A 29-Kilodalton Golgi Soluble N-Ethylmaleimide-sensitive Factor Attachment Protein Receptor (Vti1-rp2) Implicated in Protein Trafficking in the Secretory Pathway*

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Expressed sequence tags coding for a potential SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) were revealed during database searches. The deduced amino acid sequence of the complete coding region predicts a 217-residue protein with a COOH-terminal hydrophobic membrane anchor. Affinity-purified antibodies raised against the cytoplasmic region of this protein specifically detect a 29-kilodalton integral membrane protein enriched in the Golgi membrane. Indirect immunofluorescence microscopy reveals that this protein is mainly associated with the Golgi apparatus. When detergent extracts of the Golgi membrane are incubated with immobilized glutathione S-transferase α soluble N-ethylmaleimide-sensitive factor attachment protein (GST-SNAP), this protein was specifically retained. This protein has been independently identified and termed Vti1-rp2, and it is homologous to Vti1p, a yeast Golgi SNARE. We further show that Vti1-rp2 can be qualitatively coimmunoprecipitated with Golgi syntaxin 5 and syntaxin 6, suggesting that Vti1-rp2 exists in at least two distinct Golgi SNARE complexes. In cells microinjected with antibodies against Vti1-rp2, transport of the envelope protein (G-protein) of vesicular stomatitis virus from the endoplasmic reticulum to the plasma membrane was specifically arrested at the Golgi apparatus, providing further evidence for functional importance of Vti1-rp2 in protein trafficking in the secretory pathway.

Participation of NSF1 and soluble NSF attachment proteins (SNAP) in diverse transport events in the secretory and endocytic pathways is in conjunction with a superfamily of membrane proteins termed SNAP receptors (SNAREs) (1–5). The SNARE hypothesis suggests that vesicles derived from a donor compartment harbor a set of vesicle-associated SNAREs (v-SNAREs) that will interact specifically with those associated with the target acceptor membrane (t-SNAREs) (6–11). This v-/t-SNARE pairing is a key event in the docking and fusion of the vesicle with its specific target membrane (6–11). Vesicle-associated membrane proteins (VAMPs) or synaptobrevins are v-SNAREs associated with the synaptic vesicles, whereas syntaxin 1 and SNAP-25 (synaptosome-associated protein of 25 kDa) are t-SNAREs associated with the presynaptic membrane. The specific interaction of VAMPs/synaptobrevins with the syntaxin 1-SNAP-25 complex plays a fundamental role in the docking/fusion of synaptic vesicles with the presynaptic membrane (9–11).

Because of the central role of SNAREs in diverse vesicular transport steps, molecular identification, biochemical characterization, and subcellular localization of novel SNAREs constitute fundamentally important aspects of study in the field of vesicular transport. The Golgi apparatus plays a major role in the secretory pathway (1–4, 12). Currently, five distinct SNAREs have been shown to be associated with the Golgi apparatus in mammalian cells. These include syntaxin 5 (13–15), GS15 (16), GS27 (also termed membrin) (17–18), GS28 (also named GOS-28) (19–20), and syntaxin 6 (21–22). Syntaxin 5 and GS28 have both been shown to be involved in the endoplasmic reticulum (ER) to Golgi transport. GS28 has also been implicated in transport from the cis- to the medial-Golgi (19, 20). GS27 was shown to be involved in transport from the cis/medial- to trans-Golgi/trans Golgi network (18). The functional aspects of GS15 and syntaxin 6 remain to be established (16, 21–22). In this report, we describe the molecular, biochemical, and cell biological characterizations of Vti1-rp2, a novel 29-kDa SNARE associated with the Golgi apparatus. Vti1-rp2 is structurally homologous to Vti1p, a recently described yeast Golgi SNARE (23). We further show that Vti1-rp2 exists in distinct syntaxin 5- and syntaxin 6-containing SNARE complexes and is functionally important for protein trafficking in the secretory pathway.

EXPERIMENTAL PROCEDURES

Materials—Mouse EST clones (accession numbers AA016379 and W13616) were generated by the Washington University-Merck expressed sequence tag (EST) project and made available by IMAGE consortium via Research Genetics Inc. (Huntsville, Alabama). The mouse mRNA multiple tissues Northern blot was obtained from CLONTECH (Palo Alto, CA). Mouse monoclonal antibody against Golgi mannosidase II was from Babco (Berkeley, CA). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG were purchased from Boehringer Mannheim. Brefeldin A was from Epicentre Technologies.

cDNA Cloning and Sequencing—Mouse EST clones were fully sequenced by the dyeodeoxy chain termination method using a kit from U. S. Biochemical Corp. The complete coding region was assembled using the DNA Strider 1 program.

Northern Blot Analysis—A mouse multiple tissue blot of poly(A)+ mRNA was probed with the insert of the EST clone AA016379 followed by actin probe as described previously (24).

Expression of Recombinant Proteins in Bacteria—GST fusion pro-
teins were produced using the pGEX-KG vector (25) and purified as described (24–25). Oligonucleotide 1 (5’-gtcatagttgctagttgatgaga)
and oligonucleotide 2 (5’-ctttgtactggtagttgatgagta) were used to retrieve the coding region for residues 1–185 by polymerase chain reaction using the EST clone AA016379 as the template. The polymerase chain reaction product was digested with the restriction enzymes XbaI and SalI and then inserted into a similarly digested pGEX-KG vector. After transformation of the Escherichia coli strain DH5a, colonies were screened for the production of GST-Vti1-rp2 fusion protein. GST-α-SNAP has been described previously and was purified accordingly (16). GST-Vti1-rp1 (31) was produced similarly, and the details will be described elsewhere.

Preparation of Polyclonal Antibodies—Rabbits were immunized with GST-Vti1-rp2, and the specific antibodies were affinity-purified as described previously (16, 24). Affinity-purified rabbit polyclonal antibodies against syntaxin 5, syntaxin 6, syntaxin 7, and α-COP have been described previously (26–29).

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described previously (16, 24, 30).

Differential Extraction of Golgi Membranes and Immunoblot Analysis—These were performed as described previously (16, 30).

RESULTS

Vti1-rp2, a Mammalian Protein Homologous to Yeast Vti1p—Searching the EST data bases using the amino acid sequence of a novel Golgi SNARE characterized in the lab2 led to the identification of mouse EST clones (accession numbers AA016379 and W13616) that encode a putative SNARE. The EST clone W13616 was fully sequenced, and the nucleotide and the deduced amino acid sequences are shown in Fig. 1A. This protein was independently identified in three other laboratories and has been referred to as Vti1-rp2 (31), Vti1b (32), and Vti1a (33), respectively. To avoid further confusion in nomenclature, we have adopted the name Vti1-rp1 for this protein.

Vti1-rp2 is a protein of 217 residues. Although the predicted molecular weight of Vti1-rp2 is 24,971 daltons, its apparent molecular weight was about 29 kDa (Fig. 3A), because it migrates (lane 1) in between the 30-kDa marker (the marker lane) and GS28 (lane 2) (a Golgi SNARE with an apparent size of 28 kDa) (20, 30). Another mammalian protein homologous to Vti1p has also been identified and referred to as Vti1-rp1 (31), Vti1 (32), and Vti1b (33), respectively. To avoid further confusion, we have adopted the name Vti1-rp1 for the other mammalian homolog of yeast Vti1p. Since Vti1-rp2 displays significant amino acid sequence identity (about 30%) with Vti1-rp1, it is essential to establish that our affinity-purified antibodies do not cross-react with Vti1-rp1. As shown (Fig. 3A), the detection of the 29-kDa protein in immunoblot was selectively abolished by preincubation of antibodies with recombinant cytoplasmic domain of Vti1-rp2 (lane 4) but not with the cytoplasmic domain of Vti1-rp1 (lane 3), establishing that our antibodies are specific for Vti1-rp2.

Indirect immunofluorescence microscopy was used to examine the exact subcellular localization of Vti1-rp2 (Fig. 4). Affinity-purified antibodies against Vti1-rp2 specifically labeled perinuclear structures (Fig. 4A, panel a) characteristic of the Golgi apparatus (34), and this labeling colocalized well with that of Golgi mannosidase II (panel b) (35). When the Golgi apparatus was fragmented by nocodazole treatment (panels c–d), Vti1-rp2 and mannosidase II were colocalized well in the fragmented Golgi apparatus. Similar to mannosidase II and other Golgi proteins (36), Vti1-rp2 was redistributed to the ER when cells were treated with brefeldin A (e–f). These results firmly establish that Vti1-rp2 is an integral membrane protein associated preferentially with the Golgi apparatus.

Vti1-rp2 Is a Novel Golgi SNARE—To investigate whether Vti1-rp2 indeed functions as a novel SNARE of the Golgi apparatus, we examined the potential interaction of Vti1-rp2 with α-SNAP. As shown in Fig. 5A (upper panel), Vti1-rp2 in the Golgi detergent extract was specifically retained by immobilized GST-α-SNAP in a dose-dependent manner. Under identical conditions, Vti1-rp2 was not retained by immobilized GST or several other control GST fusion proteins (data not shown). Furthermore, other Golgi proteins, including α2,6-sialyltransferase, were not retained by immobilized GST-α-SNAP (Fig. 5A, lower panel). The interaction of Vti1-rp2 with α-SNAP was

\[Vti1-rp2\]
further investigated (Fig. 5B). Proteins in the Golgi extract were incubated with GST (lanes 1 and 4), GST-α-SNAP (lanes 2 and 5), and GST-γ-SNAP (lanes 3 and 6); after extensive washing, the beads (lanes 1–3) and 1/10 of the supernatants (lane 4–6) were analyzed by immunoblot to detect Vti1-rp2 (upper row) as well as GS28 (lower row), which serves as a positive control. Vti1-rp2 was retained by GST-α-SNAP as efficiently as GS28 (lanes 2 and 5). Neither Vti1-rp2 nor GS28...
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**Fig. 2. Northern blot analysis of Vti1-rp2.** The mouse mRNA multiple tissue Northern blot (CLONTECH) containing 2 μg of poly(A)⁺ mRNA from the indicated rat tissues were processed for Northern blot analysis to detect Vti1-rp2 mRNA (A) and subsequently actin mRNA (B). The lower form of Vti1-rp2 mRNA detected only in testis may represent an alternatively spliced form. SK Muscle, skeletal muscle; kb, kilobases.

**Fig. 3. A.** Vti1-rp2 is a protein with an apparent size of 29 kDa. Proteins extracted from Golgi-enriched membranes were analyzed by immunoblot with antibodies against Vti1-rp2 (lanes 1, 3, and 4) or a monoclonal antibody against GS28 (lane 2). The detection of the 29-kDa protein was specifically blocked by preincubation of the antibodies with recombinant GST-Vti1-rp2 (lane 4) but not by the recombinant cytoplasmic domain of Vti1-rp1 fused to GST (lane 3). B, Vti1-rp2 is enriched in the Golgi membranes. Proteins extracted from total membranes (TM), microsomal membranes (MM), and Golgi membranes (GM) of rat liver were processed for immunoblot analysis with antibodies against α2,6-sialyltransferase (ST) (lanes 1–5) and antibodies against Vti1-rp2 (lanes 4–6). C, Vti1-rp2 is an integral membrane protein. Golgi membranes were subjected to different extractions as indicated, and the resulting supernatants (S) and pellets (P) were processed for immunoblot analysis with antibodies against Vti1-rp2. PBS, phosphate-buffered saline.

was retained by GST (lanes 1 and 4). To lesser extents, Vti1-rp2 and GS28 was significantly retained by GST-γ-SNAP. These results establish that interaction of Vti1-rp2 with GST-α-SNAP is specific and occurs with efficiencies comparable with that of known Golgi SNAREs such as GS28. Furthermore, interaction of Vti1-rp2 with immobilized GST-α-SNAP could be abolished by NSF in conditions that promote dissociation of SNARE complexes (Fig. 5C). When Golgi extract was incubated with immobilized GST-α-SNAP in the presence of increasing amounts of NSF in conditions (assembly buffer) that promote formation of SNARE complexes (lanes 1–6), comparable amounts of Vti1-rp2 were retained. However, retention of Vti1-rp2 by immobilized GST-α-SNAP was readily abolished by NSF in conditions (lane 7–12) that promote ATP hydrolysis by NSF and disassembly of SNARE complexes. These results not only further confirmed that the interaction of Vti1-rp2 with α-SNAP is specific but also revealed that the interaction of Vti1-rp2 with α-SNAP is in the context of Vti1-rp2-containing SNARE complexes.

**Fig. 4.** Vti1-rp2 is associated preferentially with the Golgi apparatus. Control normal rat kidney cells (a and b), normal rat kidney cells treated with 10 μg/ml nocodazole for 1 h at 37 °C (c and d), and normal rat kidney cells treated with 10 μg/ml brefeldin A for 1 h at 37 °C (e and f) were double-labeled with rabbit polyclonal antibodies against Vti1-rp2 (a, c, and e) and a monoclonal antibody against Golgi mannosidase II (Man II) (b, d, and f). Bar, 10 μm.

**Comunoprecipitation of Vti1-rp2 with Syntaxin 5 and 6**—To gain additional understanding of Vti1-rp2 as a Golgi SNARE, we investigated the potential interaction of Vti1-rp2 with two Golgi t-SNAREs, the cis-Golgi syntaxin 5 (13–15, 17) and the trans-Golgi network syntaxin 6 (21–22). Golgi detergent extracts were immunoprecipitated with antibodies against syntaxin 5, syntaxin 6, or control rabbit IgG. The immunoprecipitates (beads) (lanes 1, 2, 5, and 6) and 1/10 of the supernatants (lanes 3, 4, 7, and 8) were analyzed by immunoblot analysis. As shown in Fig. 6 (lanes 1–4), syntaxin 5 was efficiently precipitated by its antibodies (lanes 1 and 3) but not by control antibodies (lanes 2 and 4). Vti1-rp2 was clearly coimmunoprecipitated by antibodies against syntaxin 5 (lanes 1 and 3) but not by control antibodies (lanes 2 and 4), suggesting that a significant fraction of Vti1-rp2 exists in a syntaxin 5-containing SNARE complex. Usually, about 9–5% of Vti1-rp2 could be coimmunoprecipitated by antibodies against syntaxin 5. Under identical conditions, syntaxin 6 was not coimmunoprecipitated by antibodies against syntaxin 5 (lanes 1 and 3). When Golgi extracts were immunoprecipitated with antibodies against syntaxin 6 (lanes 5–8), essentially all syntaxin 6 was precipitated (lanes 5 and 7). Furthermore, Vti1-rp2 was obviously coimmunoprecipitated by anti-syntaxin 6 antibodies (lanes 5 and 7) but not by control control IgG (lanes 6 and 8). About 10–15% of Vti1-rp2 is routinely coimmunoprecipitated.
by antibodies against syntaxin 6. In contrast, syntaxin 5 was not coimmunoprecipitated with anti-syntaxin 6 antibodies (lanes 5 and 7). These results suggest that significant amounts of Vti1-rp2 exist in at least two distinct SNARE complexes, one containing syntaxin 5 and the other containing syntaxin 6. This conclusion was further substantiated by our observation that significant amounts of syntaxin 5 and syntaxin 6 were coimmunoprecipitated by antibodies against Vti1-rp2 (data not shown).

A Role of Vti1-rp2 in Protein Transport in the Secretory Pathway—The association of Vti1-rp2 with the Golgi apparatus and its establishment as a SNARE suggest that it may participate in protein trafficking in the secretory pathway. To investigate this, Vero cells grown on coverslips were first infected with vesicular stomatitis virus ts045 and then microinjected with affinity-purified antibodies against Vti1-rp2. Transport of G-protein along the secretory pathway was monitored by indirect immunofluorescence microscopy. Since microinjection of antibodies against EAGE epitope of β-COP was shown previously to inhibit G-protein transport (29), cells microinjected with β-COP antibodies serve as the positive control. We have shown recently that syntaxin 7 is in the endosomal compartment (28), and syntaxin 7 is thus not expected to function in the secretory pathway. Cells microinjected with syntaxin 7 antibodies thus serve as a negative control. As shown in Fig. 7, in cells microinjected with antibodies against Vti1-rp2 (C, arrows), surface labeling of G-protein was dramatically reduced, resulting in accumulation of G-protein in perinuclear structures characteristic of the Golgi apparatus (D, arrows). This inhibitory effect is comparable with that seen in cells microinjected with antibodies against β-COP (A–B, arrows). In marked contrast, transport of G-protein to the cell surface was unaffected (E and F, arrows) in cells microinjected with syntaxin 7 antibodies. These results suggest that transport of G-protein from the ER to the plasma membrane is specifically inhibited in cells microinjected with antibodies against Vti1-rp2, and the site of inhibition seems to be at the level of the Golgi apparatus because G-protein was seen to accumulate in structures characteristic of the Golgi apparatus and the arrested G-protein colocalized well with markers of the Golgi apparatus such as 12-β-hydroxysteroid dehydrogenase (data not shown).

Although more detailed future experiments are needed to address the mechanistic aspects of Vti1-rp2 involvement in protein transport, these results clearly revealed a role of Vti1-rp2 in protein transport in the secretory pathway.

DISCUSSION

We have identified a novel 29-kDa mammalian protein (Vti1-rp2) that has characteristics of a SNARE based on the presence of a COOH-terminal hydrophobic membrane anchor and several regions that can potentially form coiled-coil structures (6–11, 16). Three observations establish that Vti1-rp2 is indeed a SNARE. First, Vti1-rp2 in Golgi detergent extract can interact with immobilized GST-α-SNAP in a specific and dose-dependent manner. Interaction of Vti1-rp2 with immobilized GST-α-SNAP occurs with efficiencies comparable with that of known Golgi SNAREs such as GS28 (20). The second line of evidence is that association of Vti1-rp2 with GST-α-SNAP could be abolished by NSF, specifically under conditions that promote disassembly of SNARE complexes. 200 μg of Golgi extract was incubated with 2 μg of immobilized GST-α-SNAP in the presence of indicated amounts of NSF in either assembly (lanes 1–6) or disassembly buffer (lanes 7–12). The amounts of Vti1-rp2 bound onto the beads were then determined by immunoblot.
double labeling to visualize microinjected antibodies (in the presence of cycloheximide, cells were then fixed and processed for incubation at 40 °C for 2 h. After a further incubation at 31 °C for 45 min matitis virus ts045 were microinjected with antibodies against taxin-like t-SNARE of the pre-vacuolar compartment (equiva-

transport from the late Golgi to the vacuole (equivalent to the mammalian lysosome) by interacting with Pep12p (39), a syn-

port. Furthermore, Vti1p has also been shown to interact with at least five distinct syntaxin-like t-SNAREs (40). Since Vti1p participate in two distinct transport events (one associated with the secretory pathway and the other in the endosomal pathway), the existence of two distinct mammalian proteins homologous to Vti1p indicates that the two equivalent transport events in mammalian cells may be mediated by two distinct proteins. The preferential association of Vti1-rp2 with the Golgi apparatus indicates that Vti1-rp2 may participate in a transport event in the secretory pathway. Consistent with this, we have shown that microinjection of antibodies against Vti1-rp2 specifically inhibited transport of G-protein to the cell surface at the level of Golgi apparatus. The extents of inhibition of G-protein transport seen in cells microinjected with Vti1-rp2 antibodies are comparable with those seen in cells microinjected with antibodies against β-coat protein. Serving as a negative control, G-protein transport to the plasma mem-

brane was unaffected in cells microinjected with antibodies against endosomal syntaxin 7. Vti1-rp2 thus plays a role in protein transport in the secretory pathway, and the role of yeast Vti1p in the secretory pathway is most likely mediated by Vti1-rp2 in mammalian cells. Furthermore, our preliminary studies with Vti1-rp1 suggests that it is preferentially associ-

ated with the trans-Golgi network and/or the endosomal compartment, indicating that the endosomal role of yeast Vti1p is most likely mediated by Vti1-rp1 in mammalian cells.

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Fig. 7. A role of Vti1-rp2 in protein transport along the secretory pathway in intact cells. Vero cells infected with vesicular stomatitis virus ts045 were microinjected with antibodies against β-COP (A and B), Vti1-rp2 (C and D), or syntaxin 7 (E and F). Cells were then incubated at 40 °C for 2 h. After a further incubation at 31 °C for 45 min in the presence of cycloheximide, cells were then fixed and processed for double labeling to visualize microinjected antibodies (A, C, and E) as well as G-protein (B, D, and F). In four independent microinjection experiments, about 95% of the cells microinjected with antibodies against Vti1-rp2 (altogether about 150 cells) showed similar defect in G-protein transport.

provides the third line of evidence to support that Vti1-rp2 is a novel SNARE. The subcellular localization of Vti1-rp2 was established by two independent results. First, Vti1-rp2 is highly enriched in a membrane fraction that is also enriched for the Golgi apparatus. Furthermore, Vti1-rp2 colocalized well with the Golgi marker mannose-6-phosphate II in both control as well as nocodazole-fragmented Golgi apparatus. Like mannose-6-phosphate II, Vti1-rp2 could be redistributed into ER-like structures by brefeldin A. It is thus firmly established that Vti1-rp2 is a novel SNARE of the Golgi apparatus.

Data base searches with Vti1-rp2 sequence revealed that Vti1-rp2 is most homologous to Vti1p. Vti1p is a recently identified v-SNARE of the yeast Golgi and has been implicated in two independent vesicular transport events (23). By interacting with the early Golgi t-SNARE Sed5p (the yeast counterpart of syntaxin 5) (37–38), Vti1p has been suggested to function as a v-SNARE for vesicles involved in retrograde intra-Golgi transport. Furthermore, Vti1p has also been shown to be involved in transport from the late Golgi to the vacuole (equivalent to the mammalian lysosome) by interacting with Pep12p (39), a syntaxin-like t-SNARE of the pre-vacuolar compartment (equiva-

lent to the mammalian late endosome). Whether Vti1-rp2 represents the mammalian counterpart of Vti1p remains to be further investigated, although another mammalian protein (Vti1-rp1) homologous to Vti1p could functionally substitute for the yeast Vti1p (32). Besides its sequence homology with Vti1p, another property of Vti1-rp2 that is similar to Vti1p is that a significant amount of Vti1-rp2 exists in a syntaxin 5-containing Golgi SNARE complex. In addition, a significant amount of Vti1-rp2 was also shown to be present in a syntaxin 6-containing SNARE complex. Since coimmunoprecipitation of syntaxin 5 and syntaxin 6 was not observed, these results suggest that Vti1-rp2 exists in distinct syntaxin 5- and syntaxin 6-containing SNARE complexes. Although the functional aspects remain to be established, syntaxin 6 has been shown recently to be enriched in the trans-Golgi network (22).

The presence of Vti1-rp2 in at least two distinct Golgi SNARE complexes indicates that it may function as a SNARE for at least two types of vesicle-mediated transport events. One will dock and fuse with cis-Golgi by interaction with syntaxin 5, whereas the other will dock and fuse with trans-Golgi network via interaction with syntaxin 6. The interaction of Vti1-rp2 with at least two syntaxin-like t-SNAREs is consistent with a recent study showing that yeast Vti1p could interact with at least five distinct syntaxin-like t-SNAREs (40). Since Vti1p participate in two distinct transport events (one associated with the secretory pathway and the other in the endosomal pathway), the existence of two distinct mammalian proteins homologous to Vti1p indicates that the two equivalent transport events in mammalian cells may be mediated by two distinct proteins. The preferential association of Vti1-rp2 with the Golgi apparatus indicates that Vti1-rp2 may participate in a transport event in the secretory pathway. Consistent with this, we have shown that microinjection of antibodies against Vti1-rp2 specifically inhibited transport of G-protein to the cell surface at the level of Golgi apparatus. The extents of inhibition of G-protein transport seen in cells microinjected with Vti1-rp2 antibodies are comparable with those seen in cells microinjected with antibodies against β-coat protein. Serving as a negative control, G-protein transport to the plasma mem-

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