Structure of GATE-16, Membrane Transport Modulator and Mammalian Ortholog of Autophagocytosis Factor Aut7p*

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The GATE-16 protein participates in intra-Golgi transport and can associate with the N-ethylmaleimide-sensitive fusion protein and with Golgi SNAREs. The yeast ortholog of GATE-16 is the autophagocytosis factor Aut7p. GATE-16 is also closely related to the GABA receptor-associated protein (GABARAP), which has been proposed to cluster neurotransmitter receptors by mediating interaction with the cytoskeleton, and to the light chain-3 subunit of the neuronal microtubule-associated protein complex. Here, we present the crystal structure of GATE-16 refined to 1.8 Å resolution. GATE-16 contains a ubiquitin fold decorated by two additional N-terminal helices. Proteins with strong structural similarity but no detectable sequence homology to GATE-16 include Ras effectors that mediate diverse downstream functions, but each interacts with Ras by forming pseudo-continuous β-sheets. The GATE-16 surface suggests that it binds its targets in a similar manner. Moreover, a second potential protein-protein interaction site on GATE-16 may explain the adapter activity observed for members of the GATE-16 family.

Over the past decade, great progress has been made in describing the structural foundation of membrane docking and fusion in vesicle trafficking. The structures of several factors that play regulatory or mechanical roles in vesicle fusion have been determined, including N-ethylmaleimide sensitive fusion protein (NSF)1 domains (1–5), the yeast ortholog of α-SNAP (6), a SNARE complex (7), and others (8–11). These data, in combination with genetic and biochemical experiments, have defined models by which these proteins function and interact (for reviews, see Refs. 12 and 13). NSF, with the aid of SNAPs, primes SNAREs for interaction with one another, and formation of the four-helix bundle-complexes of SNARE proteins may then drive membrane fusion. Rab GTPases and their effectors appear to control membrane tethering, the size and stability of the active zone of vesicle docking/fusion, and perhaps the timing of fusion. Nevertheless, vesicle transport cannot yet be reconstituted efficiently in vitro using only known components of the docking and fusion machinery, suggesting that our understanding of this complex process is not yet complete.

A protein named GATE-16 (Golgi-associated ATPase enhancer of 16 kDa) was recently isolated in a search for previously unidentified factors that aid in intra-Golgi transport (14). GATE-16 is expressed constitutively in all secretory organs tested, particularly in the brain (15), and has been localized to the Golgi apparatus (15). GATE-16 first stimulates the ATPase activity of NSF, and NSF in turn stimulates association of GATE-16 with the SNARE protein GOS-28 (15).

Independently of the discovery of mammalian GATE-16, a yeast ortholog, Aut7p (16) (also known as Apg8p (17) and Cvt5p (18)), was found to be necessary for autophagocytosis. During autophagocytosis, sections of the cell cytoplasm are engulfed by double-membrane structures and transported to vacuoles (for review see Ref. 19). The outer vesicle membrane fuses with the vacuolar membrane, and the inner compartment is thereby delivered to the vacuolar lumen for digestion. Autophagocytosis occurs on a large scale upon starvation of the cell to break down cytoplasmic proteins for emergency re-use. Similar processes occur during normal cell growth to recycle cellular constituents and during cell differentiation to remodel a cell’s internal structure.

Aut7p is the only protein known to date to be up-regulated during autophagocytosis (20, 21). Recent studies suggest that Aut7p functions structurally rather than catalytically and that it may be involved in biogenesis of autophagosomes (20, 21). Mutants lacking Aut7p display an extreme sensitivity to nutrient deprivation, and diploid Δaut7 strains are unable to sporulate (17). The importance of GATE-16 in mammalian cellular processes related to autophagocytosis remains to be determined.

GATE-16 is closely related in amino acid sequence (39 and 57% identity, respectively) to the light chain-3 subunit of the neuronal microtubule-associated protein complex (22) and to the GABA receptor-associated protein (GABARAP), which is thought to promote clustering of neurotransmitter receptors by mediating interaction with the cytoskeleton (23). Aut7p does not contact tubulin directly (16), suggesting that members of this protein family are not united simply by a microtubule-binding function but rather may have more widespread adapter activities.

We have determined the crystal structure of GATE-16 to 1.8 Å resolution. We demonstrate that the last 90 amino acids of GATE-16 adopt a ubiquitin fold. The first 27 amino acids compose two additional helices, which may be characteristic of a distinct and novel GATE-16 subgroup within the ubiquitin fold.
superfamily. Proteins that are structurally most similar to GATE-16 align over the ubiquitin fold region and include the Ras-binding domains of various Ras effectors such as RalGDS (24), c-Raf1 (25), and phosphoinositol 3-kinase (26). The most highly conserved region of the GATE-16 surface, a likely site for protein-protein interactions, corresponds to the face that Ras effectors present to Ras.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The GATE-16 coding region (14) was inserted into the pAED4 expression vector (27). The resulting construct was transformed into BL21(DE3) ployS cells, which were grown at 37 °C in LB containing 100 mg/liter ampicillin and 30 mg/liter chloramphenicol. When the cell culture reached an optical density of *A*~o~ = 0.4, isopropyl-β-D-thiogalactoside was added to 1 mM to induce protein expression. Two h later, cells were harvested by centrifugation and lysed by sonication in 10 mM NaPO₄, pH 6.8, 25 mM NaCl. The homogenate was centrifuged for 10 min at 10,000 g, and the supernatant was applied at 4 °C to a fast flow SP-Sepharose column (Sigma). The resulting solution at 100 mM calcium acetate, 22% polyethylene glycol 8000. Although the protein was concentrated to 11 mg/ml as determined by the Edelhoch procedure (28). Subsequent preparations of protein were dialyzed directly against 10 mM Tris, pH 8.0, 570 mM NaCl and then concentrated to 11 mg/ml.

**Crystallization**—Crystals were grown by the hanging drop method to a size of approximately 0.3 × 0.2 × 0.1 mm in 100 mM Tris, pH 7.5, 100 mM calcium acetate, 22% polyethylene glycol 8000. Although *M*₈*S*O generally inhibited crystal nucleation, one large crystal was observed in a solution containing the above mixture plus 10% *M*₈*S*O. This crystal diffracted to higher resolution and displayed a lower mosaicity than other crystals of equal size, and was therefore selected for the native GATE-16 data set.

**Structure Determination**—Native data were collected to 1.8 Å resolution at 100 K on the ID14 2 beamline at ESRF Phasing was performed by multi-wavelength anomalous dispersion (MAD) using a selenomethionine derivative of GATE-16 prepared according to published protocol (29), except that an induction time of 3 h was used. Selenomethionine data were collected on ESRF beamline BM30 to 2.73 Å resolution at three wavelengths around the selenium absorption edge (see Table I). All native and MAD data were processed and scaled using DENZO and SCALEPACK (30). Seven of the eight possible selenium sites were located using the program SOLVE (31). Phases obtained from SOLVE (mean figure of merit 0.64 to 2.8 Å) were modified using the program DM (32), choosing a solvent content of 50%. Two GATE-16 molecules were found per asymmetric unit, and the GATE-16 structure was built using O (33). The entire chain could be readily traced for both molecules, with the exception of the terminal phenylalanine in chain A. Refinement of the structure was done using the crystallography and NMR System (34) without non-crystallographic symmetry restraints, and the two molecules in the asymmetric unit were compared with the program lsqkab (35).

**RESULTS**

GATE-16 was expressed in Escherichia coli and purified over a cation exchange column. Crystals belonged to the space group P2₁ and contained two molecules (chain A and chain B) per asymmetric unit, were grown by the hanging drop method. Phases were obtained by MAD using selenomethionine-substituted protein, and the structure was refined to 1.8 Å resolution against data from a native crystal. The final working *R* factor was 22.9% and the free *R* factor 25.6%. A stereo representation of the Ca trace of the GATE-16 structure is presented in Fig. 1.

Comparison of the GATE-16 structure with the protein fold data base (36) revealed strong similarity to the ubiquitin domain superfold (37). Residues 28–33, 46–69, 74–95, 103–110, 111–114 of GATE-16 superpose with residues 1–6, 13–38, 40–61, 64–71, and 73–76 of ubiquitin (PDB I.D. code 1ubi) with a root mean square deviation for α-carbons of 2.2 Å and a Z-score of 6.0. In addition, Z-scores of 6.9, 6.5, and 5.4 were obtained for structural alignments with other members of the superfamily ElonginB (PDB I.D. code 1veb), RalGDS (PDB I.D. code 1ffd), and the Ras-binding domain from phosphoinositide 3-kinase (PDB I.D. code 1qmm), respectively. A superposition of GATE-16 with the Ras-binding domain of RalGDS in complex with Ras is shown in Fig. 2, left.

Like ubiquitin, the GATE-16 structure has a four-stranded central β-sheet with the middle two strands parallel to one another and the outer two strands antiparallel to the inner strands (Fig. 2, right). The sheet is backed by helices α₃ and α₄ on one side. Helices α₁ and α₂, not found in the other ubiquitin fold superfamilies, mem, pack against the opposite side of the sheet (Figs. 1 and 2). Glu-34, which forms hydrogen bonds to the backbone NH groups at the N terminus of helix α₁, and Glu-12, the N-cap residue of the α2 helix, are universally conserved (Fig. 3), indicating that the a₁ and a₂ helices are likely to be present in all GATE-16 homologs. Furthermore, two salt bridges between residues in helix a₂ and the ubiquitin fold (Arg-14 to Asp-102 and Glu-17 to Lys-48) also appear to be maintained in all homologs (Fig. 3).

The two independent copies of GATE-16 in the crystals superpose with a root mean square deviation for backbone atoms of 0.47 Å between residues 1 and 110 and a maximum displacement of 3 Å. The last seven residues then diverge (Fig. 1), with a maximum displacement in the backbone of 18 Å. This flexible C-terminal region contains a phenylalanine (Phe-115) that is conserved in all known homologs, excepting the Caenorhabditis elegans GATE-16 ortholog and GABARAP, both of which con-
tain a tyrosine at this position (Fig. 3). The next residue, Gly-116, is invariant. In chain A, residues 112–116 extend out from the globular region of the protein, and Phe-115 packs against chain B. Residues 112–117 of chain B are oriented such that Phe-115 packs against its own chain, and only Phe-117 extends into the neighboring molecule. Strong sequence conservation at positions that do not contribute structurally to the fold of a protein implies functional constraints on residues Phe-115 and Gly-116. The nature of these constraints remains to be determined.

Within the GATE-16 crystals, neighboring molecules are packed such that the Cys-15 side chains are within appropriate disulfide-bonding distance. Furthermore, we observed that dissolved crystals contained a higher fraction of disulfide-bonded dimeric protein as determined by nonreducing SDS-polyacrylamide gel electrophoresis than did the original crystallization stock solution (data not shown). The GATE-16 cysteine residue is not conserved in other family members, and we do not ascribe any functional significance to the disulfide bond seen in the crystals. However, the formation of intermolecular disulfide bonds indicates that the cysteine residue is solvent-exposed in the GATE-16 protomer.

An examination of the charge distribution at the GATE-16 surface reveals a ring of basic residues near the edge of strand β2 surrounding a region of partially exposed hydrophobic residues (Fig. 4A). The amino acids contributing to this face of GATE-16 are more highly conserved between yeast and mammals than those on the opposite side of the protein (Fig. 4B).

The basic/hydrophobic face can accommodate the packing of hydrophobic residues from other molecules, as Phe-115 from chain A docks into a pocket formed by Ile-21, Pro-30, Leu-50, the aliphatic portion of Lys-48, and Phe-104 from chain B. In turn, Phe-117 from chain B packs into the same pocket of a neighboring chain A. The same face of GATE-16 contains a second patch of partially exposed hydrophobic residues composed of Tyr-49, Val-51, Pro-52, Ile-55, Trp-62, and Ile-63. This region of chain B accommodates the exposed hydrophobic residues Val-36, Phe-77, Phe-79, and Val-84 from chain A. In
contrast, the C-terminal tail of chain B shields Val-36, Phe-77, Phe-79, and Val-84 within the same protomer, so that these residues do not form a surface-exposed hydrophobic patch and are not involved in intermolecular contacts.

The particular crystal packing interactions described above illustrate the character of the GATE-16 surface and highlight...
regions of the protein available for contact. It should be noted that GATE-16 behaves as a monomer under physiological conditions in solution as determined by gel filtration and analytical ultracentrifugation (data not shown). Its binding activity in vivo is therefore likely to be hetero-oligomeric rather than homo-oligomeric.

**DISCUSSION**

We have presented our findings that GATE-16, a protein involved in membrane-trafficking events, consists of a ubiquitin fold with two additional N-terminal helices. Like other factors with key roles in intracellular membrane fusion, GATE-16 is highly conserved from yeast to mammals. Interestingly, mammalian GATE-16 was identified by an approach distinct from the method used to assign function to its yeast ortholog, Aut7p. Whereas Aut7p was characterized as an autophagy protein mutant (16, 20, 21), GATE-16 was first described as a membrane transport modulator in the constitutive secretory pathway (14, 15). Aut7p is dispersed throughout the cytoplasm under normal growth conditions but shifts dramatically to intermediate structures in autophagosome formation under starvation conditions (21), whereas GATE-16 localizes to the Golgi apparatus in growing cells (15). Nevertheless, the functions of the proteins from mammals and yeast may be closely related, because Aut7p can partially substitute for GATE-16 in the cell-free intra-Golgi transport assay and can interact specifically with SNAREs (26).

Aut7p has been ascribed a role in formation of autophagocytic vesicles (21). The biogenesis of autophagosomes is likely to employ complexes similar to those used in other intracellular processes requiring membrane fusion, and general components of the membrane-fusion machinery may be recruited for autophagosome formation during starvation. Whereas GATE-16 lacks a transmembrane region and appears to be a soluble protein, it is associated with membrane-bound organelles in vivo (15). It also associates with the soluble hexameric ATPase NSF (15). Therefore, GATE-16 may act as an adapter protein that helps target NSF to the appropriate membranes, be they vesicle or Golgi membranes in constitutive secretion or precursors of autophagosomes during starvation. A study of GATE-16 localization under conditions that favor autophagosome formation should indicate whether mammalian GATE-16 has a comparable function to Aut7p during starvation.

Analysis of the GATE-16 structure suggests possible surfaces by which this protein can interact with its partners. Both RaGDS and ElonginB, the proteins to which GATE-16 shows the strongest structural similarity, have been studied crystallographically in complex with other factors (24, 39). RaGDS is an effector of Ras and binds to Ras by forming a pseudo-continuous β-sheet using the strand corresponding to β2 in GATE-16 (24) (Fig. 2). ElonginB forms a complex with ElonginC and VHL, a tumor-suppressor protein defective in von Hippel-Lindau syndrome and in the majority of kidney cancers. ElonginB interacts with ElonginC also by forming a pseudo-continuous β-sheet through the structural analog of GATE-16 strand β2 (38). In the ElonginB/C complex, the intermolecular strand-strand interaction is parallel, and between Ras and RaGDS it is antiparallel (Fig. 2, right). The structure of phosphoinositol 3-kinase γ (PI3Kγ) has been determined alone and not in complex with Ras (26). Nevertheless, the edge of strand β2 from the Ras-binding domain of PI3Kγ is exposed, whereas a large portion of the rest of the domain is buried. It has indeed been predicted that the PI3Kγ Ras-binding domain shares the same binding mode as its structural analogs (26).

Although it has not been demonstrated that GATE-16 contacts other proteins in a manner structurally similar to Ras effectors, it is important to note that a distinctly basic face with exposed hydrophobic patches is found on the face of GATE-16 corresponding to the Ras-binding face of Ras effectors. The top panels of Fig. 4 illustrate the character of the GATE-16 surface, viewing strand β2 edge-on, from the perspective of the binding partners of RaGDS, c-Raf1, and ElonginB. The region containing basic residues and hydrophobic pockets is also the largest conserved patch of surface between GATE-16 and yeast Aut7p. We therefore predict that this surface is functionally important in protein-protein interactions involving GATE-16 and Aut7p. The bottom panels of Fig. 4 show a region on the opposite side of GATE-16 that is smaller than the basic patch but contains the largely exposed, hydrophobic residues Phe-77, Phe-79, and Val-84. These hydrophobic residues, conserved in both GABARAP and in yeast Aut7p, may represent another binding site on GATE-16, which may extend into the C-terminal tail. It is intriguing that the C-terminal tail of GATE-16 adopts two widely differing conformations in the two independent molecules in the GATE-16 crystals. In one of these conformations (chain B), the tail shields Phe-79, and partially shields Phe-77 and Val-84, from solvent. In the other conformation (chain A), the tail leaves this hydrophobic patch entirely exposed. The putative second binding site on GATE-16 could therefore be modulated by regulating the conformation of this C-terminal tail.

A large protein complex active in membrane docking and fusion has recently been detected (40). This complex contains Rab GTPases, Rab effector proteins, NSF, and SNAREs. The activities assigned to the Rab effectors parallel certain observations regarding GATE-16 function. For example, release of Rab effectors from the complex upon ATP hydrolysis by NSF has been proposed to trigger an interaction of these effectors with SNAREs (40). GATE-16 would be comparable with the Rab effectors in this model because it stimulates NSF activity and may then be transferred to SNAREs (15). In this light, the structural similarity we observed between GATE-16 and effectors of the Rab-related protein Ras led us to speculate that Rab may interact with GATE-16.

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