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Identification and Characterization of an 18-Kilodalton, VAMP-Like Protein in Suspension-Cultured Carrot Cells[1]

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Polyclonal antibodies raised against rat vesicle associated membrane protein-2 (VAMP-2) recognized, in carrot (*Daucus carota*) microsomes, two major polypeptides of 18 and 30 kD, respectively. A biochemical separation of intracellular membranes by a sucrose density gradient co-localized the two polypeptides as resident in light, dense microsomes, corresponding to the endoplasmic reticulum-enriched fractions. Purification of coated vesicles allowed us to distinguish the subcellular location of the 18-kD polypeptide from that of 30 kD. The 18-kD polypeptide is present in the non-clathrin-coated vesicle peak. Like other VAMPs, the carrot 18-kD polypeptide is proteolyzed by tetanus toxin after separation of coatomers. Amino acid sequence analysis of peptides obtained by digestion of the 18-kD carrot polypeptide with the endoproteinase Asp-N confirms it to be a member of the VAMP family, as is suggested by its molecular weight, vesicular localization, and toxin-induced cleavage.

The vesicular transport of proteins between different membrane compartments is a highly conserved process common to all eukaryotic cells. In the early secretory pathway, which is considered the equivalent of an assembly line for newly synthesized proteins, vesicle-mediated membrane trafficking occurs between the endoplasmic reticulum (ER) and the Golgi complex. Vesicle transport is also present for retrograde transport from Golgi to the ER in a process essential for recycling of the anterograde trafficking and for retrieval of ER-resident proteins. From the trans-Golgi network (TGN), which is considered the beginning of the late phase of the secretory pathway, vesicles are formed able to sort proteins destined to the vacuole (or the lysosome) away from others intended for secretion or for localization at the plasma membrane.

In plants, a direct route between the TGN and the vacuole has not been described, but a prevacuolar compartment is known to be involved. The vesicles are formed at donor organelles through the action of several distinct coat proteins responsible for selection and packaging of their cargo (specific proteins). Specific membrane proteins termed SNAREs, which separately reside on the vesicle and target membranes, determine the target of a transport vesicle. The term SNARE is used to describe two distinct families of integral proteins: the v-SNAREs found on vesicle membranes and the t-SNAREs found mainly on the target membrane. Both v- and t-SNAREs are anchored into their respective membranes and both contain coiled-coil domains that allow interactions between v- and t-SNARE pairs. The SNARE proteins do not act alone in directing the vesicular traffic, but many other factors are necessary to regulate their function. This field has been recently reviewed by Sanderfoot and Raikhel (1999).

The docking and fusion events between the donor and target membrane were initially identified at the synapse level (Südhof, 1995). Successively, many homologs to synapse-specific proteins have been found in other cellular fusion systems, where they possibly perform analogous functions (Ferro-Novick and Jahn, 1994). Genetics and "in silico" data obtained from the entire genomic sequence show that yeast is likely to have the basic set of t-SNARE proteins (Pelham, 1998). Although the molecules that affect vesicle transport in the plant secretory pathway are still poorly characterized, recently, several plants proteins have been identified which appear to be homologous (or orthologous) counterparts of animal and yeast proteins involved in vesicle budding and fusion. Genes have been isolated in Arabidopsis that are functionally homologous to the yeast genes for SAR1 and SEC12 proteins involved in ER to Golgi traffic (d'Enfert et al., 1992; Bar-Peled and Raikhel, 1997). A large family of small GTP-binding proteins have been identified in plants (for review, see Gomord and Faye, 1996). A syntaxin homolog, PEP12, has been isolated from Arabidopsis cDNA (Bassham et al., 1995) and its encoded protein is resident on a late post-Golgi, more precisely, a prevacuolar compartment present in plant cells (da Silva Conceição et al., 1997; Sanderfoot et al., 1998). The gene product altered in Arabidopsis KNOLLE mutant codes for a syntaxin-related protein involved in the process of cytokinesis (Lukowitz et al., 1996) and represents a t-SNARE that has no counterpart in the yeast genome.

Concerning the v-SNARE proteins, vesicle-associated membrane protein (VAMP) is an 18-kD integral component of the synaptic vesicles with a single carboxy-terminal transmembrane domain (Trimble et al., 1988; Baumert et al., 1989). Evidence that VAMP may play a role in synaptic

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vesicle docking or fusion is provided by the observation that VAMP is a substrate for the zinc-endopeptidases (tetanus toxin and botulinum toxins type B, D, F, and G), each of which is able to block neurotransmitter release (Schiavo and Montecucco, 1995). VAMP homologs have been characterized from a large number of organisms (from yeast to torpedo, from *Drosophila* to rat and human; Elferink et al., 1989; Südhof et al., 1989; Protopopov et al., 1993). In addition, another member of the VAMP family was identified and named cellubrevin. mRNA transcripts of cellubrevin were detected in several organs of rat (from testis to cerebellum, from liver to kidney; McMahon et al., 1993). Recently, two isoforms of VAMP (VAMP-1 and -2), expressed in the nervous system, were shown to be present in a large number of rat tissues (Rossetto et al., 1996). In yeast, Sec22p, Bet1p, Bos1p, and Ykt6P have been identified as v-SNAREs in ER to Golgi transport (Newman et al., 1990; McNew et al., 1997). Another v-SNARE, Vti1p, which was identified in yeast, is instead involved in vesicular traffic to the vacuole (Fischer von Mollard et al., 1997). Recently, an ortholog of Vti1P has been identified in Arabidopsis cells (Zheng et al., 1999).

Members of the v-SNARE family are involved in functionally homologous roles and share common structural elements even if they show low sequence identities (18%–40%).

Using polyclonal antibodies raised against rat VAMP-2 protein we identified a VAMP-like protein in carrot cells. This protein is located in non-clathrin-coated vesicles and, in appropriate experimental conditions, can be digested by tetanus toxin.

MATERIALS AND METHODS

Plant Materials

Carrot cells (*Daucus carota* L. cv S. Valery) were grown in suspension culture in Gamborg's B5 medium with 0.5 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.25 mg/L 6-benzilaminopurine (6-BAP) at 25°C. The cell cultures were maintained on a rotary shaker at 70 rpm in a room which had a light:dark cycle of 16:8 h. Eight-day-old cells were harvested onto filter paper, frozen immediately in liquid nitrogen, and conserved at −80°C for intracellular membranes and coated vesicle preparations.

Separation of Intracellular Membranes by Density Gradient Centrifugation

Twenty-five grams of packed carrot cells was ground in a mortar with liquid nitrogen, resuspended in 2 volumes of homogenization buffer (25 mM Tris-2-[N-morpholino]-ethanesulfonic acid [MES], pH 7.5, 0.25 M Suc, 3 mM EDTA, 1 mM dithiothreitol [DTT]), 1 μg/mL leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride and centrifuged for 15 min at 10,000*g* at 4°C. The supernatant was centrifuged for 60 min at 150,000*g*, supernatant (named cytosol) was collected, and the pellet (termed total microsomes) was thoroughly resuspended (using several passes of a glass homogenizer) in 1 mL of buffer containing 5 mM Tris-MES, pH 7.5, 0.25 mM Suc, 3 mM EDTA, and 1 mM DTT and loaded into a 11-mL linear gradient of 15% to 50% (w/w) Suc buffered with 5 mM Tris-MES, pH 7.5, 3 mM EDTA, and 1 mM DTT. Suc gradients were centrifuged at 80,000*g* for 5 h at 4°C in a swinging bucket rotor. Fractions (1 mL) then were collected and stored at −80°C until analysis.

Enzyme Assays

Plasma membrane-specific vanadate-sensitive ATPase activity: Proteins (15 μg) were incubated for 20 min at 37°C in 0.5 mL of buffer containing 30 mM Tris-MES, pH 6.5, 50 mM KCl, 5 mM $MgSO_4$, 5 mM ATP (Tris salt), 0.02% (v/v) Triton X-100, 100 mM ammonium molybdate, and 3 mM NaN_3 in the presence or absence of 0.1 mM vanadate. The reaction was terminated by the addition of 1 mL of stop solution: 5% (w/v) SDS, 2% (w/v) H_2SO_4, and 0.5% (w/v) $(NH_4)_2Mo_7O_{24}$. The assay was incubated 20 min at room temperature in the presence of 50 μL of 10% (w/v) ascorbic acid and the optical density was determined at 660 nm. Golgi membrane-specific Triton-stimulated UDPase activity was determined as described by Nagahashi and Kane (1982). NADH cytochrome *c* reductase (±antimycine A) activity was determined as described by Hodges and Leonard (1974).

Purification of Coated Vesicles

All isolation steps were performed at 4°C and the manipulations on ice. Coated vesicles were purified as described by Lin et al. (1992) with some modifications.

Carrot cells (100 g) were ground in a mortar with liquid nitrogen and homogenized in 2 volumes of buffer A (0.1 M MES-NaOH, pH 6.5, 0.3 M Suc, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% [w/v] NaN_3, 1 mM DTT, 1 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/mL pepstatin) and centrifuged for 10 min at 6,000*g*. The supernatant was kept on ice while the pellet was ground in a mortar with an equal amount of acid-washed sand in 30 mL of buffer A and centrifuged for 10 min at 6,000*g*. Supernatants from two centrifugations were unified, centrifuged for 15 min at 20,000*g*. The supernatant was centrifuged at 150,000*g* for 60 min, the microsomal pellet was resuspended in 10 mL of buffer B (buffer A without Suc), and incubated with 10 mg of RNase A at 4°C for 40 min.

The incubation mixture was centrifuged at 6,000*g* for 15 min. The supernatant was loaded into a 28-mL linear gradient of 9% to 90% ^2H_2O in buffer B and centrifuged at 40,000*g* for 35 min. The supernatant was diluted with 2 volumes of buffer B and centrifuged for 60 min at 150,000*g*. The pellet was thoroughly resuspended (using several passes of a glass homogenizer) in 2 mL of buffer B and loaded into 10-mL linear gradient of 9% ^2H_2O/2% Ficoll to 90% ^2H_2O/25% Ficoll in buffer B and centrifuged at 80,000*g* for 16 h. At the end of the centrifugation, 1-mL fractions were collected from top to bottom and the pellet was resuspended in 2 mL of buffer B. All fractions from ^2H_2O/Ficoll gradient and other samples were frozen in liquid nitrogen and stored at −80°C until analysis.

Antibodies

Rat GST-VAMP-2 and rat GST-VAMP-1 were expressed as GST fusion proteins and were purified by affinity chromatography on GSH-agarose matrix according to the method of Schiavo and Montecucco (1995). Antibodies specific for VAMP-2 and VAMP-1 were generated in chicken by injecting recombinant rat GST-VAMP-2 and -1 fusion proteins. Twenty eggs were collected from each chicken and the antibodies were purified from the yolk (Jensenius et al., 1981).

Assay of Proteolytic Activity

Coated (10 μg) vesicles or 1 M Tris-HCl, pH 8.3, washed, coated vesicles (Kirsch et al., 1994) were incubated in 100 μL of HEPES buffer (10 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, and 10 μM $ZnCl_2$) with 200 nM tetanus toxin for 2 h at 37°C. Tetanus toxin was purified and activated by preincubation with 10 mM dithiothreitol in HEPES buffer for 30 min at 37°C as described by Schiavo and Montecucco (1995).

Gel Electrophoresis

For most experiments gel electrophoresis was performed with the buffer system of Laemmli (1970) and the blots were stained with antibodies and alkaline-phosphatase-conjugated secondary antibodies. When low-M_r peptides were to be separated, the Tricine buffer system was used (Schägger and von Jagow, 1987). Protein concentrations were determined according to the Bradford assay (Bradford, 1976).

Negative Staining of Coated Vesicles

Purified coated vesicles were negatively stained with uranyl acetate as previously described by Depta and Robinson (1986). Observations were made with a electron microscope (model EM 300, Hitachi) operating at 75 kV.

In-Gel Cleavage of 18-kD Vesicle Polypeptide

Total proteins of CV1 vesicles were separated on Tris-Tricine buffer system SDS-PAGE. The gel was stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 in 30% (v/v) ethanol for 30 min and destained in 5% (v/v) acetic acid for 1 h. Three stained bands around 18-kD molecular mass marker (Fig. 8A) were individually excised from the preparative SDS-PAGE gel, the small pieces of each excised protein bands were reloaded on a new gel, transblotted to the nitrocellulose paper, and immunoblotted with anti-VAMP-2 antibodies. The excised polypeptide band that gave a strong signal with anti-VAMP-2 antibodies and corresponded to the lower band of the doublet shown in Figure 4B, was reloaded on a new 10% (w/v) gel (four gel slices in each lane) to increase purification and concentration of the polypeptide. This 18-kD stained band was excised again and digested in the gel slice with the endoproteinase Asp-N (Boehringer) as described in Adessi et al. (1995). The digested peptides were extracted and separated by reverse-phase HPLC column Vydac C18 (4.6 × 150 mm, 5 μm, Separation Group, Esperia, CA). The fractions corresponding to the recorded peaks were collected and the peaks with sufficient amount of peptides were sequenced by an automated pulsed liquid protein sequencer (model 477A, Applied Biosystems, Foster City, CA).

RESULTS

Rat Anti-VAMP-2 Antibodies Recognize Three Major Polypeptides of 18, 19.2, and 30 kD in Carrot Cell Total Microsomes

Proteins from total microsomes and the cytosol were isolated from carrot cells after 3, 5, 7, and 10 d of culture (see "Materials and Methods") and analyzed by immunoblotting with the rat anti-VAMP-2 and -1 antibodies. Rat anti-VAMP-2 but not -1 (data not shown) antibodies reacted with 18- and 30-kD polypeptides in carrot cell total (10-μg) microsomes (Fig. 1A). A weak signal of 18-kD polypeptide was also present in the cytosol (Fig. 1B), suggesting that it is the result of non-sedimented light microsomes or vesicles (a soluble form seems unlikely, but cannot be excluded). When higher amounts of total microsomal protein (30 μg) were loaded on the gel two

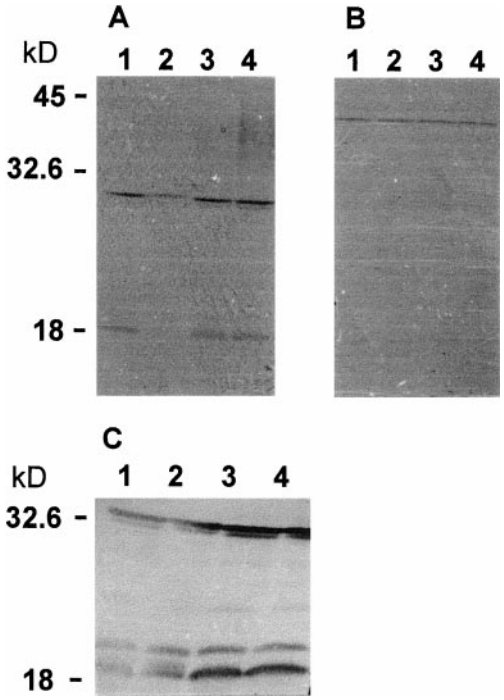

Figure 1. Antibodies against rat VAMP-2 recognize three major bands of 18-, 19.2-, and 30-kD polypeptides in carrot microsomes. A, Proteins of total microsomes (10 μg) were separated on 12.5% (w/v) SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with rat anti-VAMP-2 antibodies. B, Cytosol (10 μg) was treated as in A. C, Total microsomes (30 μg) were treated as in A. Lanes 1 to 4 correspond to protein preparations from 3-, 5-, 7-, and 10-d-old carrot cells.

polypeptides of 18 and 19.2 kD were visualized by immunoblotting with anti-VAMP-2 antibodies (Fig. 1C). As the amount of these polypeptides is higher at the end of the subculturing cycle (Fig. 1C, lanes 3 and 4), in subsequent experiments 8-d-old carrot cells were used.

Subcellular localization of 18-, 19.2-, and 30-kD polypeptides was analyzed in microsomal fractions obtained from 15% to 50% (w/w) Suc linear gradient centrifugation of total microsomes. Fractions were collected from top to bottom and analyzed for the following enzymatic activities: vanadate-sensitive ATPase, Triton-stimulated UDPase, NADH cytochrome *c* reductase as enzymatic markers for the plasma membrane, Golgi apparatus, and ER, respectively (Fig. 2). Immunoblot analysis of the microsomal fractions separated on a linear Suc gradient showed that the 18-, 19.2-, and 30-kD polypeptides recognized by anti-VAMP-2 antibodies are localized in light dense microsomes, particularly in ER-rich fractions (Figs. 2 and 3A, lanes 3 and 4). The ratios of 18- and 19.2-kD polypeptides vary in fraction numbers 3 and 4 (Fig. 3A), reflecting a non-identical distribution of these polypeptides in the two microsomal fractions. In the same fractions a 20.7-kD band was also observed (Fig. 3A), but we do not know if this is another isoform or a cross-reacting protein. Rat anti-VAMP-1 antibodies recognized the 19.2-kD polypeptide in fraction number 4 (Fig. 3B, lane 4) in linear Suc gradient, but the signal was very weak and in the subsequent experiments only anti-VAMP-2 antibodies were used.

Localization of 18- and 19.2-kD Polypeptides in CVs

Recent studies of the VAMP protein family demonstrate that these proteins are localized in transport vesicles in mammalian and yeast cells (see introduction). We purified coated vesicles from carrot cells in 9% ^2H_2O/2% Ficoll to 90% ^2H_2O/25% Ficoll gradient (see "Materials and Methods"). The protein profile of the fractions from ^2H_2O/Ficoll gradient gave three peaks (Fig. 4A) that we have named region 0 (CV0), region 1 (CV1), and region 2 (CV2). Protein aliquots from each step of vesicle purification and from ^2H_2O/Ficoll gradient fractions were analyzed by immunoblotting with anti-VAMP-2 antibodies (Fig. 4B). The 30-kD polypeptide that has the highest affinity to anti-VAMP-2 antibodies in total microsomes (Fig. 1A) and is associated with the ER (Fig. 3A) was not localized in coated vesicles (Fig. 4B). In 10 independent vesicle preparations, the 18- and 19.2-kD polypeptides were always localized in region 1 (CV1) protein peak fractions (Fig. 4B). The ratios and the intensity of the doublet depend of the amount of total vesicular protein loaded on the gel. The 19.2-kD polypeptide is not visible in immunoblots with anti-VAMP-2 antibodies if less than 10 μg of protein was loaded on the gel (data are not shown). Therefore, we decided to concentrate on the 18-kD polypeptide.

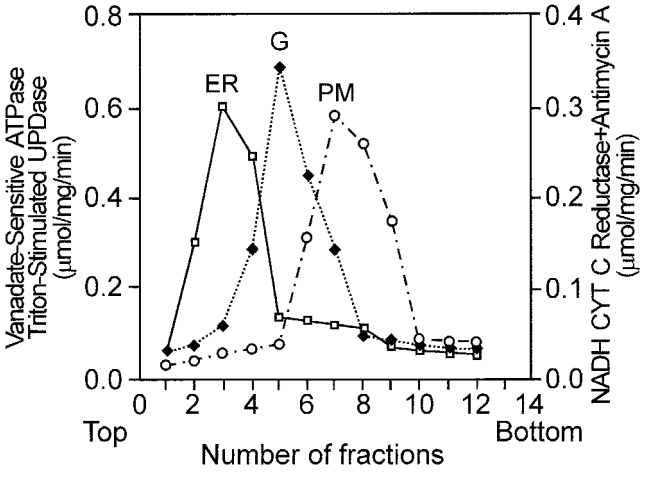

Figure 2. Distribution of membrane markers following fractionation of carrot cell membranes over a Suc density gradient. Microsomal membrane pellets were centrifuged at 80,000*g* in 15% to 50% (w/w) linear Suc gradient; fractions from top to bottom were collected and analyzed for enzymatic activities. Shown are peaks of activity for: NADH cytochrome *c* reductase, a marker for the ER; Triton-stimulated UDPase, a marker for the Golgi membranes (G); vanadate-sensitive ATPase, a marker for the plasma membrane (PM).

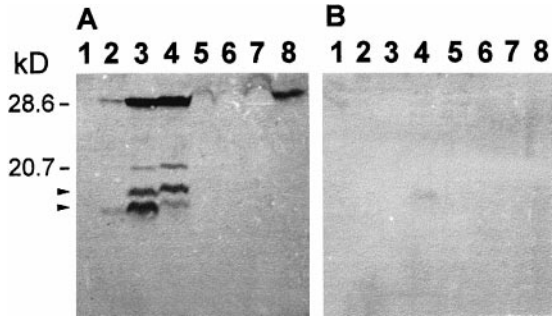

Figure 3. Intracellular localization of 18-, 19.2-, and 30-kD polypeptides in carrot cells. A, Protein from fractions 1 to 8 (20 μg) obtained from Suc linear gradient (Fig. 2) were separated on 12.5% (w/v) SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-VAMP-2. Arrowheads indicate the 19.2- and 18-kD polypeptides. B, As in A, but anti-VAMP-1 antibodies were used.

In our preparations, negatively stained coated vesicles from CV1 (Fig. 5A) can be easily distinguished from the clathrin-coated vesicles, CV2 (Fig. 5B). The latter have a significantly thicker coat and are enriched in a 190-kD band (see Fig. 8, lanes 7–9) that reacts with anti-clathrin antibodies (not shown). The diameter of CV1 vesicles varies from 40 to 100 nm and they are morphologically similar to COP1 or COP2 vesicles in mammalian cells that mediate transport from ER to the Golgi complex (Kreis et al., 1995). In plant cells the existence of COP-coated vesicles has not yet been demonstrated but, recently, the identification of probable coat proteins of plant COP vesicles has been reported (Movafeghi et al., 1999). In our preparations, the clathrin-coated vesicles (Fig. 5B) were concentrated in region 2 (CV2) protein peak fractions from the ^2H_2O/Ficoll gradient (Fig. 4A). No proteins in the CV2 vesicle peak reacted with rat anti-VAMP-2 antibodies and, in subsequent experiments, proteins from CV1 fractions were used for the biochemical characterization of the 18-kD polypeptide that we named carrot VAMP-like protein.

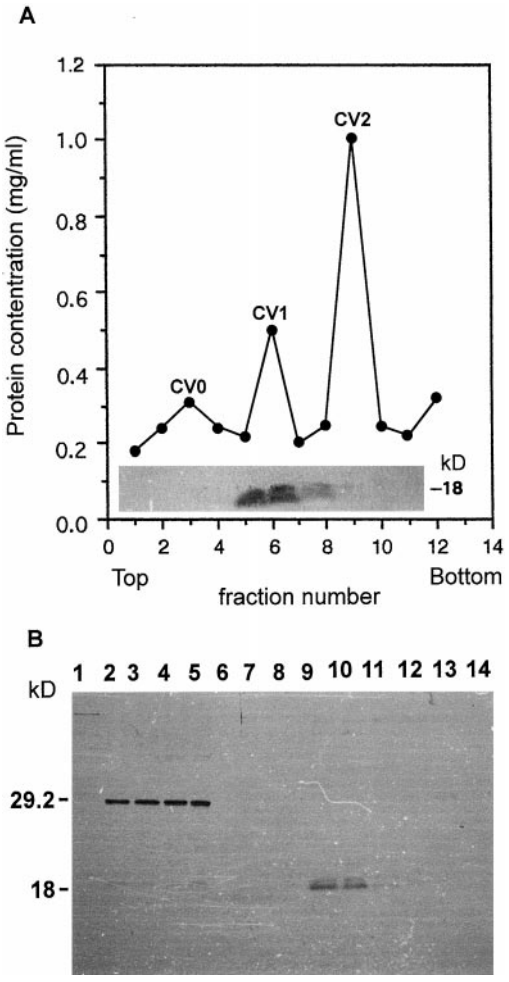

Figure 4. Eighteen- and 19.2-kD polypeptides are localized in carrot cell coated vesicles. A, Protein profile of linear isopycnic Ficoll/^2H_2O gradient. The relevant part (around 18-kD molecular mass marker) of the immunoblot shown below (B) is reported on the bottom of the figure to correlate the presence of carrot VAMP-like protein with the profile of CV1 region. B, Proteins (10 μg) from each steps of vesicles preparation were analyzed by immunoblot with anti-VAMP-2 antibodies; cytosol (1), total microsomes (2), 6,000*g* pellet after RNase incubation of microsomes (3), 9% to 90% (v/v) ^2H_2O linear gradient pellet (4), Ficoll/^2H_2O gradient pellet (5). Lanes 6 to 14 correspond to the number of fractions 2 to 10 from Ficoll/^2H_2O linear gradient (A).

Carrot VAMP-Like Protein as a Tetanus Toxin Target

Rat VAMP-2 is a target for tetanus toxin (Schiavo and Montecucco, 1995) that cleaves the protein at a single site. Functional similarities between rat VAMP-2 and carrot VAMP-like proteins could be assumed if they were similarly recognized by tetanus toxin. For this reason, we tested whether tetanus toxin also cleaves the carrot VAMP-like protein. To wash out the coatomers, the vesicles from the CV1 protein peak fractions (Fig. 4A) were diluted with an equal volume of buffer A (see "Materials and Methods") without Suc and centrifuged for 1 h at 150,000*g*. The pellet was resuspended in 1 M Tris-HCl (pH 8.3) for 30 min at room temperature and centrifuged again for 1 h at 350,000*g* (Kirsch et al., 1994). Resulting pellets were resuspended in HEPES buffer.

Ten micrograms of proteins was incubated at 37°C for 2 h in the presence of 200 nM DTT-activated (or non-activated) tetanus toxin and analyzed by immunoblot (Fig. 6A). The 18-kD VAMP-like protein was partially degraded by active tetanus toxin and the 12-kD digested peptide was recognized by rat anti-VAMP-2 antibodies. The inactive form of tetanus toxin was used as a control. We have tried different conditions (substrate-enzyme concentrations, incubation times, from 2 h to overnight) to optimize the digestion of 18-kD polypeptide by tetanus toxin. In all experiments we

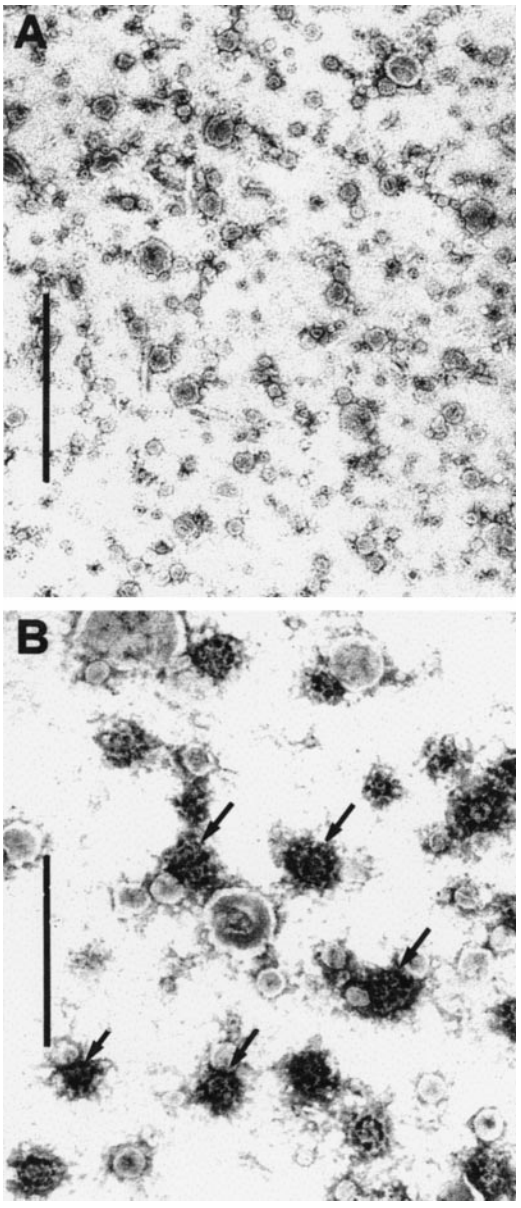

Figure 5. Coated vesicles from carrot cells. A, Electron micrographs of non-clathrin-coated vesicles (CV1 as indicated in Fig. 4A). B, Electron micrographs of clathrin-coated vesicles (CV2 as indicated in Fig. 4A). Coated vesicles were negatively stained with uranyl acetate. Bar = 0.5 μm.

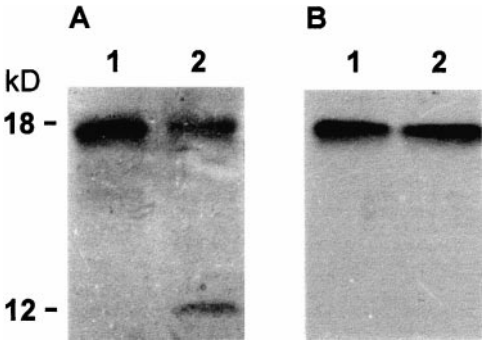

Figure 6. Eighteen-kilodalton carrot VAMP-like protein as a tetanus toxin target. Coated vesicles from CV1 protein peak (10 μg) were incubated 2 h at 37°C. with 200 nM inactivated (lane 1) or DTT-activated (lane 2) tetanus toxin. The proteins then were separated in 12% (v/v) SDS-PAGE Tris-Tricine buffer system, transferred into PVDF membrane, and immunoblotted with anti-VAMP-2 antibodies. A, High-pH-washed vesicles (1 M Tris-HCl pH 8.3). The 12-kD band represents the tetanus toxin-degraded peptide that reacts with anti-VAMP-2 antibodies. B, Intact vesicles.

performed, the polypeptide was digested partially (data not shown). The specificity of tetanus toxin for carrot 18-kD polypeptide as a VAMP-like protein was not high enough to digest it completely.

We could not test protein concentrations higher than 0.1 $\mu g/\mu L$ because the vesicles aggregated in the HEPES buffer after washing with 1 M Tris-HCl, pH 8.3. Even the human synaptobrevin II 1-93 fragment (synthesized by the solid-phase peptide synthesis technique), is not completely soluble at the approximate concentration of 1 mM, which is the range of the V_{max} for the digestion by tetanus toxin L chain (Cornille et al., 1994). On the other hand, the efficiency of the tetanus toxin for VAMP proteins, from different sources, vary in in vitro experiments (80%–25%) and depends on the amino acid sequences of the v-SNARE proteins (Yamasaki et al., 1994; Pellizzari et al., 1996).

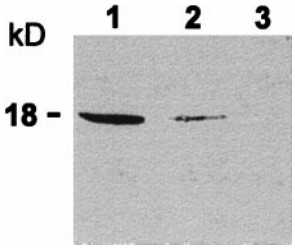

Figure 7. Eighteen-kilodalton carrot VAMP-like protein is tightly bound to the coated vesicles. CV1 coated vesicles were incubated 30 min at room temperature in 1 M Tris-HCl, pH 8.3, centrifuged at 350,000*g* for 1 h. The supernatant containing 1 M Tris-washed proteins (lane 2) was kept and the pellet was resuspended in the buffer containing 1% (w/v) CHAPS, incubated for 30 min at 25°C, and centrifuged at 350,000*g* for 1 h. Ten micrograms of protein from supernatant (lane 3) and pellet (lane 1) were analyzed by immunoblot with anti-VAMP-2 antibodies.

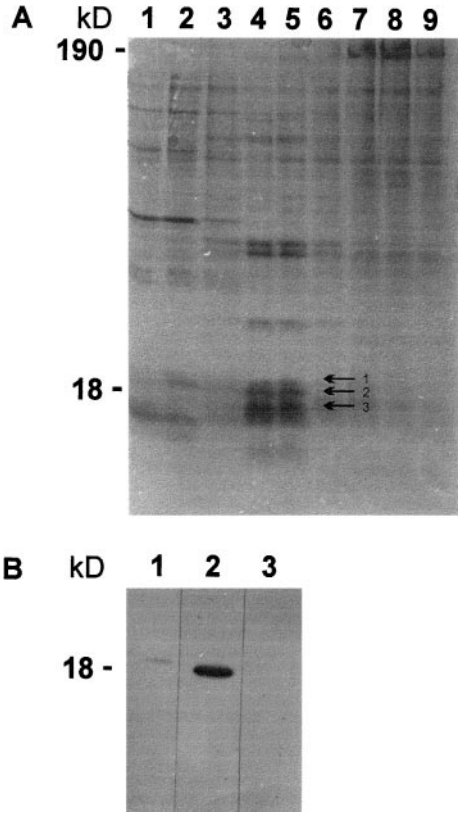

Figure 8. Isolation of 18-kD polypeptide from SDS-PAGE gel. Ten micrograms of proteins from ^2H_2O/Ficoll gradient fractions were separated by 12.5% (w/v) SDS-PAGE and visualized by staining with Coomassie Brilliant Blue G (A). Lanes 1 to 9 are corresponding to fraction number 2 to 10 in Figure 4A. Three bands around 18-kD molecular mass marker, indicated by arrows and numbered 1, 2, and 3, were excised from fraction numbers 4 and 5 (corresponding to the CV1 protein pattern), reloaded on the new SDS-PAGE gel, and immunoblotted with anti-VAMP-2 antibodies (B). Band number 2 was used for in-gel digestion by endoproteinase Asp-N and for amino acid analyses of the peptides.

The 18-kD polypeptide was not degraded by activated tetanus toxin if CV1 vesicles were not washed with high pH (8.3) Tris-HCl buffer (Fig. 6B), suggesting that the toxin cannot reach its substrate when vesicles are covered with coatomers.

18-kD Carrot VAMP-Like Protein Shares Motifs Common to VAMP

The carrot 18-kD VAMP-like protein and the 12-kD tetanus toxin digested peptide were transblotted onto PVDF membrane for microsequence analysis. Both peptides were blocked in the N-terminal domain like the other proteins of the VAMP family. The carrot 18-kD VAMP-like protein is tightly bound to the vesicle membranes and it cannot be solubilized even with 1% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid (CHAPS) detergent (Fig. 7). The CV1 vesicular proteins were separated by Tris-Tricine SDS-PAGE. Three bands around 18-kD molecular mass marker were identified by Coomassie blue

Figure 9. Peptide fragments from the 18-kD polypeptide aligned to VAMP-like proteins. Sequence fragments from yeast and animal VAMP and plant VAMP-like proteins were aligned by CLUSTAL using the algorithm of Higgins and Sharp (1989). Amino acid sequence fragments aligned to the carrot peptide fragments are boxed. Within boxes, residues are reported in red (or green) when identical (or similar) to the corresponding residue of the carrot peptide fragment. The three motifs KLXXVKA, LLVD, and DFRT, fully conserved in plant VAMP-like proteins AtEST and SAR 1, are underlined in carrot peptide fragments. The protein sequence sources are as follows: (a) d'Enfert et al. (1992), GB:ATHSAR1; (b) Protopopov et al. (1993), GB:YSCNC2G-1; (c) Chin et al. (1993), GB:DROSYBANB; and (d) Elferink et al. (1989), GB:RATVAMPB-1. Arabidopsis AtEST was obtained from the University of Minnesota's sequence analysis project (dbEST ID 656807). Sequence SAR1 from Arabidopsis was formerly known as HAT24 (Schena and Davis, 1992).

staining of the gel (Fig. 8A) and individually excised from a preparative SDS-PAGE gel.

A small piece of each gel slice from all three bands was reloaded on the new gel and immunoblotted with anti-VAMP-2 antibodies. The bands numbered 1 and 2 were recognized by anti-VAMP-2 antibodies (Fig. 8B) corresponding to the 19.2- and 18-kD polypeptides of the doublet shown in Figure 4B. Since the 18-kD polypeptide band showed the strongest signal, it was electroeluted from SDS-PAGE gel slices. Once electroeluted, the 18-kD polypeptide aggregates and forms a highly hydrophobic and insoluble pellet. Therefore, the gel slices of 18-kD polypeptide were concentrated on the new gel and subjected to the in-gel digestion procedure with endoproteinase Asp-N, which cleaves upstream of both aspartic and glutamic residues (Hagmann et al., 1995).

Digested peptides were separated by HPLC and nine peaks with sufficient amount of peptides were sequenced. The amino acid sequencing of the peptides revealed that one of them was blocked at its N-terminal end. Five of the peptides displayed identity or similarity to amino acid patterns within the conserved region of the VAMP protein family and could be aligned with animal, yeast VAMP proteins and with plant synaptobrevin related proteins (Fig. 9). As expected, the amino acid sequence of each peptide was found to start with a D or E residue. While proteins other than VAMP may show patterns similar to one or, exceptionally, two of the carrot peptides, only proteins of the VAMP family showed homology to all five regions, simultaneously. The Arabidopsis synaptobrevin-related proteins showed 100% identity to all of the four motifs identified (Fig. 9). A search of the Swissprot and translated EMBL databases, using these short motif sequences, revealed that no other proteins contain all four of these motifs regardless of what order they are placed in. Replacing the amino acids of the four motifs with functional equivalent residues in all combinations did not change that result.

DISCUSSION

Rat anti-VAMP-2 antibodies recognize major polypeptides of 18, 19.2, and 30 kD in total microsome preparations of carrot cells. Linear Suc gradients were performed to obtain fractions enriched in ER, Golgi apparatus, and PM, respectively. The three polypeptides of 18, 19.2, and 30 kD co-localized with the ER enriched fraction numbers 3 and 4 (Fig. 3A). However, there is a difference between the 18 kD on one side and the other two polypeptides. The former is essentially present in fraction number 3, whereas the two others co-localize equally in fraction numbers 3 and 4. A purification of coated vesicles from carrot microsomes allowed us to distinguish the intracellular localization of the 18- and 19.2-kD polypeptides from the 30-kD polypeptide.

The 30-kD polypeptide is localized neither in clathrin-coated nor in non-clathrin-coated vesicles; thus, it may perhaps be a SNARE protein cross-reacting with anti-VAMP-2 antibodies (Pellizzari et al., 1996). We have demonstrated that the 18- and 19.2-kD polypeptides are both enriched in purified non-clathrin vesicles (CV1). Two isoforms of VAMP have been identified in various organisms (human, rat, *Drosophila*, and yeast) (Elferink et al., 1989; Archer et al., 1990; Chin et al., 1993; Protopopov et al., 1993) and this seems also to be the case in plants.

The non-clathrin-coated vesicles are thought to be involved in anterograde or retrograde transport between the ER and Golgi complex. In plant cells, no intermediate compartment between the ER and the Golgi apparatus has yet been identified. Non-clathrin-coated vesicles have been identified in algae by electron microscopy (Noguchi and Morré, 1991); they are 50 to 70 nm in diameter and are morphologically similar to the non-clathrin-coated vesicles described in human cells (Pryer et al., 1992). Coated transition vesicles, 50 to 70 nm in diameter, mediating the ATP- and temperature-dependent transfer between the ER donor and Golgi apparatus acceptor, have been reported in etiolated seedlings of garden pea (Hellgreen et al., 1993).

On the other hand, it has been reported in plant cells that vesicles without a clathrin coat are involved in the transport of the storage proteins to the vacuoles (Hohl et al., 1996). In our preparations the diameter of non-clathrin-coated vesicles (Fig. 5A) varies between 40 and 100 nm, whereas the morphology remains similar. The yield of these vesicles is extremely low (we obtained 300–500 μg of total vesicular protein from 100 g of carrot cells), but in all preparations they were separated on ^2H_2O/Ficoll gradient

from clathrin-coated vesicles, forming a light yellow ring on top of clathrin-coated vesicles.

In this work, we concentrated on the biochemical characterization of the 18-kD polypeptide, the most abundant of the two forms localized in the non-clathrin vesicles, whose molecular mass coincides with the VAMP proteins involved in vesicular traffic in other systems. Like the other v-SNAREs, the carrot VAMP-like 18-kD protein is an integral membrane protein. It was not solubilized from membranes (neither in total microsomes nor in vesicle membranes) even with high concentrations (4%) of detergents such as CHAPS or Triton X-100 (data not shown). When electroeluted from SDS-PAGE, the carrot 18-kD VAMP-like protein precipitates in the electroelution buffer (100 mM NH_4HCO_3, 0.1% [w/v] SDS) forming an insoluble pellet, thus demonstrating that it is a highly hydrophobic protein like other members of the v-SNARE protein family.

The v-SNAREs involved in different steps of vesicle trafficking in mammalian and yeast cells show low percent (18%–40%) amino acid homology. This makes it very difficult to identify and clone the genes from different species. The conserved regions are limited to short runs of amino acids that are relatively close together and quite degenerated, making use of a molecular biological approach such as RT-PCR very difficult.

Owing to the low amount and low solubility of the 18-kD polypeptide, we thought that an "in-gel" digestion strategy was suitable. Using this method, we were able to generate the peptide sequences of nine short fragments, only five of them informative, showing homology with a conserved region. The five reported amino acid sequences present identity or similarity to the conserved region of the VAMP family proteins. The degree of conservation appears higher if the comparison is with plant synaptobrevin-related proteins, as shown for the four motifs presented in Figure 9. No other known protein shows the simultaneous presence of those four motifs, a strong indication that the 18-kD polypeptide we have identified in carrot cells is a member of the VAMP family proteins. Moreover, the fact that the 18-kD polypeptide can be hydrolyzed by the tetanus toxin is strong evidence that it belongs to the VAMP family. This biochemical similarity between rat VAMP-2 and the carrot 18-kD polypeptide suggests that the latter, too, may form a membrane fusion complex. It seems, therefore, that the carrot 18-kD VAMP-like protein (perhaps together with the 19.2-kD isoform) is a v-SNARE involved either in traffic from ER to Golgi or from Golgi to the vacuole.

Although much work on the molecular mechanisms underlying vesicle targeting and fusion has been carried out in recent years, only few SNARE proteins have been identified in plants.

In Arabidopsis, an ortholog of the yeast Pep12p has been identified as a t-SNARE that resides on a late post-Golgi or more precisely prevacuolar compartment (da Silva Conceição et al., 1997; Sanderfoot et al., 1998). The C-terminal portion of this protein displays the highest homology to the KNOLLE protein, the only plant t-SNARE known to function at the plasma membrane (Lukowitz et al., 1996; Lauber et al., 1997). Arabidopsis cells also possess an ortholog of Vti1p (Zheng et al., 1999). The yeast v-SNARE Vti1p was found to interact with two t-SNAREs, one on the cis-Golgi compartment (Sed5p) and the second on the prevacuolar compartment (Pep12p) (Fischer von Mollard et al., 1997), demonstrating that a single v-SNARE can function in two separate vesicle trafficking steps.

Our vesicle preparation allowed us to purify accurately the clathrin- and the non-clathrin-coated vesicles, providing the opportunity to perform a further detailed immunolocalization analysis of cloned plant proteins involved in vesicle trafficking as has already been performed in animal cells.

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