Bet1p plays an essential role in vesicular transport from the endoplasmic reticulum (ER) to the Golgi in yeast, and it functions as a vesicle soluble N-ethylmaleimide-sensitive factor protein receptor (v-SNARE). A mammalian protein related to Bet1p has been reported previously and was referred to as rbet1. We have now identified a new mammalian protein that is homologous to rbet1 (28% amino acid identity). mRNA for this rbet1 homologue is widely expressed in rat tissues. Affinity-purified polyclonal antibodies raised against recombinant protein specifically recognized a 15-kilodalton integral membrane protein highly enriched in Golgi membranes. Indirect immunofluorescence microscopy revealed that this protein is specifically associated with the Golgi apparatus in diverse cell types. Biochemical characterization established that this protein behaves like a SNARE and was named GS15 (Golgi SNARE with a size of 15 kilodaltons). These properties raise the possibility that GS15 is a novel SNARE mediating a yet to be defined transport event associated with the Golgi apparatus.

Protein transport along the exocytic and endocytic pathways is primarily mediated via various types of transport vesicles that bud from one membrane compartment and fuse with a specific cognate compartment (1–4). Soluble N-ethylmaleimide-sensitive factor (NSF)1 and its yeast counterpart Sec18p have been shown to participate in many different transport events (5). Membrane association and activation of the ATPase activity of NSF are mediated by soluble NSF attachment proteins (SNAPs) or the yeast counterpart Sec17p (5). Membrane recruitment of SNAPs is mediated by SNAP receptors (SNAREs) on the membranes (6–10).

To account for the specificity of vesicle transport, the SNARE hypothesis proposes that the docking and fusion of vesicles with the cognate compartment is mediated by specific pairing between vesicle-associated SNAREs (v-SNAREs) with those (t-SNAREs) associated with the target membrane (6–10). Vesicle-associated membrane proteins or synaptobrevins are v-SNAREs associated with the synaptic vesicles, while syntaxin 1 and SNAP-25 (synaptosome-associated protein of 25 kDa) are t-SNAREs associated with the presynaptic membrane. The specific pairing of vesicle-associated membrane proteins with the syntaxin 1-SNAP-25 complex plays a key role in the docking/fusion of synaptic vesicle with the presynaptic membrane (6–10).

Because of the central role of SNAREs in vesicular transport, molecular identification, biochemical establishment, and subcellular localization of novel SNAREs are of great importance. In this report, we have molecularly characterized a 15-kilodalton protein (GS15) that is homologous to rbet1 (11), a rat protein related to yeast Bet1p (12–15). Bet1p plays an essential role in vesicular transport from the ER to the Golgi apparatus in yeast. Functioning as a v-SNARE associated with vesicles derived from the ER, Bet1p is involved in docking and/or fusion of the vesicle with the early yeast Golgi subcompartment (12–15). rbet1 was identified as a rat protein that is homologous to Bet1p, and the exact subcellular localization and functional aspects of rbet1 remain to be established, although epitope-tagged rbet1 has been shown to be associated with the Golgi membrane (11, 25). Antibodies against GS15 revealed that GS15 is an integral membrane protein of the Golgi apparatus that behaves like a novel SNARE.

**Experimental Procedures**

**Materials**—MDCK II (Madin-Darby canine kidney strain II) was a generous gift from Dr. Kai Simons (EMBL, Heidelberg, Germany), and all other cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). The rat brain α-ZAP cDNA library and Pyrococcus furiosus DNA polymerase were obtained from Stratagene. The rat mRNA Multiple Tissues Northern filter was obtained from CLONTECH (Palo Alto, CA). The oligolabeling kit and glutathione-Sepharose 4B beads were purchased from Pharmacia (Uppsala, Sweden). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG were purchased from Boehringer Mannheim. Brefeldin A (BFA) was from Epixentre Technologies.

**cDNA Cloning and Sequencing**—A mouse EST clone (accession number W83047) encoding an open reading frame that is homologous to the EST clone was used to screen a rat brain α-ZAP cDNA library and Pyrococcus furiosus DNA polymerase were obtained from Stratagene. The rat mRNA Multiple Tissues Northern filter was obtained from CLONTECH (Palo Alto, CA). The oligolabeling kit and glutathione-Sepharose 4B beads were purchased from Pharmacia (Uppsala, Sweden). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG were purchased from Boehringer Mannheim. Brefeldin A (BFA) was from Epixentre Technologies.

**Expression of Recombinant Proteins in Bacteria**—GST fusion proteins were produced using the pGEX-KG vector (17) and purified as described (16, 18).

**Preparation of Polyclonal Antibodies**—Rabbits were each injected with 300 µg of GST fusion protein emulsified in complete Freund’s adjuvant. Booster injections containing a similar amount of antigen emulsified in incomplete Freund’s adjuvant were administered every 2 weeks. Rabbits were bled 10 days after the second and subsequent bleed.
booster injections. Serum was diluted twice with PBS and incubated sequentially with cyanogen bromide activated-Sepharose beads coupled with GST protein and GST fusion protein for 2 h at room temperature. The beads containing the GST fusion protein were washed extensively, and specific antibodies were eluted (16).

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described previously (16, 19). For the treatment of cells with BFA or nocodazole, cells grown on coverslips were incubated with BFA (10 μg/ml) or nocodazole (10 μg/ml) for 1 h at 37 °C, washed twice with PBSCM (PBS with 1 mM CaCl₂, 1 mM MgCl₂), and then fixed in 3% paraformaldehyde. Fixed cells were then permeabilized and incubated with antibodies against GS15 (polyclonal) and mannosidase II (monoclonal) for double labeling.

Differential Extraction of Golgi Membranes and Immunoblot Analysis—Pelleted Golgi membranes (500 μg) were extracted on ice for 1 h in 100 μl of 2 M KCl, 1% Triton X-100, 1% Nonidet P-40, 0.1 mM sodium bicarbonate (pH 12.0), 2.5 mM urea or PBS and then centrifuged at 100,000 × g for 1 h at 4 °C. The supernatants were transferred to another tube, and the pellets were resuspended in 100 μl of 1× SDS sample buffer. Aliquots (20 μl) from both the supernatants as well as the pellets were analyzed by immunoblot analysis (19).

20 S SNARE Complex Formation—This was performed as described (9, 20, 21). For immunoprecipitation of the 20 S complex, 20 S fractions were incubated with antibodies bound to protein A-Sepharose (in the presence of 1 mg/ml bovine serum albumin) at 4 °C for 2 h with agitation. Sepharose beads were washed five times with gradient buffer (100 mM KCl, 20 mM Hepes, pH 7.3, 2 mM EDTA, 2 mM dithiothreitol, 0.5 mM ATP) containing 0.5% Triton X-100 and divided into two equal aliquots. One of the aliquots was eluted with 30 μl of gradient buffer containing 0.5% Triton X-100 (assembly condition) and the other one with 30 μl of gradient buffer containing 0.5% Triton X-100 and 8 mM MgCl₂ (disassembly condition). Both the eluants as well as the beads were processed for immunoblot analysis using antibodies against NSF and α-SNAP.

In Vitro Binding Assay—Golgi enriched membranes (1 mg) were extracted in 500 μl of incubation buffer (100 mM KCl, 20 mM Hepes, pH 7.5).
7.3, 2 mM EDTA, 2 mM dithiothreitol, 0.2 mM ATP) containing 1% Triton X-100 and incubated at 4 °C for 1 h with agitation. The extracted Golgi membranes were diluted with 500 ml of incubation buffer without Triton X-100 and then centrifuged at 100,000 × g at 4 °C for 1 h. Beads containing 2 mg of GST-a-SNAP were washed twice with incubation buffer containing 0.5% Triton X-100 (1 ml each) before incubating with different amounts of extracted Golgi proteins in a total volume of 200 ml at 4 °C for 3 h with agitation. Beads were then washed twice with incubation buffer containing 0.5% Triton X-100, once with incubation buffer containing 0.1% Triton X-100, twice with incubation buffer without Triton X-100, and then processed for immunoblot analysis.

RESULTS AND DISCUSSION

GS15, a Protein Related to rbet1—Searching the EST data bases using rbet1 amino acid sequence led to the identification of a mouse EST clone (accession number W83047) that encodes a protein related to rbet1 (11). The PCR product of the EST clone was used to screen a rat brain λ-ZAP cDNA library, leading to the isolation of full-length rat cDNA clones. The nucleotide and the deduced amino acid sequence of rat GS15 are shown in Fig. 1A. GS15 is a protein of 111 residues with a 21-residue carboxyl-terminal hydrophobic region that may function as a membrane anchor. Preceding the carboxyl-terminal hydrophobic tail are several regions that have the potential to form coiled-coil structures. The alignment of rat and mouse GS15 sequences with that of rbet1 is shown in Fig. 1B. The rat and mouse GS15 are highly homologous with 96% amino acid identity, and both are related to rbet1 with amino acid identity of 28%.

GS15 mRNA Is Widely Expressed—Northern blot analysis was performed to examine the levels of GS15 mRNA in various rat tissues (Fig. 2). A major mRNA species of about 1.8 kb was detected in all the tissues examined, suggesting that GS15 may be involved in a general cellular process.

GS15 Is Associated with the Golgi Apparatus—Recombinant GST-GS15 fusion protein was produced in bacteria, purified, and used to raise rabbit polyclonal antibodies against GS15. Affinity-purified antibodies against GS15 recognized a 15-kDa protein enriched in Golgi membranes (Fig. 3A, lanes 4–6), and its enrichment was comparable to that of Golgi a2,6-sialyltransferase (lanes 1–3). When Golgi-enriched membranes were subjected to different extraction conditions, GS15 was effectively extracted by detergents but not by 2 M KCl, 2.5 M urea, or 0.1 M sodium bicarbonate (pH 12), establishing that GS15 is an integral membrane protein enriched in the Golgi fractions (Fig. 3B). Indirect immunofluorescence microscopy provided further independent evidence of the cellular localization of GS15 (Fig. 4). Affinity-purified antibodies against GS15 specifically la-
The fact that GS15 is structurally related to rbet1, in conjunction with the observation that GS15 has a carboxyl-terminal hydrophobic tail that is preceded by potential coiled-coil regions, prompted us to investigate whether GS15 is a new SNARE of the Golgi apparatus. When Golgi extract was sedimented on a glycerol gradient, GS15 was found to have a sedimentation coefficient of about 6 S (Fig. 5A, upper panel). Incubation in the presence of recombinant α-SNAP and NSF in a buffer that promotes formation of the 20 S SNARE complex caused GS15 to be shifted into the 20 S fractions (middle panel). This shift was not observed when Golgi extract was incubated with equal amounts of α-SNAP and NSF in a buffer that promotes disassembly of the Golgi SNARE complex. Under the disassembly conditions, GS15 has a sedimentation coefficient of 3 S (lower panel), indicating that GS15 may be associated with other proteins in the Golgi extract and that this association is disrupted by the disassembly conditions. When the 20 S fractions were immunoprecipitated with GS15 antibodies, both α-SNAP and NSF were released from the immunoprecipitate under the disassembly conditions (Fig. 5B, lane 4) but not in the assembly conditions (lane 3). Under the assembly conditions, immunoprecipitated NSF and α-SNAP remained associated with the beads (lane 1). Control antibodies (lanes 5–8) did not immunoprecipitate significant amounts of either NSF or α-SNAP.

Binding of GS15 to Recombinant GST-α-SNAP—When a fixed amount of recombinant GST-α-SNAP immobilized on beads was incubated with increasing amounts of Golgi extracts, it was revealed that increasing amounts of GS15 were retained by the beads (Fig. 6). Under identical conditions, GS15 was not retained by GST or several other GST fusion proteins (data not shown). Furthermore, other Golgi proteins, including α2,6-sialyltransferase, were not retained by immobilized GST-α-SNAP. These results further support the idea that GS15 is a Golgi SNARE.

Conclusions—Since SNAREs are key molecules mediating docking and fusion of vesicular transport and are present either in the vesicles and/or the target membrane, identification of new SNAREs will not only provide novel markers for a given transport step but also provide a novel approach for the molecular dissection of the respective transport events. Despite the fact that many SNAREs are known, few SNAREs have been identified in mammalian cells that are specifically associated with the Golgi apparatus (6–11, 26), the major organelle of the secretory pathway. In this report, we have molecularly identified a novel COOH-terminal tail-anchored integral membrane protein (GS15) that is significantly related to rbet1 exhibiting 28% amino acid identity. The mRNA is widely expressed in all the rat tissues examined. The subcellular localization of GS15 was clearly established in many cell types and it is ubiquitously associated with the Golgi apparatus. Biochemically, we have established that it behaves like a SNARE. Although the exact cellular function of GS15 remains to be investigated, the establishment of its being a novel SNARE associated with the Golgi apparatus suggests that it may participate in a transport event associated with the Golgi apparatus. What is the functional relationship between GS15 and rbet1? GS15 and rbet1 may either participate in a common transport event at either a...
similar stage or at different stages. Alternatively, they may participate in separate transport events. Although the functional aspects for rbet1 have not been revealed (11), recent studies suggest that it exists in a protein complex that could be immunoprecipitated by antibodies against syntaxin 5 (25). Since syntaxin 5 is involved in transport from the ER to the Golgi apparatus (27), in conjunction with the fact that yeast Bet1p is essential for ER-Golgi transport (12–15), rbet1 may also be involved in a similar transport event. Since the amino acid similarity between GS15 and rbet1 is around 28%, GS15 and rbet1 may have related but distinct properties. In this respect, we favor the possibilities that GS15 and rbet1 are involved in different transport events or distinct stages of a similar transport event. Further studies along this line will provide additional insight not only into the functional aspects of GS15 but also into the molecular and mechanistic aspects of the Golgi apparatus.

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