. Instead, ITC data most probably reflect a consistent error in estimating the concentration of MuRF-1 fragments in this study. The MuRF-1 helical domain has a low content of aromatic residues and calculation of its concentration required of the BCA colorimetric method, whose response to different proteins can vary widely. Moderate, cumulative errors in quantifying the amounts of MuRF-1 variants and/or A168-A170 could well explain the deviation of recorded ITC values (~0.1) from the expected (~0.5) titin/MuRF-1 ratio based on the oligomeric state of the individual components.

Structural and biochemical data here presented indicate that MuRF-1 and titin interact through an extensive contact area. However, in A168-A170 the unique loop insertion of IgA169 is located at the midpoint of its surface groove (Fig. 2B, C) and it could be expected to play a central role in MuRF-1 binding or function. Effectively, SPOTS blots confirmed that this motif is involved in MuRF-1 interaction (Fig. 5A). To further assess the relevance of this feature in the binding, we constructed a tetra-mutated variant of A168-A170, where residues 102-KTLE-105 had been exchanged for 102AAAAA105, and tested its interaction to MuRF-1(166–315) using pull-down assays (Fig. 5D). Results confirmed that alteration of this unique motif abolishes the interaction with MuRF-1 and, therefore, that this is central to its molecular recognition by A168-A170.

DISCUSSION

The muscle filament titin, despite its colossal dimensions, has a simple and repetitive architecture consisting of linear tandems of Ig and FnIII modules. The structure of A168-A170 reveals the molecular details of Ig-Ig and Ig-FnIII interfaces, where the latter are likely to be generic to Ig-FnIII domain pairs along titin. A168-A170 has a pronouncedly extended conformation and exhibits a well-defined long-range order with limited conformational freedom. This seems to result from extremely short linker sequences and the presence of small hydrophobic clusters that restrict the modular orientations. Specific contacts are not found across any of these domain pairs. Hydrophobic interactions include groups from similar structural elements in Ig and FnIII modules, with residues around the linker region and the FG β-turn being involved in both cases (Fig. 3C). The observed Ig-Ig contacts are poorly conserved (Fig. 3C). In agreement, domain orientations in IgA168, IgA169 and ZI22 differ somewhat (Table 2). On the contrary, sequence conservation at the Ig-FnIII interface indicates that this modular conformation might be common to equivalent pairs from the A-band. It is yet to be established whether the conservation of interface groups in this case, in particular the FG β-turn of FnIII domains, might play an additional role in interactions to other sarcomeric proteins—such as MyBP-C (25).

A168-A170 selectively recruits MuRF-1 to the M-line region of titin. The specificity of the interaction appears achieved through two idiosyncratic features of this fragment of titin: i) the topography of a shallow groove spanning the length of its surface and defined by the long-range domain arrangement of this distinct tandem; and ii) the unique loop insertion of IgA169. ITC data show that the titin/MuRF-1 interaction is of high affinity, where the α-helical region of MuRF-1 (residues 166–315) is sufficient for binding. Based on the oligomeric state of individual components, we propose the stoichiometry of the titin/MuRF-1 complex to be 1:2. SPOTS blots support the view that the groove in A168-A170 constitutes the binding interface to MuRF-1 as predicted from the crystal structure. The groove could host up to 70% of the maximal hypothetical coiled-coil length achievable by MuRF-1, if its full helical region was to form such motif. The fact that the interaction involves more than one modular unit in titin suggests that this filament uses long-range steric determinants, provided by its interdomain arrangements, to achieve specificity in the recruitment of other proteins to distinct locations of its otherwise repetitive frame. Finally, we have identified a 102KTLE105 insertion motif in IgA169 as primary molecular determinant of MuRF-1 interaction by pull-down assays on a A168-

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A170 mutated variant. So that even if the interacting surface in the MuRF-1/titin complex appears extensive, the binding could be modulated by targeting this key motif. The high affinity of the interaction and the fact that this is primarily governed by a defined sequence motif suggest that the A168-A170 region of titin could serve as a potential therapeutic target against muscle atrophy, where binding of small molecules to its distinctive structural features could block the access of MuRF-1.

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