Identification of a minimal myosin Va binding site within an intrinsically unstructured domain of melanophilin

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Myosin V is a molecular motor that transports a variety of cellular cargo, including organelles, vesicles, and messenger RNA. The proper peripheral distribution of melanosomes, a dense pigment-containing organelle, is dependent on actin and the activity of myosin Va. The recruitment of myosin Va to the melanosome and proper transport of the melanosome requires melanophilin, which directly binds to myosin Va and is tethered to the melanosome membrane via Rab27a. Here we use highly purified proteins to demonstrate that the globular tail domain of myosin Va binds directly to an intrinsically unstructured domain of melanophilin. The myosin Va-binding domain of melanophilin lacks stable secondary structure, and 1H-NMR measurements indicate that the protein is unfolded. This domain is extremely sensitive to mild proteolysis, and has a hydrodynamic radius that is consistent with a random coil-like polypeptide. We show that myosin Va binding does not induce the global folding of melanophilin. Truncations of melanophilin were utilized to define a short peptide sequence (26 residues) within melanophilin that is critical for myosin Va binding. We demonstrate that a peptide corresponding to these residues binds directly to the globular tail domain with the same affinity as melanophilin. We discuss the possible implications of protein intrinsic disorder in recruitment and maintenance of myosin Va on melanosome membranes.

Myosin V is a processive, actin-based molecular motor that transports a variety of organelles and other cargo. The biological function of myosin V in the transport of melanosomes has been studied in great detail.
transport and membrane localization of myosin Va but may function to stabilize the complex in vivo.

In addition to its role in the localization of the motor to its cargo, the globular tail domain can also regulate the activity of the myosin Va motor. In the absence of free Ca\(^{2+}\), the globular tail domain inhibits the activity of the motor domain (13). Under these conditions the addition of purified melanophilin partially restores the activity of myosin Va in vitro (14). However, melanophilin does not activate myosin Va as well as the addition of Ca\(^{2+}\) (V\(_{\text{max}}\) = 6.6 sec\(^{-1}\) head\(^{-1}\) versus 20 sec\(^{-1}\) head\(^{-1}\), respectively). It is not clear whether melanophilin and Ca\(^{2+}\) activate the myosin Va motor by the same mechanism, but melanosome transport and activation of the myosin Va motor require the direct interaction of the globular tail domain and melanophilin. To date, a detailed biochemical study of the interaction of myosin Va and melanophilin has not been reported. Indeed, biochemical characterization of the binding of any motor to its cargo has been very limited.

Fukuda et al. (2004) demonstrated that melanophilin contains several predicted PEST-like motifs and that these sites are sensitive to proteolysis (15). PEST (proline-, glutamate-, serine-, and threonine-rich) motifs are sites of targeted protein proteolytic degradation and are often localized to regions of protein disorder (16). While most cellular proteins must fold into a single well-defined structure in order to fulfill their biological function, modern genomic and proteomic approaches have led to the discovery of proteins lacking a characteristic compact, globular conformation (17,18). These intrinsically unstructured proteins (IUPs) perform vital cellular functions yet could not have been identified by traditional biochemical approaches due to their inherent proteolytic sensitivity. IUPs are often part of signal transduction pathways and often interact with many binding partners (19,20). Understanding the function of unstructured proteins may also have medical significance. IUPs are overrepresented in genes associated with diseases such as cancer and cardiovascular disease (21,22).

Here we use a variety of biochemical and biophysical approaches to demonstrate that melanophilin, specifically its myosin Va-binding domain, is an intrinsically unstructured protein. We show that it lacks any stable secondary structure, and \(^1\)H-NMR measurements indicate that it does not have a stable tertiary fold. Purified melanophilin is very sensitive to mild proteolysis, and its hydrodynamic radius is inconsistent with a compact, globular protein. These conclusions are supported by an analysis of its primary sequence and are consistent with the previously published observation that melanophilin expressed in mammalian cells is sensitive to proteolysis. In addition, we do not find any evidence that melanophilin folds into a compact, globular structure when bound to myosin Va. Finally, we identify a 26 amino acid peptide that is both necessary and sufficient to bind to the myosin Va globular tail domain.

**EXPERIMENTAL PROCEDURES**

Cloning and recombinant protein expression and purification – The globular tail domain of mouse myosin Va was amplified from a spleen mRNA library (Clontech). The sequence encoding the globular tail domain (Accession number Q99104, amino acids 1446-1853) was cloned into a modified pET28b vector in which the thrombin recognition sequence was replaced with a tobacco etch virus (TEV) protease recognition sequence. N-terminally His-tagged myosin Va globular tail domain (M5-GTD) was overexpressed in Rosetta 2 (DE3) cells (Novagen). Overnight cultures were diluted 100-fold into 2xYT media containing 30 μg/ml kanamycin and 34 μg/ml chloramphenicol. Protein expression was induced with the addition of 1 mM IPTG when the OD\(_{600}\) reached ~ 0.6. After 3 hours at 37°C the cells were harvested and stored at -80°C.

For the preparation of His-tagged M5-GTD, cells were resuspended in lysis buffer (30 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, 10% glycerol, and 0.02% β-mercaptoethanol, pH 8.0) including Complete EDTA-free Protease Inhibitors (Roche Applied Science). The cells were lysed by two passes through a French pressure cell with a cell pressure of 16,000 psi. The lysate was clarified at 37,000 x g for 30 min and then combined with 2 ml of washed Ni-NTA agarose per gram of cell pellet. This slurry was mixed for 45 min at 4°C and then washed with 10 column volumes of lysis buffer. Bound protein
was eluted with lysis buffer containing 300 mM imidazole, pH 8.0. Eluted protein was dialyzed into TEV buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02\% β-mercaptoethanol, 1 mM EDTA, pH 8.0) and then digested at 4°C with 1 mg His-TEV-Arg per 200 mg of recombinant protein overnight. His-TEV-Arg was expressed as a maltose binding protein fusion and purified as previously described (23). Uncleaved M5-GTD and the TEV protease were removed by passing the sample again over a Ni-NTA column. The flow-through was further purified by gel filtration chromatography in 25 mM HEPES-KOH, 150 mM NaCl, 1 mM DTT, pH 7.5. The concentration of M5-GTD was determined by measuring its absorbance at 280 nm using the extinction coefficient calculated from its amino acid sequence (ε = 31400 M⁻¹ cm⁻¹).

Melanophilin (Mlph) was amplified from a full-length cDNA obtained from the Mammalian Gene Collection (MGC-11931). Mlph residues 147-403 were cloned either into pGEX-4T or a variant of pGEX-4T that contains a TEV protease recognition sequence. Nonsense mutations were introduced into the coding sequence of GST-Mlph147-403 by site-directed mutagenesis to create truncations. For pulldown experiments GST fusion proteins were purified according to the manufacturer’s instructions using glutathione-sepharose 4B (GE Healthcare).

Untagged Mlph147-403 and Mlph147-240 were purified according to the following procedure: cells expressing GST-Mlph147-403 (or GST-Mlph147-240) were resuspended in PBS containing 5 mM DTT and Complete EDTA-free Protease Inhibitors. The cell suspension was sonicated on ice and clarified by centrifugation at 10,000 x g for 10 min followed by filtration through a 0.45 μm membrane. Filtered lysate was applied to a GSTPrep column (GE Healthcare). The column was washed with 15 column volumes of PBS containing 1 M NaCl and 5 mM DTT. GST-Mlph147-403 was eluted with 50 mM Tris-HCl pH 8.0 containing 20 mM reduced glutathione. Overnight treatment of the GST-Mlph147-403 fusion protein with recombinant His-TEV-Arg resulted in site-specific cleavage. The TEV-treated sample was passed over a GSTPrep column equilibrated in 20 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, pH 8.0 to remove uncleaved fusion protein and GST. The flow-through was loaded on a Mono Q 10/10 column and eluted with a linear gradient to 1 M NaCl. The purified proteins migrated anomalously on SDS-PAGE gels, for example Mlph147-403, which has a predicted molecular weight of 28 kDa migrated with an apparent molecular weight of 50 kDa. MALDI mass spectrometry was performed at the Stanford Protein and Nucleic acid facility (PAN). The observed mass of Mlph147-403 (28023.7 m/z) and Mlph147-240 (10690.3 m/z) were consistent with their predicted molecular weights. The concentrations of the cleaved Mlph proteins were determined from their absorbance at 280 nm as described above (Mlph147-403 ε = 2980 M⁻¹ cm⁻¹ and Mlph147-240 ε = 1490 M⁻¹ cm⁻¹).

Limited proteolysis – Mlph147-403 and monomeric BSA were dissolved to 0.5 mg ml⁻¹ in 50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, pH 8.0. Trypsin (Roche Applied Science) or proteinase K (New England Biolabs) was added to 1 μg ml⁻¹ to initiate the reaction. Proteolysis was performed on ice. The reaction was stopped at the indicated time points by the addition of an equal volume of Laemmli sample buffer and immediately boiling the sample. The samples (2 μg) were then separated on NuPage 12% Bis-Tris gels (Invitrogen Corporation) with MOPS-SDS running buffer. The observed bands were excised from the gel and tandem mass spectrometry was performed at PAN to identify the products of the proteolysis reaction.

Circular dichroism – Circular dichroism spectra were recorded on an AVIV model 68S spectrometer in a 0.1 cm pathlength cuvette. Proteins were exchanged into 20 mM potassium phosphate pH 7.5 and diluted to 6 μM. To measure spectra of the complexes both proteins were mixed (6 μM each) in CD buffer and incubated overnight on ice. For each experiment at least three scans were taken from 270 to 190 nm in 0.5 nm increments with 2 second averaging time. The scans were averaged and then blank scans were subtracted. Mean residue ellipticity ([Θ]) was calculated from according to equation 1, where Θ is the raw signal in millidegrees, l is pathlength in cm, n is the number of amino acids, and c is the concentration of the protein in moles per liter.
The theoretical spectra were calculated from the spectra of the individual proteins according to equation 2; where \([\Theta]\) is the observed mean residue ellipticity for the indicated protein and \(n\) is the number of residues for the indicated protein(s). In both cases a 1:1 stoichiometry was assumed based on observations from other experiments.

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eq 1. \quad [\theta] = \frac{\theta}{(10 \times l \times n \times c)}
\]

Measurement of hydrodynamic properties – The hydrodynamic radius of Mlph147-403, M5-GTD and the complex were measured by size-exclusion chromatography and dynamic light scattering (DLS). Purified Mlph147-403 or M5-GTD, 10 nanomoles each, was loaded on a Superdex 200 HR column equilibrated in 25 mM HEPES-KOH, 150 mM NaCl, 1 mM DTT, pH 7.5, at a flow rate of 0.5 ml min\(^{-1}\). Fractions (0.5 ml each) were collected and analyzed on NuPage 12% Bis-Tris gels run with MOPS-SDS running buffer. The hydrodynamic radius \(R_h\) was determined by comparing its elution volume to several standard proteins of known \(R_h\). To measure \(R_h\) of the complex, 10 nanomoles of Mlph147-403 was mixed with 10 nanomoles M5-GTD and incubated overnight at 4°C. The complex was then applied to the Superdex 200 HR column as described above.

For DLS measurements the peak fractions were pooled, concentrated to 15-20 \(\mu\)M, and clarified for 5 minutes at 14,000 \(x\) g. The clarified samples were analyzed in a DynaPro Titan (Wyatt Technology Corporation) instrument. In all cases the samples were observed to contain a single monodisperse scattering source. The diffusion coefficient for each sample was determined from the 2\(^{nd}\) order autocorrelation function. The hydrodynamic radius was then calculated according to the Stokes-Einstein equation.

The oligomerization state of Mlph147-403 and M5-GTD was measured by size-exclusion chromatography with inline multiple angle light scattering (SEC-MALS). Briefly, 100 \(\mu\)l of protein samples at 3 mg ml\(^{-1}\) were injected onto a Shodex Protein KW-803 HPLC column at a flow rate of 0.5 ml min\(^{-1}\). The columns were equilibrated in 25 mM HEPES-KOH, 150 mM NaCl, 1 mM DTT, pH 7.5. Protein concentration was determined with an Optilab rEX refractive index detector and scattering was detected with a Dawn 18 angle MALS light scattering instrument (Wyatt Technology Corporation). In all cases detectors 7-15 were used for the final determination of the molecular mass.

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eq 2. \quad [\theta] = \frac{[\theta]_{\text{GTD}} \times n_{\text{GTD}}}{n_{\text{complex}}} + \frac{[\theta]_{\text{GTD}} \times n_{\text{Mlph}}}{n_{\text{complex}}}
\]

\(^1\)H-NMR Spectra - Protein samples were exchanged into 20 mM sodium phosphate, 75 mM NaCl, pH 7.0, by passing the samples over a Superdex 200 16/60 column. The proteins were then concentrated by ultrafiltration. D\(_2\)O was added to 10\% just prior to recording proton spectra. Spectra were acquired on 400 \(\mu\)M protein samples as well as 10 fold dilutions. One-dimensional \(^1\)H spectra of Mlph147-403 and Mlph147-240 were acquired on a 600 MHz Varian Inova spectrometer running VNMR 6.1C at the Stanford Magnetic Resonance Laboratory. A variable temperature controller was used to regulate samples at 25 °C. Presaturation water suppression experiments (1 s presaturation) and WET water suppression experiments (24) were acquired with 8192 points, 8384.86 Hz spectral width, and 1.49 s recycle delay. 64 scans were acquired per experiment for the 400 \(\mu\)M samples, and greater than 350 scans for the 40 \(\mu\)M samples. Spectra were processed with 1 Hz line-broadening and manually baseline corrected.

GST pulldowns – GST-Mlph fusion proteins, 1 or 10 \(\mu\)M, were mixed with a 5 fold excess of M5-GTD and 50 \(\mu\)l glutathione-agarose in 500 \(\mu\)l of 25 mM HEPES-KOH, 75 mM NaCl, 1 mM DTT, 0.05% Triton X-100, pH 7.5. The beads were washed three times with 500 \(\mu\)l of the same buffer and bound proteins were eluted with Laemmli sample buffer. The amount of GST fusion loaded in each well was normalized in Figure 5B for clarity.

To compare the relative affinity of the GST-Mlph truncations for M5-GTD, 1.6 \(\mu\)M of the GST-Mlph fusion proteins were incubated with increasing concentrations of M5-GTD (0.3-10 \(\mu\)M) and 50 \(\mu\)l glutathione-agarose as above. The amount of M5-GTD retained on the glutathione-agarose beads was
quantified using Image J. Briefly, the observed integrated density value (IDV) of M5-GTD was normalized to the average IDV of the GST fusion proteins to correct for gel-to-gel variation in Coomassie staining intensity. Then the amount of M5-GTD bound was plotted as a function of total M5-GTD and fit to the rectangular hyperbola in equation 3, where c is a constant that describes the maximum observed signal and $K_{GTD}$ is the concentration of M5-GTD in μM that produced half-maximal saturation of the binding equation. For GST-Mlp147-403, GST-Mlp147-220, and GST-Mlp147-201 both c and $K_{GTD}$ were free parameters. Since neither GST-Mlp147-192 nor GST-Mlp147-176 binding curves approached saturation, c was fixed in these fits as the average of the other fits.

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\text{eq. 3. } M5-GTD_{\text{bound}} = \frac{[M5-GTD] \times c}{[M5-GTD] + K_{GTD}}
\]

**Competitive displacement assay** - Peptides corresponding to melanophilin residues 176-201 and 193-201 were synthesized at the Stanford Protein and Nucleic Acid Facility (PAN). Unpurified peptides (80-90% pure as judged by analytical HPLC) were dissolved in 20 mM Tris-HCl pH 8.0 containing 10% DMSO. Note that because these peptides were not purified and the concentrations used in the competition experiments are based on the mass dissolved, the concentrations of peptide in the experiment are approximate. Competitive binding experiments were performed with 1 μM GST-Mlp147-403, 5 μM M5-GTD, and 2.5-40 μM of Mlp-derived peptides. The amount of M5-GTD nonspecifically retained by the beads was subtracted from each data point.

**Isothermal titration calorimetry** - ITC measurements were performed on a VP-ITC calorimeter (Microcal Inc.) at 30°C. In all cases the titration experiment was initiated with 2 x 2 μl injections followed by 28 x 8 μl injections. Untagged M5-GTD and Mlp147-403 were prepared as described above and both were dialyzed extensively into 20 mM MES-KOH, 100 mM NaCl, 1 mM DTT, pH 6.5. Mlp147-403 (150 μM) was injected into a 15 μM solution of M5-GTD. To measure the affinity of Mlp176-201 for M5-GTD, a peptide corresponding to these residues plus a C-terminal tyrosine for accurate concentration determination, was purchased at > 95% purity (Genscript Corporation) and dissolved in 20 mM HEPES-KOH, 100 mM NaCl, 2 mM TCEP, pH 7.5 to a final concentration of 115 μM. Mlp176-201 was injected into a 10 μM solution of M5-GTD that had been dialyzed into the same buffer. Each sample was degassed immediately before use. The dissociation constant (Kd), the stoichiometry (n), and the enthalpy change (ΔH) were determined by fitting the data to a single-site interaction model using the Origin software package provided by the manufacturer.

**RESULTS**

**Primary sequence analysis of melanophilin** - Melanophilin contains an N-terminal Rab-binding domain, a myosin Va-binding domain, and a C-terminal end binding protein-1 (EB1)/actin-binding domain (Figure 1A). We used the Predictors Of Natural Disordered Regions (PONDR) to identify potential unstructured domains in melanophilin (25,26). As shown in Figure 1B, much of melanophilin is predicted to be unstructured, which is consistent with the high fractional composition of disorder-promoting amino acids (A, R, G, Q, S, P, E, and K) relative to order-promoting amino acids (W, C, F, I, Y, V, and L). A notable exception is the N-terminal Rab-binding domain. This domain shares high sequence identity with the N-terminus of rabphilin 3A, which forms a well-ordered structure bound to Rab3A (27). These analyses suggest that both the myosin binding domain and the C-terminal domain – or more than two-thirds of the total protein – probably lack a well-defined, compact fold.

**Evaluating the affinity of the globular tail domain-melanophilin interaction** - Melanophilin residues 147-403 or 147-241 (Mlp147-403 or Mlp147-241, respectively) were expressed as GST fusion proteins and purified by affinity chromatography. The Mlp fragments were cleaved from GST as described above and purified to homogeneity. The myosin Va globular tail domain (M5-GTD) was expressed with an N-terminal His-tag and purified to homogeneity. The myosin Va globular tail domain (M5-GTD) was expressed with an N-terminal His-tag and purified to homogeneity. The myosin Va globular tail domain (M5-GTD) was expressed with an N-terminal His-tag and purified to homogeneity. Subsequently the His-tag was removed leaving only two non-native residues (Gly-His) at the N-terminus of the recombinant protein.

We tested the ability of M5-GTD to interact directly with Mlp147-403 and Mlp147-241 using
purified proteins. The thermodynamics of the interaction were measured by isothermal titration calorimetry (ITC). A typical ITC experiment is shown in Figure 1C. We found that M5-GTD binds directly to M1ph to form a 1:1 complex, and that M5-GTD binds to both M1ph147-403 and M1ph147-240 with a dissociation constant of ~ 0.5 μM. This is the first time the stoichiometry and affinity of this protein-protein interaction has been measured.

The myosin Va-binding domain of melanophilin is intrinsically disordered - Circular dichroism (CD) spectroscopy is a sensitive means to assess the secondary structural features of a protein in solution. To investigate the secondary structure of M1ph147-403, CD spectra were recorded at 25°C in 20 mM potassium phosphate buffer (Figure 2A). The spectrum of M1ph147-403 exhibits a large minimum at ~ 200 nm and a shallow trough between 215-235 nm. There is no evidence for any stable secondary structure; distinct minima at 208 and 222 nm, characteristic of α-helices, are not present. Nor do we observe a minimum at 217 nm, which would indicate the formation of some stable β-sheet secondary structure. We monitored the helicity at 222 nm as a function both of temperature and urea concentration. Neither increasing temperature (up to 80°C) nor urea concentrations to 8 M had a significant effect on the observed helicity, which is consistent with an unstructured protein. Spectra recorded in phosphate buffered saline, a more physiological buffer, were very similar to those reported here (data not shown).

The observed minimum at 200 nm is consistent with polyproline type II helix (PII) backbone conformations. Previous studies argue that PII backbone conformations are a predominant conformation in unfolded proteins (reviewed in 28) and PII helices have been observed in other IUPs (29,30). However, model peptides that adopt PII conformations typically display a weak positive peak at 220 nm, in addition to the 200 nm minimum, which we do not observed here. This suggests that PII structure is a minor and perhaps transient backbone conformation of M1ph147-403. We conclude that stable secondary structural elements are not formed in M1ph147-403. This conclusion is consistent with secondary structural predictions such as PHDsec and JPRED (31,32), which predict the presence of very little secondary structure within M1ph147-403.

Because M1ph might in principle have a regular folded conformation that lacks any stable secondary structure, we examined both M1ph147-403 and M1ph147-240 by one-dimensional 1H-NMR at 25°C. Consistent with an unstructured protein the amide and aliphatic protons showed very little dispersion (Figure 2B). The amide peaks were clustered between 7.8 and 8.6 ppm, typical of unfolded proteins. And we do not observe any up-shifted methyl peaks (< 1 ppm) that would be indicative of buried alkyl groups present at the hydrophobic core of a properly folded protein. Additionally, we observed that the presaturation water suppression noticeably reduced the signal from the amide protons compared to the WET suppression method (data not shown). This suggested that most, if not all, of the amide protons are readily exchanging with solvent, and not protected by hydrogen bonding due to secondary structure or buried in a stable solvent inaccessible core. Together the NMR data strongly argue that M1ph147-403 and M1ph147-240 are unstructured in solution.

While folded, globular proteins tend to be largely resistant to mild treatment with proteases, unfolded proteins are very susceptible to proteolysis. Fukuda and colleagues have reported that recombinant melanophilin expressed in mammalian cells is extremely sensitive to both trypsin and calpain proteases (15). We found that purified melanophilin is also very sensitive to proteolysis. Purified M1ph147-403 was treated with limiting amounts of trypsin and proteinase K; as a control BSA was also treated with protease under identical conditions. M1ph147-403 is clearly much more sensitive to mild proteolysis as compared to BSA (Figure 2C). Both trypsin and proteinase K degrade M1ph147-403 within 15 minutes under these conditions, while the globular BSA protein is largely resistant to the protease treatment even after 30 minutes. However, several relatively stable products resulted both from proteinase K and trypsin proteolysis (Figure 2C, asterisks). These bands were excised from the gel and the identity of these products was determined by tandem mass spectrometry. The peptides identified by this approach encompass M1ph residues 299-322 (data not shown). The
observation that some stable products result from mild proteolysis suggests that some structured elements could be present within Mlph147-403. However, we conclude that Mlph147-403 as a whole is sensitive to protease digestion, consistent with a largely disordered protein.

The hydrodynamic radii ($R_h$) of folded, globular proteins are characteristically smaller than denatured proteins of the same molecular weight. We assessed the value of $R_h$ for Mlph147-403 and Mlph147-240 under non-denaturing conditions by size-exclusion chromatography and dynamic light scattering (DLS). The observed $R_h$ of Mlph147-403 was 4.9 nm by size-exclusion chromatography and 4.6 nm by DLS (Figure 2D, and Table 1). For comparison, a 260 residue globular protein would have an $R_h$ of approximately 2.4 nm (18). The observed value of $R_h$ is inconsistent with a monomeric globular, folded protein, but is consistent with a random coil-like natively unfolded polypeptide. The results obtained for Mlph147-240 showed a similar discrepancy between the observed and expected $R_h$ (Table 1). Size-exclusion chromatography with inline multiple angle light scattering (SEC-MALS) measurements show that Mlph147-403 is a monomer under these conditions (Table 1). Thus, the unusually large hydrodynamic radius of the melanophilin myosin Va-binding domain is an intrinsic property of the molecule that is not due to oligomerization of the molecule under the conditions tested.

To summarize, the primary sequence, CD spectrum, $^1$H-NMR spectrum, proteolytic sensitivity, and hydrodynamic properties of Mlph147-403 all indicate that it is an intrinsically disordered protein. Furthermore, we have shown that recombinant Mlph147-403 binds directly to M5-GTD. This indicates that the lack of stable structure described here is compatible with the function of this molecule.

Melanophilin is not induced to fold upon binding to the myosin Va globular tail domain - The binding of an IUP to another protein stabilizes the conformation of the IUP at the interface between the two proteins. Binding is often concomitant with the formation of stable secondary or tertiary structures in the IUP; several examples of coupled folding and binding events are reviewed in Dyson and Wright (2005) (17). In some cases binding is associated with large-scale folding as is the case when two natively unfolded proteins, CBP/p300 and human ACTR, fold cooperatively to form a heterodimer (33). To assess the global conformation of the M5-GTD-Mlph147-403 complex we first measured its hydrodynamic radius. The hydrodynamic radius of the complex measured by size-exclusion chromatography and DLS, is 5.4 nm (Figure 3 asterisk and Table 1). Assuming Mlph147-403 were to fold completely upon binding, the complex should have an $R_h$ of ~ 3.4 nm. The measured radius is consistent with complex formation without Mlph147-403 folding.

When equimolar amounts of Mlph147-403 and M5-GTD are mixed and injected onto a size-exclusion column, we observe a single peak that contains equivalent amounts of the proteins (Figure 3). The addition of a 2 fold molar excess of M5-GTD results in two observed peaks of approximately equal absorbance: the complex and the free M5-GTD peaks. This result supports our previous conclusion that M5-GTD and Mlph147-403 form a 1:1 complex. The binary complex was purified by size-exclusion chromatography and subjected to proteolysis as before. We do not observe any protection of Mlph147-403 in the presence of M5-GTD (data not shown), again supporting our conclusion that formation of the binary complex does not induce major structural changes in either protein, but in melanophilin in particular.

While M5-GTD binding does not induce any global structural changes in Mlph147-403, it may stabilize the folding of local secondary structure, as has been observed for many other similar protein-protein interactions. To test whether binding stabilizes any secondary structure in Mlph147-403, we compared CD spectra for the two components with a CD spectrum of the complex. The observed spectrum of the complex is indistinguishable from a calculated spectrum for a theoretical non-interacting mixture (Figure 4A). This is also true for M5-GTD bound to Mlph147-240 (Figure 4B). Note that at the concentrations of the proteins used in this experiment we expect that > 90% should form a stable binary complex. More sensitive techniques (e.g. heteronuclear NMR) may detect the stabilization of short elements of secondary structure in the complex. From the data presented here, however, we see no evidence that binding causes the
formation or stabilization of any significant secondary structure.

**GST-Mlp147-403 truncations reveal the minimal M5-GTD binding site** - Because Mlp147-403 is disordered in solution and M5-GTD binding does not induce large structural changes, we hypothesized that the molecular interface between the two proteins consists of a linear segment of amino acids from melanophilin bound to M5-GTD. Therefore, we introduced a series of nonsense mutations into GST-Mlp147-403 in order to identify sequences that are required for binding to M5-GTD. The mutations were designed to preserve highly conserved sequences and to separate groups of charged residues as indicated in Figure 5A.

Each of the purified GST fusion proteins (10 or 1 μM, as indicated in Figure 5B) was incubated with an excess of M5-GTD; proteins retained on the beads were analyzed by SDS-PAGE. As shown in Figure 5B, the first two truncations (GST-Mlp147-220 and GST-Mlp147-202) bound M5-GTD with affinities similar to full-length GST-Mlp147-403. Deletion of another 9 amino acids (GST-Mlp147-192) led to a noticeable reduction in the amount of M5-GTD retained by the GST-Mlp fusion at 1 μM. Removal of an additional 16 amino acids led to an almost complete loss of binding (GST-Mlp147-175).

The GST pulldown assay was modified slightly to quantitatively evaluate the effects of the truncations described above. In this assay a fixed concentration of the GST-Mlp fusion protein (generally 1.6 μM) was incubated with a range of M5-GTD concentrations from 0.1 μM to 10 μM. The amount of bound M5-GTD was observed by SDS-PAGE and quantified using Image J. The resulting data (Figure 5C) was fit as described in Experimental Procedures to determine the amount of M5-GTD required for half-maximal binding to each GST fusion protein. GST-Mlp147-403, GST-Mlp147-220, and GST-Mlp147-201 reach half-maximal observed binding at less than 1 μM M5-GTD, consistent with the measured dissociation constant of ~ 0.5 μM. M5-GTD binding to GST-Mlp147-192 is at least 10 fold weaker as measured by this assay, reaching half-maximal binding at greater than 15 μM M5-GTD. A reasonable fit for GST-Mlp147-176 was not possible given the insignificant amount of bound M5-GTD observed at all concentrations tested. Clearly, melanophilin residues 176-201 are absolutely required for M5-GTD binding.

**Mlp176-201 binds directly to M5-GTD** - The GST pulldown assay described above identified the residues that are required for high affinity binding to M5-GTD. However, this assay does not address whether these residues are sufficient for this interaction. We sought to determine whether synthetic peptides corresponding to melanophilin residues 176-201 (Mlp176-201) or 193-201 (Mlp193-201) could bind to M5-GTD. To address this question we set up a competitive displacement assay. Excess M5-GTD was mixed with GST-Mlp147-403 and increasing concentrations of peptide. The amount of M5-GTD retained on the beads was analyzed by SDS-PAGE (Figure 6A & B). The results were quantified and we found that Mlp176-201 but not Mlp193-201 effectively displaces M5-GTD (Figure 6C).

To directly assess the affinity of this interaction another Mlp176-201 peptide was synthesized with a C-terminal tyrosine. The tyrosine allowed us to accurately measure the concentration of the peptide in solution. We then measured the thermodynamics of the peptide binding to M5-GTD by ITC. A representative titration is shown in Figure 6D. We found that Mlp176-201 binds to M5-GTD with a ~ 0.5 μM dissociation constant and that the peptide forms a 1:1 complex with M5-GTD. Therefore, we conclude that melanophilin residues 176-201 constitute the myosin Va globular tail domain binding site.

**DISCUSSION**

Unconventional myosin motors in general, and myosin V in particular play a critical role in the intracellular transport of a wide variety of molecular and organelle cargoes. The myosin Va-based transport of melanosomes has been a useful model system for the study of these transport processes because many of the molecular components have been identified and the transport process itself is readily observed with a simple light microscope. However, the interactions between the individual molecular players have not been studied in detail. In this study we have demonstrated that the myosin Va-binding domain of melanophilin is intrinsically unstructured. Additionally, we have shown that
melanophilin does not adopt a more compact or folded conformation when bound to the myosin Va globular tail domain. There are three known receptors for the yeast myosin V homologue Myo2p; two of these, Vac17p and Mmr1p, may contain large potentially disordered regions based on a PONDR analysis of their primary sequence (our unpublished observations). It is conceivable that intrinsic disorder is a common feature of receptors for molecular motors.

We have, for the first time, measured the affinity of the interaction between melanophilin and the myosin Va globular tail domain (Kₐ ~ 0.5 μM). We have also shown that these molecules form a 1:1 complex as determined by analytical size-exclusion chromatography and titration calorimetry. In order to make sense of the observed dissociation constant, we would like to know the intracellular concentration of the proteins involved. While we do not have any information on the cellular concentration of melanophilin, Cheney and colleagues reported that myosin Va constitutes approximately 0.3% of the total protein in brain cells (34). From this we can estimate the cellular concentration of myosin Va in the brain to be about 3 μM. However, myosin Va is expressed at much lower levels in other tissues. It has been estimated that kinesin represents 0.04% to 0.3% of the total cellular protein (35), so we can expect the cellular concentration of myosin Va to vary at least ten fold depending on the cell type, or between 0.3 μM and 3 μM. Although the globular tail domain of myosin Va has a relatively high affinity for melanophilin in vitro, it is clear that the globular tail domain alone is not sufficient to interact with melanophilin in vivo. As described in the Introduction the interaction between myosin Va and melanophilin involves at least one additional interaction, between exon F and melanophilin. This second interaction may contribute significantly to the overall binding affinity. Additionally, Hume et al. (2006) describe a putative coiled-coil in melanophilin that is required for melanosome transport which suggests that dimerization of melanophilin might be required for the interactions of myosin Va and melanophilin in vivo.

Using a series of melanophilin truncations, we narrowed down the binding site of the globular tail domain to a 26 amino acid region of melanophilin. We demonstrated that a synthetic peptide corresponding to melanophilin residues 176-201 is sufficient to displace the myosin Va globular tail domain from melanophilin and it directly binds to the globular tail domain as measured by ITC. Taken together these results begin to describe more completely the recruitment of the myosin Va to melanosomes. Specifically, the globular tail domain of myosin Va binds to a short linear sequence of residues within melanophilin. This peptide is readily accessible for binding as the myosin Va-binding domain of melanophilin is largely unstructured in solution.

Melanophilin is another example of a growing class of unstructured proteins that perform critical cellular functions, yet it remains unclear what “unstructured” means. The traditional understanding of unfolded proteins, for example those exposed to high concentrations of strong denaturants (e.g., 6 M guanidine hydrochloride or 8 M urea), is that they behave as linear random coils (36). However, it was not long before it was recognized that even in these denaturing conditions proteins retain some residual structure (reviewed in (37,38). And heteronuclear multidimensional NMR has enabled investigators to observe that a number of IUPs contain residual secondary and even tertiary structure (39-41).

In some cases transient secondary structural elements, observed by heteronuclear NMR in IUPs, are stabilized at protein-protein interfaces. For example p27, which regulates nuclear cyclin-dependent kinases is partially unstructured in solution but contains some transient helical structures that are further stabilized by binding to Cdk2-cyclin A (42). These types of interaction sites have been dubbed Molecular Recognition Features (MoRFs). One of the key characteristics of MoRFs is that they behave as linear random coils (MoRFs) for elements, observed by heteronuclear NMR in IUPs, and are stabilized at protein-protein interfaces. For example p27, which regulates nuclear cyclin-dependent kinases is partially unstructured in solution but contains some transient helical structures that are further stabilized by binding to Cdk2-cyclin A (42). These types of interaction sites have been dubbed Molecular Recognition Features (MoRFs). One of the key characteristics of MoRFs is a short (< 60 residues) region of predicted order flanked on both sides by disorder regions (43). Interestingly, the Mlph176-201 peptide that binds to the myosin Va globular tail domain overlaps a region of the PONDR prediction for melanophilin that meets these criteria (Figure 1A). Based on an analysis of the RCSB Protein Data Bank, MoRFs were classified as α-MoRFs, β-MoRFs, or α-MoRFs for elements that form α-helical, β-sheet, or irregular secondary structures respectively (44). Some examples of MoRFs include the transactivation domain of p53 bound to MDM2, the Cdc42-binding
domain of WASP, and a short peptide derived from Grim bound to DIAP1. Future work will reveal whether the formation of the binary complex described here induces the formation of secondary structure with the myosin Va-binding domain of melanophilin.

The frequency of proteins that contain long (> 30 residue) unstructured segments is much higher in eukaryotic genomes than in eubacterial or archaean genomes (20). Proteins containing large segments of predicted disorder appear to function in complex cellular processes such as transcriptional regulation, cell signaling, and processes involving the cytoskeleton. While we know that many intrinsically disordered proteins serve these critical cellular functions, it is not immediately evident why evolution has selected unstructured proteins for those functions.

Intrinsically unstructured proteins may have functional advantages such as the capacity to bind multiple proteins, or to form large protein-protein interfaces without requiring extremely large proteins. Unstructured proteins, or unstructured regions within folded protein, are also excellent substrates for regulated degradation. Degradation could be used to rapidly turn off some cellular process. Such a mechanism is employed in the transport of the yeast vacuole to the daughter cell. Vac17p contains a PEST site between its Myo2p and vacuole localization domains and Vac17p is rapidly degraded in the bud. Deletion of the

PEST site disrupts vacuole localization to the daughter cell (45).

Another possibility is that the thermodynamics of protein-protein interactions involving intrinsically unstructured proteins are desirable for some biological functions. As described above binding is often coupled to the folding of an unstructured element of the protein. Spolar and Record (1994) suggested that the coupling of folding and molecular recognition has very characteristic thermodynamic consequences (46). Specifically, the entropic penalty incurred by the folding of an unstructured protein chain must be compensated for by the formation of favorable binding interactions, while avoiding unfavorable interactions. In this situation highly specific complexes can be formed, but as a result of the entropic penalty the overall thermodynamic binding affinity is weakened. As such, IUPs may be able to form complexes with high specificity and low affinity, a property that could be advantageous where cargo must be released at the appropriate site within the cell. That is, low affinity (i.e., micromolar as opposed to nanomolar) may serve a physiological purpose. In this case, the ability to release cargo at the appropriate time, location, or in response to a cellular signal may be as important as the movement of the cargo. Any of these properties of IUPs may be employed alone or in combination to carry out complex biological processes such as the transport of molecular cargo.

REFERENCES

46. Spolar, R. S., and Record, M. T., Jr. (1994) Science 263(5148), 777-784

ACKNOWLEDGEMENTS

We thank Corey Liu of the Stanford Magnetic Resonance Laboratory (SMRL) for assistance collecting the 1H-NMR spectra. The SMRL is supported in part by the Stanford University School of Medicine. This work was financially supported by NIH PO1 AR42895 to JAS. NCG was supported by a Howard Hughes Medical Institute Predoctoral Fellowship.

FIGURE LEGENDS

Figure 1. A, Schematic of the known functional domains of melanophilin, including the N-terminal Rab-binding domain, the myosin Va-binding domain, and the EB1/F-actin-binding domain. Residue numbers above the schematic define the boundaries between these domains. B, Predictors Of Natural Disordered Regions (PONDR) analysis of mouse melanophilin. Note that melanophilin contains large segments of predicted disorder, including most of the myosin Va-binding domain (indicated with thick black bars). C, An example isothermal calorimetric titration of Mlph147-430 into a 15 μM solution of M5-GTD. The raw data (inset) was integrated and fit to a single-site binding model (solid line) using the Origin software package. The observed dissociation constant (K_d) for each titrant is shown.

Figure 2. The myosin Va-binding domain of melanophilin is intrinsically unstructured. A, The CD spectra of Mlph147-403 (black line) and Mlph147-240 (gray line) recorded at 25°C are consistent with an unfolded protein. B, 1H-NMR spectrum of 400 μM Mlph147-403 shows that all of the amide proton peaks are clustered between 7.8 and 8.6 ppm and we do not observe any up-shifted methyl peaks. Both results are consistent with an unfolded protein. C, BSA and Mlph147-403 were digested with either trypsin or proteinase K. Mlph147-403 is rapidly degraded under these conditions. D, Mlph147-403 was applied to a Superdex 200 HR column as described. The elution volume of Mlph147-403 was compared to several globular protein standards whose elution volumes are indicated with arrows. The standards used were, from left to right: dextran (void volume), apoferritin (R_h = 6.1 nm), alcohol dehydrogenase (R_h = 4.5 nm), ovalbumin (R_h = 3.05 nm), and ribonuclease A (R_h = 1.64 nm). We conclude that Mlph147-403 has hydrodynamic volume of 4.9 nm, see Table 1. As noted in the text Mlph147-403 is monomeric under these conditions.

Figure 3. M5-GTD (closed arrowhead), Mlph147-403 (open arrowhead), or the indicated mixtures were subjected to size-exclusion chromatography. The retention volume of the complex (*) corresponds to a calculated hydrodynamic radius of 5.2 nm. As described in the text this radius is inconsistent with a folded, globular protein-protein complex. Additionally, when a 2 fold molar excess of M5-GTD was mixed with Mlph147-403 we observe both the complex and the M5-GTD
monomer peaks. Fractions corresponding to the peak fractions were collected and separated on NuPage 12% Bis-Tris gels.

Figure 4. CD spectra of M5-GTD-Mlph binary complexes suggest that conformational changes do not accompany binding. Each protein was added to a final concentration of 6 μM, approximately 12 fold above the observed dissociation constant, to ensure sufficient complex formation. A, CD spectra of Mlph<sub>147-403</sub> (green), M5-GTD (blue), an equimolar complex (red), and a theoretical spectrum of a non-interacting mixture of the two proteins (cyan, dashed) are overlaid. B, CD spectra of Mlph<sub>147-240</sub> (green) and the others as in A. Note that in each case the observed spectrum of the complex is identical to the theoretical spectrum.

Figure 5. Truncations of Mlph<sub>147-403</sub> were designed to define the minimal binding site for M5-GTD. A, ClustalW sequence alignment of melanophilin residues 147-240 (mouse) where identical and similar residues are highlighted in green and cyan, respectively. The sequences used in the alignment are: Mus musculus (M.m.), Homo sapiens (H.s.), Rattus norvegicus (R.n.), and Canine familiaris (C.f.). Conserved charged positions are also indicated below the sequence alignment. Nonsense mutations were inserted at the sites indicated with arrowheads. B, GST pulldowns indicate that residues 176-201 are critical for M5-GTD binding. The indicated GST fusion proteins at either 10 or 1 μM, as indicated, were incubated with an excess of M5-GTD. The protein retained on the beads was analyzed by SDS-PAGE. The amount of GST fusion protein loaded in each well was normalized for clarity. C, The amount of M5-GTD bound to each of the GST fusion proteins was quantified as described in Experimental Procedures. Error bars are omitted for clarity; the standard error for each measurement was less than 15% of the observed signal.

Figure 6. Mlph<sub>176-201</sub> is sufficient for M5-GTD binding. A, A synthetic peptide that corresponds to melanophilin residues 193-201 does not displace M5-GTD from GST-Mlph<sub>147-403</sub>. B, However, a peptide that is identical to melanophilin residues 176-201 does displace M5-GTD from GST-Mlph<sub>147-403</sub>. The amount of M5-GTD retained on the glutathione-agarose in the absence of the GST fusion is shown in the last lane (bkgd). C, The displacement of M5-GTD in the competitive binding experiments was quantified as described. These results were from at least three independent measurements. D, The dissociation constant for Mlph<sub>176-201</sub> binding to M5-GTD was found to be 0.5 μM by ITC.
Table 1. Summary of hydrodynamic measurements.

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<th></th>
<th>Predicted molecular weight (kDa)</th>
<th>Observed molecular weight (kDa)</th>
<th>Apparent molecular weight (kDa)</th>
<th>Hydrodynamic radius (nm)</th>
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<tr>
<td>Mlp147-403</td>
<td>28</td>
<td>28 $^{a,b}$</td>
<td>185 $^c$</td>
<td>4.9 $^c$ 4.6 $^d$</td>
</tr>
<tr>
<td>M5-GTD</td>
<td>46</td>
<td>46 $^{a,b}$</td>
<td>59 $^c$</td>
<td>3.5 $^c$ 3.8 $^d$</td>
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<tr>
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<td>n.d.</td>
<td>262 $^c$</td>
<td>5.3 $^c$ 5.4 $^d$</td>
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<tr>
<td>Mlp147-240</td>
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<td>10 $^{a,b}$</td>
<td>35 $^c$</td>
<td>2.8 $^c$</td>
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<tr>
<td>Binary complex</td>
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<td>n.d.</td>
<td>112</td>
<td>4.3 $^c$</td>
</tr>
</tbody>
</table>

$^a$ MALDI mass spectrometry  
$^b$ SEC-MALS  
$^c$ Size-exclusion chromatography  
$^d$ Dynamic light scattering  
n.d. (not determined)
Figure 1.

A. Melanophilin

1 147 403 590
Rab binding domain Myosin Va binding domain F-actin/EB1 binding domain

147 240

B. PONDR Score

PONDR Score

Residue Number

C. Thermal shift curve

kcal/mole of injectant

Molar Ratio

<table>
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<tr>
<th>Injectant</th>
<th>K_d</th>
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<tr>
<td>MlpH\textsubscript{147-403}</td>
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</tr>
<tr>
<td>MlpH\textsubscript{147-240}</td>
<td>0.60 μM</td>
</tr>
</tbody>
</table>
Figure 2.
Figure 3.

M5-GTD

Mlp_{147-403}

M5-GTD:Mlp_{147-403}, 1:1

M5-GTD:Mlp_{147-403}, 2:1
Figure 4.
Figure 6.

A 193-DVDFEEDSD-201

B 176-RQPLNSKKKRLSSFRDVDFEEDSD-201

C

D

% Binding (vs. no competitor)

Micromolar peptide

Kcal/mole of injectant

Molar Ratio
Identification of a minimal myosin Va binding site within an intrinsically unstructured domain of melanophilin
Nathan C. Geething and James A. Spudich

J. Biol. Chem. published online May 19, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M701932200

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