Three-dimensional Structure of the rSly1 N-terminal Domain Reveals a Conformational Change Induced by Binding to Syntaxin 5

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Sec1/Mun18-like (SM) proteins and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) play central roles in intracellular membrane fusion. Diverse modes of interaction between SM proteins and SNAREs from the syntaxin family have been described. However, the observation that the N-terminal domains of Sly1 and Vps45, the SM proteins involved in traffic at the endoplasmic reticulum, the Golgi, the trans-Golgi network and the endosomes, bind to similar N-terminal sequences of their cognate syntaxins suggested a unifying theme for SM protein/SNARE interactions in most internal membrane compartments. To further understand this mechanism of SM protein/SNARE coupling, we have elucidated the structure in solution of the isolated N-terminal domain of rat Sly1 (rSly1N) and analyzed its complex with an N-terminal peptide of rat syntaxin 5 by NMR spectroscopy. Comparison with the crystal structure of a complex between Sly1p and Sed5p, their yeast homologues, shows that syntaxin 5 binding requires a striking conformational change involving a two-residue shift in the register of the C-terminal β-strand of rSly1N. This conformational change is likely to induce a significant alteration in the overall shape of full-length rSly1 and may be critical for its function. Sequence analyses indicate that this conformational change is conserved in the Sly1 family but not in other SM proteins, and that the four families represented by the four SM proteins found in yeast (Sec1p, Sly1p, Vps45p and Vps33p) diverged early in evolution. These results suggest that there are marked distinctions between the mechanisms of action of each of the four families of SM proteins, which may have arisen from different regulatory requirements of traffic in their corresponding membrane compartments.

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Keywords: membrane traffic; Sly1; SM proteins; SNAREs; protein NMR

Introduction

Several families of proteins are involved in most types of intracellular membrane traffic, suggesting that a common mechanism underlies membrane fusion at most cellular compartments in organisms from yeast to humans.1,2 However, members of some of these families also exhibit conspicuously distinct properties that suggest key differences in their mechanisms of action.3–9 Particularly important for membrane fusion are the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and the proteins from the Sec1/Munc18 (SM) family.10–13 The SNAREs are characterized by 60–70 residue sequences that are called SNARE motifs and usually precede C-terminal transmembrane regions.14 SNAREs from the opposing membranes assemble via their SNARE motifs into highly stable bundles of four α-helices known as core or SNARE complexes,
bringing the membranes together. Such membrane approximation might be sufficient for membrane fusion, but this notion is still the subject of intense debate. Some SNAREs contain N-terminal regulatory domains such as the Habc domain characteristic of SNAREs from the syntaxin family, which forms a three-helix bundle. These domains can fold back onto the SNARE motifs, forming a “closed conformation” that prevents core complex assembly, but this property is not conserved among all syntaxins.

SM proteins are 600–700 residue cytosolic proteins that exhibit homology throughout their entire sequence and include seven members in vertebrates (munc18-1, munc18-2, munc18-3, Sly1, Vps45, Vps33a and Vps33b) and four in yeast (Sec1p, Sly1p, Vps45p and Vps33p). X-ray diffraction studies of munc18-1 and Sly1p revealed similar structures, with the domains arranged in an arch shape that forms a central cavity of ca 15 Å while NMR experiments showed that the N-terminal domain of SM proteins constitutes an autonomously folded module. In contrast to the relatively well-understood function of the SNAREs, the role(s) of SM proteins in membrane fusion is largely unclear. The complete blocks in traffic caused by mutations in SM proteins have emphasized their critical importance for fusion, but an inhibitory role has also been suggested by over-expression and microinjection experiments. Proposed functions for SM proteins include a role in vesicle docking or in assisting core complex assembly, but it is unclear how such potential functions may be performed, and some evidence has suggested a late role for Sec1p in secretion, after core complex assembly.

The function of SM proteins appears to be related, at least in part, to their interactions with SNAREs from the syntaxin family, but these interactions exhibit a striking diversity. Neuronal munc18-1 forms a tight complex with syntaxin 1, which involves binding of the closed conformation of syntaxin 1 to the central cavity of munc18-1 and competes with core complex formation. However, yeast Sec1p binds to assembled core complexes containing the plasma membrane syntaxin Sso1p rather than to isolated Sso1p. On the other hand, the yeast syntaxins from late endosomes and vacuoles (Pep12p and Vam3p) do not adopt close conformations and do not appear to interact directly with the corresponding SM proteins (Vps45p and Vps33p). Finally, the yeast and mammalian syntaxins from the endoplasmic reticulum (ER) (Ufe1p and syntaxin 18), the Golgi (Sed5p and syntaxin 5), the trans-Golgi network (TGN) and early endosomes (Tlg2p and syntaxin 16) bind to the corresponding SM proteins (Sly1p for the ER and the Golgi; Vps45p for the TGN and early endosomes) via short N-terminal sequences that precede the Habc domain. These syntaxin N-terminal sequences bind to the N-terminal domains of the corresponding SM proteins, at a site that is opposite to the central cavity where neuronal syntaxin 1 binds to munc18-1. Hence, these interactions are very different from those involved in neuronal exocytosis and are compatible with core complex formation. This is the most widespread mode of syntaxin/SM protein coupling and its functional relevance has been demonstrated by the observation that abrogation of Sly1/syntaxin 5 binding leads to a complete block in ER-to-Golgi transport, and disruption of the Golgi structure.

The overall similarity between the crystal structures of isolated squid munc18-1, rat munc18-1 bound to syntaxin 1 and yeast Sly1p bound to a Sed5p N-terminal peptide suggests that syntaxin binding does not cause substantial conformational changes that could regulate the function of SM proteins. NMR studies of the N-terminal domain of rat Sly1 (rSly1N) also indicated that its interaction with syntaxin 5 does not induce major changes in its secondary structure, but the widespread changes in the H_15N heteronuclear single quantum correlation (HSQC) spectrum of rSly1N induced by syntaxin 5 binding suggested the possibility of a significant conformational change. To investigate the consequences of syntaxin 5 binding on the tertiary structure of rSly1N, we have now solved the three-dimensional structure of isolated rSly1N and further analyzed its complex with a syntaxin 5 N-terminal peptide using NMR spectroscopy. We find that syntaxin 5 binding in fact causes a striking conformational change in rSly1N involving a shift in the register of a β-strand that may result in a substantial alteration of the overall shape of the molecule. A multiple sequence alignment indicates that this conformational change is conserved in all Sly1 homologues but not in other SM proteins, thus suggesting an unexpected difference between the modes of coupling of Sly1 and Vps45 homologues with syntaxins. The sequence alignment also suggests an early evolutionary divergence that led to four SM protein families and resulted in functional distinctions that may be reflected in their different mechanisms of interaction with syntaxins.

Results

Binding to syntaxin 5 induces a conformational change in rSly1N

We previously described the assignment of the backbone resonances of isolated rSly1N (residues 2–147) and of its complex with a peptide encompassing residues 1–33 of syntaxin 5, which includes its rSly1N binding sequence and we will refer to as Syx5(1-33). For both the isolated rSly1N and the rSly1N/Syx5(1-33) complex, the secondary structure deduced from the deviations of the observed Cα and Cβ chemical shifts from random-coil values was consistent with the α-β-α fold observed in the N-terminal domains of munc18-1 and yeast Sly1p, which includes a central five-stranded parallel β-sheet flanked by α-helices. However, as mentioned above, the 1H-15N HSQC
spectrum of rSly1N exhibited widespread cross-peak shifts upon binding to Syx5(1-33), 29 which led us to hypothesize that the interaction may cause a significant conformational change in rSly1N.

To explore this possibility, we acquired 3D 1H-15N nuclear Overhauser effect spectroscopy (NOESY)-HSQC spectra of 15N-labeled rSly1N in isolation and bound to unlabeled Syx5(1-33), and compared the observed backbone NOE patterns with those predicted on the basis of the structure of yeast Sly1p bound to an Sed5p peptide, 28 and the sequence homology between rat Sly1 and yeast Sly1p. The NOE patterns observed for both rSly1N and the rSly1N/Syx5(1-33) complex were generally those expected. However, numerous unexpected NOEs between protons from the two C-terminal β-strands (strands 4 and 5) were observed for isolated rSly1N (Figure 1(a)). For instance, Y109 NH/T136 Hα, L111 NH/V138 Hα, and A139 NH/L111 NH NOEs were observed, whereas several expected NOEs (e.g. L111 NH/K140 Hα and A139 NH/Y109 NH) were not present. In contrast, the expected interstrand NOEs were observed for the rSly1N/Syx5(1-33) complex (Figure 1(b)). Note also the strikingly different NOE pattern observed for the Q137 NH proton in rSly1N and the rSly1N/Syx5(1-33) complex (Figure 1). The homologous residue in the Sly1p/Sed5p complex forms part of a short 310 helix that precedes strand 5 and, correspondingly, Q137 NH exhibits strong NOEs with T136 NH and V138 NH, as well as a weak NOE with T136 Hα, in the rSly1N/Syx5(1-33) complex (Figure 1(b)). However, an NOE pattern characteristic of a β-strand, including interstrand NOEs with L111 NH and Y110 Hα, is observed for Q137 NH in isolated rSly1N (Figure 1(a)). All these observations indicate that the structure of the rSly1N/Syx5(1-33) complex is analogous to that of the yeast Sly1p/Sed5p complex, which was supported further by numerous intermolecular NOEs between hydrophobic residues of rSly1N and a key phenylalanine residue of syntaxin 5 (F10; see below) that are consistent with the structure of the yeast Sly1p/Sed5p complex (see Figure 6 of Dulubova et al. 29). However, the register between strands 4 and 5 appears to be shifted by two residues in isolated rSly1N, with a concomitant structural change in the sequences preceding strand 5.

It should be noted that substantial resonance broadening was observed for the rSly1N/Syx5(1-33) complex and, to a lesser extent, for the isolated rSly1N. Such broadening, which is manifested in the low intensity of some of the NOE data (Figure 1), most likely arises from chemical exchange between the free and bound conformations of rSly1N. We considered the possibility that the different conformation of isolated rSly1N might be an artefact resulting from truncation of the fragment too close to the C terminus of the domain. However, this possibility was ruled out by the observation that the 1H-15N HSQC spectrum of an rSly1 N-terminal fragment with seven additional residues at the C terminus (residues 2–154) is
practically identical with that of rSly1N, with a few additional cross-peaks in the center of the spectrum that correspond to the unstructured (additional) C-terminal residues (data not shown).

Three-dimensional structure of isolated rSly1N

To better define the nature of the conformational change experienced by rSly1N upon binding to syntaxin 5, we determined the three-dimensional structure in solution of isolated rSly1N using multidimensional heteronuclear NMR experiments. Final structures were obtained using a total of 2493 experimental restraints that included 738 long-range NOEs. Figure 2(a) shows a backbone superposition of the 20 structures with the lowest energy, and a ribbon diagram of a representative structure is shown in Figure 2(b). The structural statistics are summarized in Table 1. As expected, isolated rSly1N exhibits an α–β–α fold with a central parallel β-sheet that has a 2-1-3-4-5 topology and is surrounded by five helices. The structure of rSly1N is well defined throughout most of the domain, with an overall backbone rms deviation of 0.72 Å. Only the long loop connecting helix 1 and strand 1 is poorly defined, which correlates with the flexibility suggested by the observation that this region exhibits sharp resonances and fast amide proton exchange rates. The average backbone rms deviation decreases to 0.42 Å when this loop is excluded, and to 0.37 Å when only the secondary structure elements are superimposed. The quality

![Figure 2. Three-dimensional structure of rSly1N. (a) Backbone superposition of the 20 structures with the lowest energy. (b) Ribbon diagram of the lowest-energy structure with β-strands colored cyan and α-helices colored orange. The strands are labeled s1–s5 and the helices are labeled h1–h5. N and C indicate the N and C termini, respectively. The diagrams were prepared with Insight II (MSI, San Diego, CA) and MOLSCRIPT.]

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<tr>
<th>Table 1. Structural statistics for the 20 structures of the N-terminal domain of ratSly1 with the lowest energies</th>
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<tr>
<td><strong>Average rms deviations from experimental restraints (2493 total)</strong></td>
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<td>NOE distance restraints (Å)</td>
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<td>Improper angles (deg)</td>
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<td><strong>Ramachandran plot statistics</strong></td>
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<td>Residues in most-favored regions (%)</td>
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<td>Residues in generously allowed regions (%)</td>
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<td>Residues in non-allowed regions (%)</td>
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<tr>
<td><strong>Average rms deviations of atomic coordinates (Å)</strong></td>
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<td>Backbone residues 8–147</td>
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<td>Backbone residues without the flexible loop</td>
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<td>Heavy atoms residues without the flexible loop</td>
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All 20 structures have NOE energies below 30.3 kcal/mol. There was no NOE violation larger than 0.2 Å or dihedral angle violation larger than 2°. 

*Calculated using the program PROCHECK.*

b Residues 8–23, 38–147.
of the structure is also illustrated by small deviations from idealized covalent geometry and good Ramachandran map statistics (Table 1).

**Syntaxin 5-induced conformational change in rSly1N**

The substantial resonance broadening in the NMR spectra of the rSly1N/Syx5(1-33) complex prevented a full determination of its atomic structure. However, as explained above, abundant NOE data that could be assigned (e.g. Figure 1(b))\(^2^9\) indicate that the binding mode of syntaxin 5 to rSly1N is analogous to that observed in the yeast Sly1p/Sed5p complex. Thus, comparison of the structure of the isolated rSly1N described here with that of the yeast Sly1p/Sed5p complex\(^2^8\) allowed us to uncover the nature of the conformational change induced by syntaxin 5 binding to rSly1N.

Superposition of rSly1N and the N-terminal domain of yeast Sly1p in the complex using the program DALI\(^4^3\) yielded an rms deviation of 2.9 Å for 130 equivalent \(\alpha\) carbon atoms. The superposition (Figure 3(a)) shows that the core of the two structures is very similar, and that major differences exist in two regions. The superposition (Figure 3(a)) shows that the core of the two structures is very similar, and that major differences exist in two regions. The first region corresponds to the loop connecting helix 1 and strand 1, but the observed structural differences may arise, in part, from the very low level of sequence homology in this region. Moreover, these structural differences may not be meaningful, since this loop is flexible in rSly1N and its conformation may be influenced...
strongly by crystal contacts in the Sly1p/Sed5p complex. The second region exhibiting major structural differences between the isolated rSly1N and the Sly1p/Sed5p complex contains the syntaxin 5/Sed5p binding site. Hence, these structural differences reflect the conformational change required for binding of syntaxin 5 to rSly1N (and probably for binding of Sed5p to Sly1p; see below).

In the Sly1p/Sed5p complex, Sed5p binds at a groove formed between helix 5 and strand 5. The interaction involves multiple hydrogen bonds and extensive hydrophobic contacts, particularly between residues of the Sly1 groove and a key phenylalanine residue of Sed5p that is conserved in syntaxin 5 (F10; see Figure 3(b)). The superposition of Figure 3(a) shows that, in order to accommodate the Sed5p peptide, helix 5 is more separated from strand 5 in the Sly1p/Sed5p complex than in the rSly1N structure. This is illustrated by the stick models shown in Figure 3(b) and (c) and by the surface representations in Figure 4(a) and (b). Note that the F10 side-chain from Sed5p binds to a cavity surrounded by hydrophobic residues from helix 5 and strand 5 of Sly1p (Figures 3(b) and 4(a)) and that, as a consequence, the corresponding hydrophobic residues from helix 5 do not pack against those from strand 5. In contrast, helix 5 and strand 5 are packed tightly against each other in isolated rSly1N, occluding the binding site for syntaxin 5 (Figures 3(c) and 4(b)). This tight packing is made possible by the fact that the sequence connecting helix 5 and strand 5, which helps to separate these two elements of secondary structure in the Sly1p/Sed5p complex, is shorter in the isolated rSly1N. Part of this sequence forms a short 3\(_{10}\) helix in the bound form of Sly1p and part of strand 5 in free rSly1N (Figure 3(b)) and the two C-terminal residues of this sequence (T136 and Q137 in rSly1N) move upward, forming instead the N terminus of strand 5 (Figure 3(c)). This is also illustrated in Figures 4(a) and (b) and 5, where the backbone of the sequence that forms the 3\(_{10}\) helix in the complex is colored blue.

Concomitant with this conformational change is a two-residue shift in the overall register of strand 5 with respect to strand 4 (Figure 5). However, the shift in register increases from two to three residues in the middle of the strand because of a \(\beta\)-bulge in C terminus of strand 5 in isolated rSly1N (labeled with a star in Figure 5(a)), while a bulge in the C terminus of strand 5 in isolated rSly1N (labeled with a star in Figure 5(b)) brings the
shift in register back to two residues. Note also that, as a result of these bulges, only the C-terminal half of strand 5 in the complex has the usual geometry and hydrogen bonding pattern of a parallel β-sheet, while this standard geometry is present in the N-terminal half of strand 5 only for the isolated rSly1N. It seems likely that these features may facilitate the conformational change required to form the complex.

As a result of the overall two-residue shift in interstrand register, the two hydrophobic residues from strand 5 that are involved in hydrophobic contacts with F10 of syntaxin 5 in the complex (V138 and V141) are displaced ca 7 Å toward the top of the domain in the isolated rSly1N (Figures 3(c) and 5(b)). Hence, these residues cannot form the hydrophobic binding pocket in this structure (Figure 3(c)), and strand 5 must “slide down” two residues, with the concomitant formation of the 3₁₀ helix and opening of the groove between helix 5 and strand 5, in order to bind to syntaxin 5. It is most likely that the conformational change is required also to establish multiple additional interactions, including numerous hydrogen bonds that are observed in the Sly1p/Sed5p complex. An important consequence of the two-residue shift in interstrand register induced by binding of syntaxin 5 to rSly1 is that two additional residues emerge at the C terminus of the N-terminal domain of isolated rSly1, compared to the complex (Figures 3(a) and (b) and 5). Since this C-terminal sequence connects with domain 2 of rSly1, the conformational change induced by syntaxin 5 binding is expected to cause a substantial alteration in the relative orientation of the domains 1 and 2 of rSly1, and hence in the size of the cavity and the overall shape of the molecule.

Conservation of the syntaxin 5-induced conformational change in the Sly1 family

An immediate question that arises from the observation that binding of syntaxin 5 induces a striking conformational change in rSly1 is whether this conformational change is conserved in other SM proteins. Among the four SM proteins found in yeast (Sec1p, Sly1p, Vps45p and Vps33p) and their mammalian homologues (munc18-1, munc18-2, munc18-3, Sly1, Vps45, Vps33a and Vps33b), only those from the Sly1 and Vps45 families have shown to bind to syntaxin short N-terminal sequences. The munc18s also bind directly to syntaxins, but this interaction involves the syntaxin H₃c-domain and SNARE motif folded into a closed conformation that binds to the munc18 central cavity, and the surface of the munc18 N-terminal domain involved in binding is opposite to the Sed5p/syntaxin 5-binding surface of the Sly1 N-terminal domain. Hence, the conformational change induced by syntaxin 5 binding is conserved in these proteins and is distinct from the changes that are observed in other SM proteins.
The finding that several families of proteins such as the SNAREs and SM proteins are involved in most types of intracellular membrane traffic has led to the general belief that intracellular membrane fusion is controlled by a conserved core machinery. As is common in biological research, the components of this fusion machinery were initially assumed to have conserved functions and to perform them by similar mechanisms. Thus, the finding that neuronal munc18-1 binds to the closed conformation of syntaxin 5 led initially to the general assumption that this interaction is conserved in different organisms and different membrane compartments. However, the observations that yeast Sec1p binds to assembled SNARE complexes rather than to the closed conformation of the plasma membrane syntaxin Sso1p, and that the yeast vacuolar syntaxin Vam3p does not adopt the closed conformation of syntaxin 5 to rSly1, is a distinctive feature of the Sly1 family. These observations suggest that the conformational change induced by binding of syntaxin 5 to rSly1 is conserved across the Sly1 family but does not occur in SM proteins from other families.

Discussion

The finding that several families of proteins such as the SNAREs and SM proteins are involved in most types of intracellular membrane traffic has led to the general belief that intracellular membrane fusion is controlled by a conserved core machinery. As is common in biological research, the components of this fusion machinery were initially assumed to have conserved functions and to perform them by similar mechanisms. Thus, the finding that neuronal munc18-1 binds to the closed conformation of syntaxin 5 led initially to the general assumption that this interaction is conserved in different organisms and different membrane compartments. However, the observations that yeast Sec1p binds to assembled SNARE complexes rather than to the closed conformation of the plasma membrane syntaxin Sso1p, and that the yeast vacuolar syntaxin Vam3p does not adopt a
closed conformation, unveiled an unexpected diversity in the modes of interaction between SM proteins and syntaxins, and indicated that there are fundamental differences in the mechanisms by which these proteins mediate or regulate membrane fusion. This diversity was emphasized further by the finding that Sly1 and Vps45, the SM proteins that function in the ER, the Golgi, the TGN and the endosomes, bind to short N-terminal sequences of their cognate syntaxins. Nevertheless, these latter results suggested at the same time that there is a unifying theme for syntaxin/SM protein interactions in most internal membrane compartments. Here, we show that syntaxin 5 binding causes a striking conformational change in the rSly1 N-terminal domain involving a two-residue shift in the register of its C-terminal β-strand. Together with the prediction from sequence comparisons that this conformational change is conserved only in Sly1 homologues, our results uncover a new level of divergence in syntaxin/SM protein coupling that distinguishes the Sly1 and Vps45 families, and suggest that there are distinctions between the functions and/or mechanisms of actions of each of the four families of SM proteins. Moreover, our sequence analysis indicates that these distinctions likely emerged early in evolution.

The reasons for the divergence in syntaxin/SM protein interactions are unclear. The emergence of distinct intracellular membrane compartments during the early evolution that led to eukaryotic cells is likely to have required (or have been the result of) the evolution of membrane resident proteins that conferred a particular identity to such compartments, and of proteins that could mediate specific fusion in these compartments. Such specificity in membrane fusion might have arisen from subtle mutations that altered interactions between the components of the membrane fusion machinery, but it is tempting to speculate that drastic changes in the nature of these interactions may have provided a more efficient route toward specific differentiation of the membrane compartments. It is also possible that such drastic changes in the modes of coupling within the fusion machinery may have responded to different regulatory requirements of traffic in distinct membrane compartments. Clearly, establishing whether these or other possibilities are correct will require a much more thorough characterization of the mechanisms of fusion and its regulation than is currently available.

The functional importance of the rSly1/syntaxin 5 interaction in mammalian cells was demonstrated by the observations that abrogation of this interaction leads to a complete disruption of the Golgi complex, and to acute inhibition of ER-to-Golgi transport. On the other hand, experiments in yeast have suggested recently that Sly1p and Sed5p mutants bearing single-residue substitutions that disrupt their mutual interaction are fully functional, although it was unclear from the results of the biochemical experiments described whether the mutations abolish binding completely. Thus, it remains to be established whether the lack of overt functional defects in these mutant yeast strains arises from residual Sed5p/Sly1p binding or from differences in the functional importance of this SNARE/SM protein interaction between yeast and mammals, which would be surprising given the evolutionary conservation of the interaction. Further research will also be necessary to explore the biological relevance of the conformational change caused by binding of syntaxin 5 to rSly1N, as well as to understand its mechanistic consequences. From a thermodynamic point of view, the conformational change cannot influence the specificity of rSly1 for its cognate syntaxins, whose N-terminal sequences are very similar to those that bind Vps45. However, it could be envisaged that the conformational change imposes a kinetic barrier for syntaxin binding and selects for sequences that are able to bind in a biologically relevant time-scale. A more attractive possibility is that the conformational change induced by syntaxin 5 binding is required for activation of rSly1, since the shift in the register

Figure 7. An evolutionary tree of selected SM proteins. The sequence set is the same as that in the alignment of Figure 6. The tree is rooted in such a way that the family of Vps33p is separated from the other three families. The local bootstrap supporting values are shown at each branch. Supporting values above 90 are highlighted in bold. The species name abbreviations are the same as in Figure 6.
of the C-terminal β-strand of the N-terminal domain upon binding is likely to change the relative orientation of this domain with respect to domain 2 and hence to alter the overall shape of the molecule substantially. This model is based on the notion that the cavity of the arch formed by SM proteins may be critical for their function, perhaps providing a mold for SNARE complex assembly or for an as yet unidentified activity. Key predictions of this model are that free rSly1 exists in an inactive conformation that is unique for the Sly1 family, while the overall structural change caused by syntaxin 5-binding leads to the active conformation that is common to all SM proteins.

Independently of its biological role, the conformational change of rSly1 constitutes a striking example of protein malleability and is somewhat reminiscent of the structural rearrangement involved in activation of ARF1 GTPase.46 In this case, GTP binding to ARF1 causes a shift in the register of strands 2 and 3 with respect to the rest of the strands of a β-sheet. This structural change leads to exposure of the myristoylated N terminus, explaining how ARF-GTPases couple the GTP-GDP conformational switch to membrane binding. The conformational changes of both rSly1 and ARF1 seem to result from an ability of β-strands to pack against β-strands with different sequences. Such ability is reflected in other reported protein conformational changes involving β-strand swaps or insertions, including for instance those observed in activation/inactivation of serpins,47 in binding of the mitotic spindle checkpoint protein Mad2 to its upstream activator and downstream target,48 or in Ca2+ binding to the C2-domain of the active zone protein Piccolo.49 The increasing number of examples of promiscuity in β-strand/β-strand interactions suggests that this is a widespread theme in biological signaling mediated by protein conformational changes.

Materials and Methods

Protein expression and purification

The construct for expression of the rat Sly1 N-terminal domain (residues 2–147) has been described.29 To generate uniformly 15N-labeled or 15N,13C-labeled samples for NMR studies, bacteria were grown in minimal medium supplemented with 15NH4Cl and with or without 13C]glucose (CIL, Andover,MA) as the sole nitrogen and carbon sources. The fusion protein was affinity-purified on glutathione-Sepharose (Pharmacia), cleaved from the GST moiety with thrombin (Sigma) and affinity-purified on glutathione-Sepharose (Pharmacia), cleaved from the GST moiety with thrombin (Sigma) and purified further by gel-filtration in 40 mM Tris (pH 8.1), 150 mM NaCl, 1 mM DTT. The synthetic peptide of the N-terminal fragment of syntaxin 5 (residues 1–33) was purchased from the Center for Biomedical Inventions (University of Texas Southwestern Medical Center).

NMR spectroscopy

All NMR experiments were acquired at 27 °C on Varian Inova500 or Inova600 spectrometers with samples of rat Sly1 N terminal domain (residues 2–147) dissolved in 20 mM Mes (pH 5.7), 200 mM KCl, 1 mM DTT. One 15N-labeled sample of rSly1N (1.2 mM) with or without 2 mM Syx5(1-33) peptide was used to acquire initial 3D 1H-15N-NOESY-HSQC spectra. Another 15N-labeled sample and a 15N,13C-labeled sample of isolated rSly1N (both 1.2 mM) were used to determine the structure using a suite of pulsed-field gradient enhanced NMR experiments,50–53 as described.54–56 Briefly, these included 3D 1H-15N total correlated spectroscopy (TOCSY)-HSQC, HNCO, HNCAcb, CBCab(COJ)NH, (HC)(CO)NH-TOSY, H(C)(CO)-NH-TOSY, and HCH-TOSY spectra for resonance assignments, and 2D NOESY, 3D 1H-15N-NOESY-HSQC, and 3D 1H,15N-NOESY-HSQC experiments (100 ms mixing times) to measure NOEs for structure determination. Protection of amide protons from the solvent was measured from the intensities of exchange cross-peaks with the water resonance in 3D 1H-15N-TOCSY-HSQC experiments. Stereoe specific assignments of Val and Leu methyl groups were obtained from a 1H-13C HSQC spectrum acquired on a 1 mM sample of 10% 13C-labeled rat Sly1 N terminal domain. All data were processed with the program NmrPipe,72 and analyzed with the program NMRview.58

Structure calculations

NOE cross-peak intensities were classified as strong, medium, weak, and very weak, and assigned to restraints of 1.8–2.8 Å, 1.8–3.5 Å, 1.8–5.0 Å and 1.8–6.0 Å, respectively, with appropriate pseudoatom corrections. Phi and psi torsions angle restraints were included based on analysis of HN, 15N, 13Cα, 13CO, and 13Cβ chemical shifts using the program TALOS.59 Dihedral angles were restrained to the maximum of 22.5° or 1.5 times the standard deviation observed in the TALOS database matches. To restrain hydrogen bonds, the H/O distances were restrained to 1.3–2.5 Å and the N/O distances to 2.3–3.5 Å. Structures of the N-terminal domain of rSly1N were initially calculated and refined using torsion angle simulated annealing with the program CNS.60 A total of 1400 structures were calculated with the final set of restraints, and the 20 structures with the lowest NOE energy were selected.

Protein Data Bank accession code

The structures have been deposited in the Protein Data Bank with accession code 1V9J.

Sequence analysis

PSI-BLAST61 was used to search for homologues of SM proteins. Starting from the query of syntaxin binding protein 1 from Rattus norvegicus (NCBI gene identification number 6981602), PSI-BLAST iterations were performed to convergence on the nr database (May 2001, 686,213 sequences; 216,043,563 total letters) with e-value cutoff 0.001. The homologues that were found were grouped by single-linkage clustering (1 bit per site threshold, about 50% identity) as implemented in the SEALS package,62 and the representative sequences from each group were used as new queries for subsequence PSI-BLAST iterations. After two rounds of these extensive searches, about 100 SM proteins were identified. These sequences were aligned using T-Coffee,44 followed by manual adjustment. The full-length alignment is shown in Figure 1 of the Supplementary Data. A maximum-likelihood tree was built for representative sequences from six selected...
species using the MOLPHY package (version 2.3).\(^6\) Only positions with gap fraction less than 20% were selected for tree building. The local estimates of bootstrap percentages were obtained by the RELL method,\(^5\) as implemented in the program ProtML of MOLPHY.

**Acknowledgements**

This work was supported by a grant from the Welch Foundation and by NIH grant NS37200 (to J.R.).

**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2004.12.004

**References**


*Edited by M. F. Summers*

(Received 25 September 2004; received in revised form 1 December 2004; accepted 1 December 2004)