Structural Insights into Functional Overlapping and Differentiation among Myosin V Motors*§

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Background: MyoVs are molecular motors widely distributed in eukaryotic cells responsible for membrane trafficking and intracellular transport.

Results: The cargo-binding domain from human MyoV paralogs was structurally and biophysically characterized.

Conclusion: We identified singular structural changes and molecular events conferring functional differentiation and modulating cargo binding.

Significance: This work provides structural insights into cargo recognition and regulatory mechanisms in MyoVs.

Myosin V (MyoV) motors have been implicated in the intracellular transport of diverse cargoes including vesicles, organelles, RNA-protein complexes, and regulatory proteins. Here, we have solved the cargo-binding domain (CBD) structures of the three human MyoV paralogs (Va, Vb, and Vc), revealing subtle structural changes that drive functional differentiation and a novel redox mechanism controlling the CBD dimerization process, which is unique for the MyoVc subclass. Moreover, the cargo- and motor-binding sites were structurally assigned, indicating the conservation of residues involved in the recognition of adaptors for peroxisome transport and providing high resolution insights into motor domain inhibition by CBD. These results contribute to understanding the structural requirements for cargo transport, autoinhibition, and regulatory mechanisms in myosin V motors.

Myosins V (MyoVs)§ are actin-based molecular motors, widely distributed in eukaryotic organisms from yeast to human, which play crucial roles in membrane trafficking and in transporting of organelles, secretory vesicles, mRNA, lipids, and proteins (1–4). In human, three class V members are found, named myosin Va, Vb, and Vc. Myosin Va (MyoVa) is commonly present in brain and melanocytes, whereas myosin Vb is ubiquitously expressed, and myosin Vc is abundant in exocrine tissues and epithelial cells (5–8). Class V myosins have been demonstrated as key molecular motors on the maintenance of cell homeostasis, because they mediate intracellular traffic and translocation of several membrane receptors and cell growth/proliferation-related proteins such as PTEN (4, 9–11). In addition to the well known MyoV-related diseases, such as Griscelli syndrome type I and microvillus inclusion disease, recent studies have been shown the involvement of MyoVs in cancer and metastasis process (12–16).

The C-terminal globular tail domain, commonly referred to as the cargo-binding domain (CBD), is the region involved in the selection, recognition, and binding of cargoes that will be transported (17–20) and is directly involved in the autoinhibition of MyoV by interacting with the motor domain (MD) (21–23). Despite the importance of this region for MyoV function in higher eukaryotes, structural information is scanty, and until recently the only two MyoV-CBD-related structures solved were Myo2p and Myo4p from Saccharomyces cerevisiae with ~20% of identity with mammalian MyoV-CBDs (24, 25). The lack of experimentally determined atomic coordinates for MyoV-CBDS from higher eukaryotes, particularly from mammals, has limited the interpretation of both functional and cryo-EM data. Thus, here, we report the crystal structures of the CBDS from the three human MyoV members: Va, Vb, and Vc at 2.20, 2.07, and 2.95 Å resolution, respectively, revealing the structural (dis)similarities among yeast and mammalian MyoV-CBDS, the effects of phosphorylation on protein conformation, and a new redox mechanism involved in the coiled-coil independent dimerization process of the MyoVc-CBD that is unique to this subclass. In addition, the interaction between the MD and CBD was analyzed at high resolution, providing insights into how the ATPase activity is inhibited in the MyoV folded (inactive) conformation.
**EXPERIMENTAL PROCEDURES**

**Molecular Cloning and Site-directed Mutagenesis**—The MyoVa-CBD sequence (Met1448 to Val1851, UNIPROT accession code Q9Y411) was amplified by PCR from a human fetal brain cDNA library (Clontech) and cloned into the pET28a tobacco etch virus vector between EcoRI and Sall restriction sites. The MyoVb-CBD (Leu1452 to Val1848, UNIPROT accession code Q9ULV0) and MyoVc-CBD (Asp1319 to Leu1743, UNIPROT accession code Q9NQX4) sequences were amplified by PCR from a Mammalian Gene Collection clone (IMAGE: 5162990) and Source Biosciences clone (IMAGE: 100068220), respectively. The CBDs from MyoVb and Vc were cloned into bacterial expression vector pNIC28-BsaI as described (26). The site-directed mutagenesis (MyoVa-CBD S1652E and S1652A and MyoVc-CBD C1600A, C1608A, and C1600A/C1608A) was performed based on the QuikChange site-directed mutagenesis kit (Stratagene).

**Protein Expression and Purification**—The MyoVa-CBD was expressed in *Escherichia coli* RosettaII strain in LB medium at 25 °C for 4 h. The MyoVb-CBD and MyoVc-CBD were expressed in *E. coli* RosettaII strain in Terrific Broth medium at 18 °C for 16 h (overnight). Cell lysis was performed by sonication (Vibra-cells; Sonics) and buffer composition varied according to the protein: MyoVa-CBD (and mutants), 25 mM Tris, 500 mM NaCl, 5% (v/v) glycerol, pH 7.4; MyoVb and MyoVc CBDs, 25 mM HEPES, 500 mM NaCl, 5% (v/v) glycerol, pH 7.5. In all cases, the buffer was supplemented with 0.1 mM TCEP, 1 mM PMSF, and 5 mM benzamidine. All proteins were purified by two chromatographic steps at 4 °C using a HiTrap Chelating column (GE Healthcare) and a HiLoad Superdex 200 16/60 column (GE Healthcare). Preparative size-exclusion chromatography (SEC) was carried out with the following buffers: MyoVa-CBD, 20 mM HEPES, 100 mM NaCl, 5% (v/v) glycerol, pH 7.4; MyoVb and MyoVc CBDs, 10 mM HEPES, 200 mM NaCl, 5% (v/v) glycerol, 0.5 mM TCEP, pH 7.5. Sample homogeneity was confirmed by SDS-PAGE and dynamic light scattering. The molecular masses of the constructs overproduced are 50.35, 48.16, and 51.46 kDa for MyoVa, MyoVb, and MyoVc CBDs, respectively.

**Oligomerization and Stability Analyses**—To determine the oligomerization state of MyoVc-CBD as well as the effects of the treatment with oxidizing and reducing agents, the protein was submitted to analytical SEC analysis. The MyoVc-CBD was treated with 10 mM H$_2$O$_2$ or 10 mM TCEP at 4 °C for 1 h under gentle agitation. After the treatment, the samples were immediately injected in the SEC column, and results were compared with untreated samples. To estimate the molecular mass and Stokes radius of each sample, the analytical SEC was performed with standard molecules (gel filtration calibration kit; GE Healthcare). All analytical SEC runs were performed in a Superdex 200 10/300 GL column (GE Healthcare) with 0.3 ml/min flow rate using the same buffer from MyoVc-CBD SEC purification without TCEP.

The thermal shift assays were performed using SYPRO Orange (Invitrogen) as probe, where 2 μM MyoVa-CBD was incubated with 1–500 mM NaCl for 30 min at 18 °C. The stability was verified from 20 to 90 °C using an adapted thermocycler (7500 real time PCR system; Applied Biosystems).

The dynamic light scattering experiments were carried out on a DynaPro 810 dynamic light scattering instrument (Wyatt Technology Corporation). The measurements were carried out at 90 ° detection angle at 18 °C. All samples were centrifuged at 20,000 × g for 10 min prior to the analysis. A total of 100 measurements at intervals of 5 s were recorded and averaged for further calculations of hydrodynamic parameters using the software DYNAMICS v.6.10.1.2.

**Strategies for Limited Proteolysis and Crystallization**—MyoVb-CBD was crystallized by the sitting drop vapor diffusion method at 4 °C using *in situ* proteolysis. Chymotrypsin in a mass ratio of 1:250 (chymotrypsin:MyoVb-CBD) was mixed to 9.8 mg/ml MyoVb-CBD and immediately transferred into a crystallization plate. Best crystal grew in the condition containing 20% (w/v) PEG-3350 and 200 mM Na$_2$SO$_4$.

The same strategy did not work for MyoVa and MyoVc CBDs. Thus, different enzymes and times were tested, and the best cleavage condition was scaled up for further crystallization experiments.

The MyoVb-CBD proteolysis was performed with 10 μg/ml trypsin at 4 °C for 3 h under gentle agitation, and the reaction was stopped by the addition of 5 mM benzamidine. The cleaved sample was repurified with a HiLoad Superdex 200 16/60 column. The MyoVb-CBD at 6.0 mg/ml crystallized by the hanging drop vapor diffusion method at 18 °C in 100 mM Bis-tris, pH 5.5, 22% (w/v) PEG-3350 and 200 mM Na$_2$SO$_4$.

The MyoVc-CBD proteolysis was performed with an approximated ratio of 1:100 (w/w) of chymotrypsin and 1:100 (w/w) of thermolysin at 4 °C for 2 h under gentle agitation and the reaction was stopped by adding 1 mM PMSF/10 mM EDTA. The proteolysis product was immediately purified by SEC using the same conditions described in the previous section. The MyoVc-CBD at 44 mg/ml was crystallized by hanging drop vapor diffusion method at 18 °C in the condition containing 100 mM CAPS, pH 10.5, 2 M NH$_4$SO$_4$, and 200 mM Li$_2$SO$_4$.

**Data Collection and Structure Solution**—Native data of MyoVa-CBD and MyoVc-CBD crystals were collected at 100 K on the W1B-MX2 Beamline at the Brazilian Synchrotron Light Laboratory (Campinas, Brazil) (27), and MyoVb-CBD native data were collected at 100 K on the I02 beamline at the Diamond Light Source (Didcot, United Kingdom). The data were processed and merged with XDS (28) using CC$_{1/2}$ cutoffs (29).

Different strategies were attempted to solve the phasing problem for MyoV-CBDs including standard molecular replacement methods, selenomethionine-substituted crystals for MyoVa-CBD and heavy atom derivatization. However, all these procedures were unsuccessful. A solution for MyoVb-CBD crystal data were only obtained using advanced molecular replacement methods combined with Rosetta *ab initio* modeling as developed by DiMaio et al. (30) and implemented in the Phenix package (phenix.mr_rosetta) (31, 32). The Myo2p tail structure (Protein Data Bank accession code 2F6H) was used as input model based on an HHpred alignment (33) and a fragment-based library, derived from the sequence information, generated by the Robetta server (34). Then the refined MyoVb-
The Cargo-binding Domain from Human MyoV Paralogs

TABLE 1
Data processing and refinement statistics for the MyoVa-, MyoVb-, and MyoVc-CBD structures

|                      | MyoVa-CBD | MyoVb-CBD | MyoVc-CBD |
|----------------------|-----------|-----------|-----------|
| Data collection      |           |           |           |
| Space group          | P2₁       | C222₁     | P4₂2₂     |
| Cell dimensions      |           |           |           |
| a, b, c (Å)          | 57.80, 79.49, 94.14 | 69.09, 78.20, 151.82 | 116.40, 116.40, 114.75 |
| α, β, γ (°)          | 90.00, 105.77, 90.00 | 90.00, 90.00, 90.00 | 90.00, 90.00, 90.00 |
| Resolution range (Å) | 40.0–2.20 | 49.0–2.07 | 50.0–2.95 |
| CCₜ₁₂₆₈ | 0.134 (48.6) | 0.098 (0.610) | 0.138 (2.347) |
| R₁ (free)           | 8.56 (0.99) | 8.69 (1.56) | 12.49 (0.91) |
| Completeness (%)     | 99.4 (97.7) | 97.1 (90.3) | 99.6 (99.4) |
| Redundancy           | 5.25 (4.17) | 2.31 (1.97) | 9.55 (9.16) |
| Refinement           |           |           |           |
| Resolution range (Å) | 39.36–2.20 | 40.00–2.07 | 47.07–2.95 |
| No. reflections      | 41,574    | 25,402    | 17,073    |
| No. atoms            |           |           |           |
| Protein              | 5,242     | 2,967     | 2,950     |
| Water                | 92        | 206       | 7         |
| B-factors            |           |           |           |
| Protein              | 64.00     | 37.70     | 159.00    |
| Water                | 129.60    | 46.20     | -         |
| Root mean square deviations | 58.20 | 40.60 | 113.70 |
| Bond lengths (Å)     | 0.002     | 0.009     | 0.004     |
| Bond angles (°)      | 0.598     | 0.900     | 0.754     |
| Ramachandran (%)     | 96.91, 2.95, 0.14 | 97.2, 1.65, 0.83 | 92.2, 6.13, 1.67 |

* As defined in XDS (29).

CBD structure was used as template to determine the MyoVa-CBD and MyoVc-CBD structures by molecular replacement using Phaser (35, 36). All structures were refined using Refmac5 (36, 37) or phenix.refine (30, 38). The models were improved by iteratively model rebuilding in Coot (39). Translation/libration/screw refinement was included in the final cycles of refinement using translation/libration/screw parameters from the TLSMD server (40). The data collection and refinement statistics are given in Table 1.

Small Angle X-ray Scattering (SAXS)—SAXS data for all proteins were collected at 6 °C on both Beamlines D02A/SAXS2 and D01A/SAXS1 at the Brazilian Synchrotron Light Laboratory. All samples were prepared in several concentrations (1–5 mg/ml) and centrifuged at 20,000 × g, 30 min, and 4 °C, before the data collection. Buffer scattering was recorded before each sample scattering and then subtracted from the corresponding sample scattering. The integration of SAXS patterns was performed using Fit2D software (41) and data analysis using GNOM (42). Low resolution envelopes of each construction formed using Fit2D software (41) and data analysis using SUPCOMB (45). For the flexibility analysis (46, 47), the data were scaled using the program PRIMUS (48).

Molecular Docking Simulations—A customized docking routine was developed to gather further understanding of the interaction between the CBD (this study) and MD (Protein Data Bank accession code 1W7J) of mammalian MyoV. Using previous experimental data (site-directed mutagenesis) as docking restrictions, we applied a shape complementarity algorithm (49) followed by an energetic evaluation step (50). Complementary conformational solutions that do not fulfill the geometrical restrictions were discarded. Top ten-ranked decoys of the CBD-MD interaction were submitted to a docking procedure with no restraints using the Monte Carlo algorithm and scoring decoys based on their interaction energy. Then the obtained decoys were filtered to obtain complexes that were within the most favorable energetic quartile and that presented distance lengths for the experimental restraints of 5.0 Å (Asp₁₃₆–Lys₁₇₈₁) and 10.0 Å (Asp₁₃₆–Lys₁₇₈₁) (sequence numbering based on human MyoVa). Selected decoys were undergone to energy minimization and side chain optimization using the YAMBER3 force field (51). The energy-minimized MD-CBD complex was then fitted into the cryo-EM electron-density map (Electron Microscopy Data Bank accession code 1201) using Situs (52) and compared with available models (22, 23, 53).

RESULTS AND DISCUSSION

The Molecular Architecture of the Cargo-binding Domain from Mammalian MyoVs—Mammalian MyoV-CBDs display a conserved structural scaffold consisted of two four-helical bundles (helices α₂–α₅ and α₇–α₁₀) connected by a long α-helix (α₆ or cervical helix) and stabilized by a C-terminal extension (Fig. 1). From helix α₁₀ arises a three-helical motif (α₁₁–α₁₃), which is stabilized by extensive contacts with the α₇–α₁₀ bundle. The C-terminal extension (Asp¹₈₁⁰–Ile¹₈₅₇), analogously to yeast CBDs, embraces both helical bundles with a number of hydrophobic and polar contacts conferring conformational stability to the tertiary arrangement. At the α₁₂–α₁₃ bundle, the C-terminal extension is guided into the N terminus by steric restrictions imposed by the loop between helices α₅ and α₆ and forms an anti-parallel β-sheet (β₁–β₁₄) with the N-terminal segment as a molecular zipper (Fig. 1a). This β-sheet, along with the α₁₂–α₁₃ bundle, the first half of the cervical helix, and...
the last 22 residues of the C-terminal extension (Phe\textsuperscript{1831}–Ile\textsuperscript{1852}), comprise lobule I, whereas the second half of the cervical helix, the $\alpha$7–$\alpha$10 bundle, the three helical motif and the first 20 amino acid residues of the C-terminal extension (Asp\textsuperscript{1810}–Pro\textsuperscript{1830}) constitute lobule II (Fig. 1). Lobule II is the most conserved from yeast to humans, preserving all secondary structural elements and the three-helical motif. In addition, the faces of the elongated structure can be named according to the function, wherein the M face is related to the interaction with the MD (21) and characterized by the cervical helix exposed to the solvent (Fig. 1a), and the C face has helices $\alpha$7 and $\alpha$9–$\alpha$11, forming a large recognition surface area that contains most of the known cargo-binding sites (54).

Structural Integrity Is Essential for Stability and Function of the Cargo-binding Domain—The expression of multiple designed constructs of MyoVa-CBD (Fig. 2) showed that the isolated lobules or the removal of the C-terminal extension yielded misfolded proteins, indicating that the structural integrity of mammalian MyoV-CBDs is essential for protein stability and consequently to proper function (data not shown). The N-terminal fragment (\textsuperscript{1473}FQGMLEYKE\textsuperscript{1482}) preceding lobule I also proved to be essential for protein stability, and
crystal analysis revealed its role in forming an antiparallel β-sheet (β1 and β14) with the C terminus of mammalian MyoV-CBDs (Fig. 1).

MyoVa-CBD thermal tolerance was enhanced with increasing ionic strength, revealing the importance of the hydrophobic core in the domain stability, including the C-terminal extension that makes a number of hydrophobic contacts with both lobules (Fig. 3, a and b). In fact, the two CBD fragments obtained by limited proteolysis (Fig. 3c), corresponding to lobules I and II (MS-MS data not shown), did not dissociate in solution, as shown by analytical SEC and SAXS analyses (Fig. 3, d–f). These observations are in full agreement with the Myo2p-CBD behavior (55), highlighting that, despite the high sequence divergence, MyoV members, from yeast to human, conserve the hydrophobic interactions responsible for the CBD stability.

FIGURE 2. Schematic representation of all constructs designed for human MyoVa-CBD (I–VII). Construct III was used to design constructs for MyoVb and MyoVc CBDs. ABCDE(F), the exon region; N, N-terminal region important for protein solubility; bd I, β1 and helical bundle I; α6, cervical helix; bd II, helical bundle II; P, phospho loop; hm, three-helical motif; C, C-terminal region, including β14. The proximal, medial, and distal tails refer to the different regions of the MyoVa tail domain.

FIGURE 3. The key role of hydrophobic interactions in MyoVa-CBD stability. a, thermal shift analysis of MyoVa-CBD showing the increase of thermal stability with the increase of NaCl concentration (1–500 mM). With 500 mM NaCl, a ΔTm of −6 °C was observed. b, this behavior in solution is associated with a greater number of hydrophobic interactions in the inner core of the MyoVa-CBD (yellow spheres), as well as between the C-terminal extension (cyan spheres) and helices from both lobules. Residues involved in hydrophobic interactions (spheres) were defined by the Protein Interaction Calculator Server, using the interaction distance cutoff of 4 Å. c, electrophoretic pattern of human MyoVa/b/c-CBD, uncleaved and cleaved by limited proteolysis using trypsin (MyoVa-CBD), chymotrypsin (MyoVb-CBD), or chymotrypsin/thermolysin (MyoVc-CBD). The numbers below the arrows indicate the time of incubation in hours. M, molecular mass marker. N, uncleaved protein. d, analytical SEC of MyoVa-CBD before and after limited proteolysis showing that the proteolysis products elute as a single entity. e and f, SAXS analysis of the cleaved MyoVa-CBD indicating a typical scattering (e) and p(r) curve (e, inset) of an elongated-globular protein resulting in an envelope (f) into which the entire crystallographic model of the MyoVa-CBD (green) was fitted.
Lobule Arrangements Differs among Mammalian MyoV Members—As expected because of high sequence conservation (Table 2), the MyoVb-CBD is very similar to that from MyoVa (root mean square deviation value for all Ca atoms: 0.49 Å), conserving all secondary structural elements and lobule arrangement. Interestingly, however, the MyoVc-CBD showed distinct lobule geometry with an angular shift of 10.5°, resulting in a more curved structure (root mean square deviation value for all Ca atoms: 1.47 Å) (Fig. 4a). This angular difference is caused by a cooperative effect of extensive interactions across the interface between the lobules, and the most noticeable conformational divergence resides in the loop connecting helices α2 and α3 (Fig. 4a). In MyoVc-CBD, this loop is tied to helix α7 from lobule II by a number of polar and van der Waals contacts, whereas in MyoVa-CBD, it is shifted into lobule I with stabilizing interactions with helix α5 (Arg1398—Glu1552 electrostatic interaction). This permutation of interactions from lobule I to

TABLE 2
Sequence identity and similarity among yeast and human class V myosins
Mammalian MyoV-CBDs share medium to high sequence identity and similarity, whereas yeast Myo2p- and Myo4p-CBDs share low sequence identity and similarity.

|            | MyoVa | MyoVb | MyoVc | Myo2p | Myo4p |
|------------|-------|-------|-------|-------|-------|
| CBD sequence similarity (%) |       |       |       |       |       |
| MyoVa      | 100   | 70.6  | 63.5  | 22.7  | 19.1  |
| MyoVb      | 70.6  | 100   | 60.1  | 22.3  | 21.4  |
| MyoVc      | 63.5  | 60.1  | 100   | 24.3  | 22.5  |
| Myo2p      | 22.7  | 22.3  | 24.3  | 100   | 24.3  |
| Myo4p      | 19.1  | 21.4  | 22.5  | 24.3  | 100   |

CBD sequence identity (%)

low | medium | high

The Cargo-binding Domain from Human MyoV Paralogs
lobe II in MyoVc-CBD contributes to the curvature of the molecule and may contribute to functional differentiation.

**A Novel Redox Mechanism Regulates the CBD Dimerization in MyoVc Members**—Although a monomer is found in the asymmetric unit of MyoVc-CBD crystal, interface analysis identified an energetically stable dimeric arrangement (buried area = 2320 Å² and ΔG^int = −18.7 kcal/mol) with a Y-shape conformation in the unit cell (Fig. 4b). In addition, in vitro studies showed that MyoVc-CBD oligomerization is responsive to oxidizing or reducing agents (Fig. 4c). In the presence of oxidizing agents, the MyoVc-CBD behaved exclusively as monomers in solution, whereas oxidizing compounds triggered dimerization. Structural analysis of the dimeric configuration revealed the presence of two cysteine residues at the interface, Cys1600 and Cys1608, which are present only in the CBD of MyoVc members. Both cysteine residues are located in helix α9 and spaced by −14 Å, which prevents the formation of any intramolecular disulfide bond, suggesting that both could be involved in the stabilization of the dimeric form. Indeed, mutational studies confirmed that the presence of at least one of those cysteines is sufficient for the redox dimerization process. Only the double mutant C1600A/C1608A became irresponsive to oxidizing conditions, remaining monomeric even in the presence of H₂O₂ (Fig. 4d). On MyoVa/Vb-CBDs, Cys1600 and Cys1608 are replaced by a Trp and Tyr, respectively, supporting that this redox mechanism is unique to MyoVc members. In fact, analytical SEC and crystal analyses did not show any self-dimerization in a coiled coil-independent manner for the CBDs of MyoVa and MyoVb.

Interestingly, this dimeric arrangement involves exclusively lobule II in such an orientation that the M face and most of the C face are solvent-exposed, permitting the binding to the MD in the autoinhibited state or even the recognition of cargoes. In addition, the 2-fold symmetry in this dimeric configuration results in the equivalent faces positioned at opposite sides, which is important to prevent steric impediments for MD interaction or the recognition of typical large cellular cargoes. Furthermore, this configuration yields a large space between lobule I of both subunits that, combined with a more curved structure, would allow the accommodation of the 45-kDa medial tails. Collectively, these observations suggest that this dimeric form is physiologically feasible and represents a novel (redox) regulatory mechanism, unique to MyoVc, and the most divergent MyoV member found in mammals.

**Phosphorylation Affects Conformational Flexibility of the Cargo-binding Domain**—Phosphorylation of the mammalian MyoV-CBD has been proposed to down-regulate the organelle transport during mitosis (56) and to trigger the MyoV migration to the nucleus, being the phosphorylated form found exclusively in nuclear speckles (57). It was demonstrated that MyoVa-CBD is phosphorylated in the motif 1647RKRT(S)SpS1652 (Fig. 5); however, the structural implications of this post-translational modification remain elusive.

The phosphorylation site is fully conserved in all mammalian MyoV-CBDs with a canonical signature RKRT(S)SpS, permitting conservative substitutions in the basic residues and serine to threonine (Fig. 5a). The CBD structure revealed that the phosphorylation site is strategically located at the tip of a long loop (His1632 to Thr1660 in MyoVa-CBD) connecting α6 and α7 (phospho loop), and it might trigger conformational changes by interacting with one of the two lobules (Fig. 1a). The geometric disposition of the helices forming the ends of the phospho loop suggests that, once phosphorylated, this loop is more likely to interact with the M face of lobule II, where a conserved cluster of positively charged residues, involved in motor recognition and binding, is located (Fig. 6). Another cluster of basic residues, present in lobule I, is conserved in mammals. However, because of structural organization, it is less likely to be the binding site of the phosphorylated loop. A large scale molecular modeling and surface charge analysis of 54 mammalian MyoV-CBDs with sequence identity ranging from 45 to 95% (data not shown) revealed that both positively charged clusters on the surface of the M face are conserved, whereas the C face is variable (Fig. 6), supporting the correlation between the long loop, with the signature RKRT(S)SpS, and the charge distribution in the M face.

Interestingly, the wild-type MyoVa-CBD and the mutant S1652A showed a higher flexibility when compared with the phospho-mimetic mutant S1652E according to Porod-Debye plot of MyoVa-CBD SAXS data showing a decrease of flexibility in the phospho-mimetic mutant S1652E by the formation of a plateau in comparison with the wild type.
region that may be related to the recognition of and binding to nuclear proteins according to colocalization data (57). Moreover, the conservation of the M face is related to the autoinhibition event, which seems to be present in several myosin classes, suggesting that interaction of the phospho loop with this region may interfere with the autoinhibition event.

On the Structural (Dis-)Similarities between Yeast and Mammalian MyoV-CBDs—Despite the conserved overall fold including the two helical lobules and C-terminal extension, the molecular surfaces of yeast and mammalian MyoV-CBDs are very distinct in terms of amino acid composition, charge distribution, and loop arrangements (Figs. 6 and 7). Lobule II, also improperly named the dilute domain because it does not constitute a domain itself, is relatively conserved, whereas in lobule I reside the most significant differences (Fig. 7). The angular curvature between the two lobules is also diverse, even among the three mammalian MyoV paralogs. All human CBDs present a more curved structure in comparison with the yeast Myo2p-CBD (Fig. 7a). The open conformation of Myo2p-CBD can be assigned to the loop connecting helices $\alpha 2$ and $\alpha 3$ that is 10 residues longer than the human orthologs and is located at the convex part of the curvature making several stabilizing contacts with lobule II (Fig. 7a, zoom of the C face). The other yeast MyoV, Myo4p, also showed a distinct angular geometry between the lobules (Fig. 7b); however, the structure lacks a significant segment of the C terminus, which is known to be essential for conformational stability and displays lower sequence identity than Myo2p in comparison with mammalian MyoV-CBDs (Table 2). This variability in the angular curvature between the two lobules indicates a molecular plasticity of MyoV-CBDs that is an inherent feature from yeast to human.

Moreover, the loop connecting the lobules in yeast MyoV-CBDs is shortened and lacks the consensus RKRT(S)SpS for phosphorylation, suggesting that yeast orthologs do not undergo this post-translational modification as a regulatory mechanism. The molecular zipper is also present in yeast Myo2p-CBD; however, instead of an antiparallel $\beta$-sheet between the N and C termini present in mammalian MyoV-CBDs, it contains a pair of antiparallel $\alpha$-helices involving the same corresponding regions (Fig. 7a, zoom of the M face). The human MyoV loop connecting helices $\alpha 5$ and $\alpha 6$ may contribute to the variance of the molecular zipper between yeast and mammalian orthologs, because it adopts a totally different conformation in relation to yeast Myo2p-CBD, driving the C-terminal extension to the N terminus and stabilizing the molecular zipper. On the other hand, lobule II is very conserved, including the four-helical core and the three-helical motif preceding the C-terminal extension.

Structural Assignment of Cargo-binding Sites—The cargo-binding sites in yeast MyoVs are distributed in the two lobules of the CBD, although on opposite faces. On the M face, the binding sites for Vac17 and Mmr1 in yeast are overlapped in lobule I with several residues found in helix $\alpha 6$ (54); however, these residues are poorly conserved in mammalian MyoV-CBDs, indicating a nonconserved function (Fig. 8a). The MD binding site in mammalian MyoV-CBDs is at lobule II in the same face as the Vac17 and Mmr1 binding sites, driving the C-terminal extension to the N terminus and stabilizing the molecular zipper. On the other hand, lobule II is very conserved, including the four-helical core and the three-helical motif preceding the C-terminal extension.
in mammalian MyoVa/Vb-CBDs. This organelle is virtually found in all eukaryotic cells, and it suggests that the mammalian MyoVa and MyoVb members may be involved in the transport of peroxisomes, representing a highly conserved function among MyoV motors from yeast to human.

The tumor suppressor PTEN, involved in the control of PI3K signaling pathway, was also identified as a partner of MyoVa in its phosphorylated form by interacting with three basic residues \(1752\)QLLQV\(1756\)KKK\(1758\)

\(TDD\) located in the loop connecting helices \(\alpha10\) and \(\alpha11\) (11) (Fig. 9, a–c). The structure suggests that the residues at both ends from the sequence \(1757\)KKK\(1759\) are most likely involved in the interaction to the phosphorylated form of PTEN because they are exposed to the solvent, whereas the inner Lys\(1758\) residue is turned to the protein core (Fig. 9, d and e). The substitution of Lys\(1759\) by a threonine residue in MyoVc did not impair the basic character of the region, supporting the binding of PTEN to MyoVc observed in vitro (11) (Fig. 9, f and g). However, in the yeast Myo2p-CBD, the residue corresponding to Lys\(1759\) is replaced by a tyrosine (Fig. 9, h and i), and it significantly modifies the charge distribution and may

FIGURE 8. The conservation of cargo-binding sites from yeast to human. a, the binding sites for Vac17 and Mmr1 in the yeast Myo2p are located at lobule I (M face) and poorly conserved in mammalian MyoV-CBDs. b, lobule II (C face) concentrates most of the known cargo binding sites in yeast Myo2p including for Kar9, Sec4, Ypt11, Ypt31/32, and Inp2 (54). Interestingly, all residues involved in the binding to Inp2 (highlighted with dashed lines on Myo2p), an adaptor to peroxisome transport, are conserved in mammalian MyoVa and MyoVb CBDs. In MyoVc-CBD, two of the conserved residues (Trp\(^{1713}\) and Tyr\(^{1721}\)) are replaced with cysteines (Cys\(^{1600}\) and Cys\(^{1608}\)), supporting the notion that the redox dimerization is a regulatory mechanism unique to this subclass.

FIGURE 7. Structural (dis-)similarities between yeast and mammalian MyoV members. Myo2p-CBD (a) and Myo4p-CBD (b) superposition on the human MyoVa-CBD highlighting the major differences. Myo2p (Protein Data Bank accession code 2F6H) and Myo4p (Protein Data Bank accession code 3MMI) root mean square deviations for aligned Ca atoms are 2.5 and 4.8 Å, respectively. a, the left inset shows a zoom in to the M face illustrating the antiparallel N- and C-terminal β-sheet in mammalian MyoV-CBDs in contrast to the α-helices at the corresponding region in the yeast Myo2p. The right inset shows a zoom in to the C face highlighting the α2/α3 loop that contributes to changes in the angular curvature of Myo2p in relation to human MyoVa/Vb-CBDs. b, zoom of lobule I highlighting the structural differences among Myo4p and human MyoV-CBDs (left) and the shortened loop corresponding to the phospho loop in mammals (right). The dotted lines in all panels illustrate portions of disordered or cleaved loops absent from the crystallographic models.
promote steric impediments in the corresponding region, suggesting a nonconserved function.

**Structural Basis for Inability of MyoVb to Bind RILPL2**—The RILPL2 (Rab interacting lysosomal protein-like 2) is a centrosomal and ciliary protein that regulates the protein content of the primary cilium (58). RILPL2 differs from RILP by lacking the Rab7-interacting region and therefore is not involved with lysosomal morphology (59). Class V myosins and RILPL2 interaction was first described by Lise et al. (60), whereupon the MyoVa was shown to be the specific MyoV member performing this function. The interface involves the CBD and the 113-residue-long N terminus of RILPL2, which contains the RILP-homology region-1 (RH1) (58). On the other hand, the MyoVb did not interact with RILPL2, and MyoVc was not investigated. A recent study describing the mouse MyoVa-CBD structure in complex with RILPL2-RH1 (61) provided more details about this interaction but did not provide the structural basis for the inability of MyoVb-CBD to bind RILPL2 because of the lack of structural data for MyoVb-CBD.

The interaction occurs between lobule I of MyoVa-CBD (α2-α3 link region and a segment of the C-terminal extension preceding the β14) and the helical bundle region of RILPL2 (α2 and α3N) mainly by hydrophobic interactions (61). Interestingly, within the highly conserved structure of MyoVb-CBD in relation to MyoVa-CBD, there is a noticeable singular difference located exactly at the α2-α3 link region, which is considered essential for recognition and binding of RILPL2 (Fig. 10a, zoom). However, despite the greater divergence of MyoVa-CBD to MyoVc-CBD, this region is conserved with the same topological arrangements and the presence of two hydrophobic residues (Val1387 and Met1391) on the surface of the short helix within the α2-α3 link region (b, zoom).
The Cargo-binding Domain from Human MyoV Paralogs

MyoVa-CBD (Fig. 10b). This structural conservation suggests that in mammals MyoVc can compensate for the lack of MyoVa to perform this function.

High Resolution Insights into the MD-CBD Interaction—Despite the multilayered regulatory mechanisms found in MyoVs, the autoinhibition is the most intriguing one in which the CBD interacts with the MD inhibiting the ATPase activity and resulting in a compact structure with weak affinity to actin (62). This mechanism is paradoxically regulated by Ca\(^{2+}\) ions, but the competition between cargoes and MD to CBD might be the physiological mechanism to convert MyoV from its inactive conformation to its active state (reviewed in Ref. 63). Electron microscopy studies shed light on the inactive form of MyoV, revealing a folded conformation with a triangular shape caused by the interaction between MD and CBD (21–23). Moreover, site-directed mutagenesis revealed that the acidic residue Asp\(^{136}\) from MD and the basic residues Lys\(^{1708}\) and Lys\(^{1781}\) from CBD (corresponding to residues Lys\(^{1706}\) and Lys\(^{1779}\) in mouse MyoVb, respectively) from CBD are essential for stabilization of the inactive folded conformation (21, 23, 62).

The high resolution view of the CBD-MD interaction was rather limited because of the lack of structural information of the MyoV-CBD from higher eukaryotes. Thus, to better understand the structural determinants involved in the inhibition of ATPase activity by CBD, we performed in silico molecular docking simulations using the available restraints to model the CBD-MD interaction (Fig. 11 and supplemental Fig. S1).

The best modeled interface between MD and CBD had a binding energy of 4,327 kJ/mol with a buried area of 1,523 Å\(^2\) being stabilized by a number of hydrophobic, polar, and electrostatic interactions (Fig. 11a and Tables 3 and 4) involving highly conserved residues among human MyoV paralogs. This interface involves almost exclusively the N-terminal subdomain of MD and the M face of lobule II, leaving the actin-binding site free.

As proposed by Thirumurugan et al. (23), the surface comprising the amino acids 117–137 with helix-loop-helix topology is an important region for interaction with CBD. Helices a6, a8, a12, and a13, including a segment of the C-terminal extension of CBD, are interfacing the MD N-terminal domain (Fig. 11b). Moreover, the MD-CBD interaction does not seem to block the ATP-binding site but probably interferes in the recognition and binding via interactions with the Ile\(^{111}\)–Glu\(^{121}\) loop (amino acid numbering based on the MyoVa MD structure; Protein Data Bank accession code 1W7J) that orientates the adenine base of ATP. Interestingly, the three-helical motif seems to act as a physical barrier to the converter subdomain of MD, with an electrostatic interaction between the Arg\(^{709}\) and Glu\(^{1791}\) (Fig. 11, a and b). These observations suggest that the binding of CBD to MD perturbs the coordination of the ATP at the base region and also blocks any conformational changes related to the converter subdomain, keeping the actin-binding site accessible.

Furthermore, the CBD-MD complex was compared with the current available models for the inhibited state of MyoV proposed by Liu et al. (22) and Thirumurugan et al. (23). The superposition of the CBD-MD complex on the domain-swap modeling (22) showed no agreement with the cryo-EM density map (Electron Microscopy Data Bank accession code 1201, at 24 Å resolution), suggesting a different arrangement of the dimeric MyoV into the rosette-like architecture (Fig. 12a). The best result for rigid body fitting (CC = 0.817) of the CBD-MD complex into the cryo-EM electron density map indicated a configuration that resembles the folded confor-

| TABLE 3 |
| MyoVa MD-CBD hydrophobic and electrostatic interactions defined by docking analysis |
| --- |
| Hydrophobic interactions |
| Domain | Position | Residue | Domain | Position | Residue |
| MD | 69 | Val | CBD | 1798 | Val |
| MD | 91 | Val | CBD | 1798 | Val |
| MD | 98 | Leu | CBD | 1817 | Leu |
| MD | 98 | Leu | CBD | 1780 | Val |
| MD | 98 | Leu | CBD | 1785 | Leu |
| MD | 116 | Leu | CBD | 1817 | Leu |
| MD | 116 | Leu | CBD | 1815 | Pro |
| MD | 117 | Pro | CBD | 1818 | Leu |
| MD | 118 | Ile | CBD | 1818 | Leu |
| MD | 118 | Ile | CBD | 1817 | Leu |
| MD | 123 | Ile | CBD | 1706 | Leu |
| MD | 123 | Ile | CBD | 1818 | Leu |
| MD | 126 | Ala | CBD | 1706 | Leu |
| MD | 127 | Tyr | CBD | 1706 | Leu |
| MD | 670 | Phe | CBD | 1815 | Pro |
| MD | 670 | Phe | CBD | 1817 | Leu |

| Electrostatic interactions |
| --- |
| Domain | Position | Residue | Domain | Position | Residue |
| MD | 71 | Glu | CBD | 1708 | Lys |
| MD | 71 | Glu | CBD | 1795 | Arg |
| MD | 73 | Asp | CBD | 1708 | Lys |
| MD | 73 | Asp | CBD | 1795 | Arg |
| MD | 95 | Asp | CBD | 1802 | Arg |
| MD | 95 | Asp | CBD | 1809 | Arg |
| MD | 121 | Glu | CBD | 1822 | Lys |
| MD | 134 | Asp | CBD | 1707 | Arg |
| MD | 136 | Asp | CBD | 1781 | Lys |
| MD | 97 | Lys | CBD | 1813 | Asp |
| MD | 709 | Arg | CBD | 1791 | Glu |
According to our docking results, instead of the domain-swapping configuration suggested by Liu et al. (22), intramolecular CBD-MD complexes are formed, and each dimeric subunit is tilted in relation to the adjacent molecule to compose the rosette of six molecules as proposed by Sellers et al. (53) (Fig. 12d). This interpretation satisfies all experimental restraints including atomic coordinates of MD (Protein Data Bank accession code 1W7J) (64) and CBD (this study), mutational data (21, 23), single-particle images (23), and the electron-density map obtained by electron tomography (22). These findings support that the biological assembly of the inhibited state of MyoV consists of a folded dimeric MyoV as shown by single-particle EM images (23) that is tilted and laid over the adjacent molecule to form the rosette of six molecules observed in two-dimensional crystals (53).

Concluding Remarks—Despite high fold conservation, including the two discrete helical bundles connected by the long α-helix and stabilized by the C-terminal extension, yeast and mammal MyoV-CBDs display very low residue conservation on the molecular surface, reflected by contrasts in charge distribution, molecular topography, lobule arrangements, and, consequently, cargo selection and binding. However, some functions have been preserved through evolution, as suggested by the conservation in yeast and mammal CBDs of the Inp2-binding site.

Among the mammalian MyoV-CBDs, there are significant differences probably related to functional differentiation. Two examples are the redox-sensitive dimerization of MyoVc, which involves cysteine residues (Cys1600 and Cys1608) only conserved in this subclass, and the local divergence between MyoVa and MyoVb, at the α2-α3 link region, which confers RILPL2 binding specificity to MyoVa (and likely to MyoVc). On the other hand, the high conservation observed between mammalian MyoVa and MyoVb CBDs supports a possible functional overlapping considering other cargoes. Indeed, recent studies have demonstrated a functional compensation by MyoVb with the loss of MyoVa in signaling networks regulating cell growth (11).

The phospho-mimetic state of the long loop connecting lobules I and II was demonstrated to trigger structural changes in the CBD structure, conferring a lower conformational flexibility that could be related to the interaction of the phosphorylated loop with the MD-binding site (residues Arg1707, Lys1708, Lys1781, and Arg1795) affecting the formation of the inhibited folded conformation. Previous studies showed the localization of phosphorylated MyoVa to the nucleus, and these phosphorylation-induced conformational modifications might be implicated in the generation of new functional sites for the recognition and binding of nuclear proteins. Additionally, a high resolution analysis of the CBD binding to MD indicates a major role for the MD N-terminal subdomain in the stabilization of

| TABLE 4 |
| Cargo-binding Domain from Human MyoV Paralogs |

| Domain | Position | Residue | Atom | Domain | Position | Residue | Atom | Distance Å |
|--------|----------|---------|------|--------|----------|---------|------|-----------|
| Main chain-main chain bonds | 136 | Asp | N | CBD | 1706 | Leu | O | 2.89 |
| | 1708 | Lys | N | CBD | 134 | Asp | O | 2.96 |
| | 1709 | Asp | N | MD | 134 | Asp | O | 2.99 |
| | 1798 | Val | N | MD | 66 | Asp | O | 3.03 |
| | 1798 | Val | N | MD | 69 | Val | O | 3.44 |
| Main chain-side chain bonds | 1784 | Asn | ND2 | MD | 70 | Gly | O | 3.05 |
| | 1816 | Gln | NE2 | MD | 115 | Gln | O | 3.12 |
| Side chain-side chain bonds | 96 | Ser | OG | CBD | 1784 | Asn | OD1 | 3.32 |
| | 96 | Ser | OG | CBD | 1784 | Asn | ND2 | 3.26 |
| | 97 | Lys | NZ | CBD | 1813 | Asp | OD1 | 2.89 |
| | 122 | Asp | OD1 | CBD | 1622 | Gln | NE2 | 3.92 |
| | 709 | Arg | NH1 | CBD | 1791 | Glu | OE1 | 2.75 |
| | 709 | Arg | NH1 | CBD | 1791 | Glu | OE1 | 3.25 |
| | 709 | Arg | NH1 | CBD | 1791 | Glu | OE2 | 2.89 |
| | 1622 | Gln | NE2 | MD | 122 | Asp | OD1 | 2.92 |
| | 1707 | Arg | NH1 | MD | 130 | Gln | OE1 | 3.34 |
| | 1707 | Arg | NH1 | MD | 130 | Gln | OE1 | 3.12 |
| | 1707 | Arg | NE | MD | 134 | Asp | OD2 | 2.85 |
| | 1707 | Arg | NH2 | MD | 134 | Asp | OD2 | 3.00 |
| | 1707 | Arg | NH2 | MD | 71 | Glu | OE1 | 2.98 |
| | 1707 | Arg | NH2 | MD | 71 | Glu | OE2 | 2.96 |
| | 1707 | Arg | NH2 | MD | 73 | Asp | OD2 | 2.89 |
| | 1781 | Lys | NZ | MD | 136 | Asp | OD1 | 3.23 |
| | 1781 | Lys | NZ | MD | 136 | Asp | OD2 | 2.76 |
| | 1784 | Asn | OD1 | MD | 96 | Ser | OG | 3.32 |
| | 1784 | Asn | ND2 | MD | 96 | Ser | OG | 3.26 |
| | 1795 | Arg | NE | MD | 71 | Glu | OE2 | 2.97 |
| | 1795 | Arg | NH2 | MD | 71 | Glu | OE2 | 2.89 |
| | 1799 | Ser | OG | MD | 66 | Asp | OD2 | 3.16 |
| | 1802 | Arg | NH1 | MD | 95 | Asp | OD2 | 3.06 |
| | 1809 | Arg | NH2 | MD | 95 | Asp | OD1 | 2.90 |
| | 1822 | Lys | NZ | MD | 121 | Glu | OE1 | 2.84 |
| | 1822 | Lys | NZ | MD | 121 | Glu | OE2 | 3.13 |
the inhibited folded conformation through extensive electrostatic interactions with the CBD lobule II that may promote steric impediments to the movement of the MD converter subdomain by ATP hydrolysis. Based on the CBD-MD complex, a structural model for the folded conformation was proposed (Fig. 12) that corroborates all experimental data and supports the reinterpretation of the cryo-EM electron density map by Sellers et al. (53).

The provision of all three MyoV-CBD structures from humans sheds light into the complex structural puzzle of MyoV members from higher eukaryotes, permitting an in-depth molecular view of the enormous amounts of functional data concerning this myosin class that is the most studied unconventional myosin. Furthermore, our data highlight the MyoVc subclass with unique features of its CBD such as particular lobule arrangement and self-dimerization ability in a coiled coil-independent manner regulated by a redox mechanism.

FIGURE 12. A structural model for the inhibited conformation of MyoVa. a, superposition of the CBD-MD complex (this study) on the 2DFS model proposed by Liu et al. (22) indicating a disagreement with EM experimental data (electron density map shown in gray). b, the best fit of the CBD-MD complex into the cryo-EM electron density map (gray) obtained by rigid body refinement that resembles the single-particle images (23). c, a structural model for the folded conformation of MyoVa based on the CBD-MD complex docked into the cryo-EM map in a similar orientation observed in the single-molecule studies (23), the fitting of the six MyoVa molecules into the rosette arrangement (upper panel) according to our results with the CBD-MD interface formed by intramolecular contacts without domain swapping, which is in agreement with the reinterpretation proposed by Sellers et al. (53). In this configuration, each molecule is tilted and laid over the adjacent molecule to form the rosette architecture as schematically represented (lower panel).

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