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Syntaxin 7 Complexes with Mouse Vps10p Tail Interactor 1b, Syntaxin 6, Vesicle-associated Membrane Protein (VAMP)8, and VAMP7 in B16 Melanoma Cells*

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Syntaxin 7 is a mammalian target soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) involved in membrane transport between late endosomes and lysosomes. The aim of the present study was to use immunoaffinity techniques to identify proteins that interact with Syntaxin 7. We reasoned that this would be facilitated by the use of cells producing high levels of Syntaxin 7. Screening of a large number of tissues and cell lines revealed that Syntaxin 7 is expressed at very high levels in B16 melanoma cells. Moreover, the expression of Syntaxin 7 increased in these cells as they underwent melanogenesis. From a large scale Syntaxin 7 immunoprecipitation, we have identified six polypeptides using a combination of electrospray mass spectrometry and immunoblotting. These polypeptides corresponded to Syntaxin 7, Syntaxin 6, mouse Vps10p tail interactor 1b (mVti1b), α-synaptosome-associated protein (SNAP), vesicle-associated membrane protein (VAMP)8, VAMP7, and the protein phosphatase 1M regulatory subunit. We also observed partial colocalization between Syntaxin 6 and Syntaxin 7, between Syntaxin 6 and mVti1b, but not between Syntaxin 6 and the early endosomal t-SNARE Syntaxin 13. Based on these and data reported previously, we propose that Syntaxin 7/mVti1b/Syntaxin 6 may form discrete SNARE complexes with either VAMP7 or VAMP8 to regulate fusion events within the late endosomal pathway and that these events may play a critical role in melanogenesis.

In eukaryotic cells, proteins are transported between intracellular organelles by a series of membrane transport steps. The ability of discrete organelles to fuse in a highly specific way is central to all membrane-trafficking events and relies on a series of molecular events. One event is the formation of a protein complex between sets of molecules found within the transport vesicle (v-SNAREs)[1] and the target membrane (t-SNAREs). Much of the work that has led to the formulation of this hypothesis has been performed in the mammalian synapse (1). Here the R- or v-SNARE, VAMP2, forms a complex with two Q- or t-SNARE proteins, Syntaxin 1a and SNAP25. This ternary complex consists of a four-α-helical bundle containing one helix from both Syntaxin 1a and VAMP2 with the remaining two helices being contributed by SNAP25 (2). SNARE complexes that regulate traffic to the cell surface in both mammalian and yeast cells contain three distinct proteins, whereas most intracellular SNARE complexes appear to be comprised of four separate proteins: one v-SNARE and three t-SNAREs (3, 4). For example, in *Saccharomyces cerevisiae* endoplasmic reticulum to Golgi transport is regulated by the Sed5p·Bos1p·Sec22p·Bet1p complex, whereas vacuolar transport is regulated by a complex comprising Vam3p·Vam7p·Vti1p·Nyv1p (3). The Syntaxin isoform, or t-SNARE heavy chain (3), associated with each complex appears to be highly specific to a particular vesicle transport step, whereas the light chain t-SNAREs associate with multiple complexes. Vti1p, for example, interacts with Syntaxin homologs involved in Golgi/endosome, intra-Golgi, vacuolar, and prevacuolar transport steps (5).

Sequencing projects are revealing the existence of an ever increasing number of SNARE family members (6). In the yeast *S. cerevisiae* there are eight different Syntaxin-like proteins, and in mammalian cells, at least 20 have been identified to date (7, 8). A major challenge is to establish which transport steps each of these proteins regulates. This will require establishing the intracellular location of these proteins as well as their binding partners. Syntaxins 1, 2, 3, and 4 localize to the plasma membrane (9); Syntaxin 5 operates between the endoplasmic reticulum and the Golgi apparatus; and Syntaxins 6, 7, 8, 13, and 16 are found in the endosomal system of mammalian cells (10–14).

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[1] The abbreviations used are: SNARE(s), soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor(s); t-, target; v-, vesicle; VAMP, vesicle-associated membrane protein; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; SNAP25, synaptosomal-associated protein of 25 kDa; Sed5, suppressor of the erd2 deletion mutant; Bos1, Bet one suppressor; Sec, secretion; Bet, blocked early in transport; Vam, vacuolar morphology; Vti, Vps10p tail interactor; Nyv, new yeast v-SNARE; GST, glutathione *S*-transferase; Syn, Syntaxin; EEA1, early endosomal antigen 1; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; eGFP, enhanced green fluorescent protein; HPLC, high performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; Tlg, t-SNARE of the late Golgi; Pep, peptidase-deficient gene; mVti1, mouse-Vti1.

The present study focuses on Syntaxin 7. Syntaxin 7 is localized to compartments within the endosomal system where it regulates transport between the late endosome and the lysosome (14–16). We have reported previously that Syntaxin 7 interacts with at least one v-SNARE, VAMP8 (14). Recent analysis of VAMP8 has demonstrated a requirement for this v-SNARE in the homotypic fusion of both early endosomes and late endosomes (17). Another v-SNARE, VAMP7, has also been implicated in late endosomal transport (16, 18), but its relationship to Syntaxin 7 has not yet been explored. This highlights the need for a more thorough characterizaton of Syntaxin 7 binding partners. The aim of the present study was to identify Syntaxin 7-binding proteins using a biochemical approach. To this end, we took advantage of the fact that Syntaxin 7 is expressed at very high levels in the melanoma cell line, B16. Using B16 cells as a source of Syntaxin 7, we used an immunoprecipitation approach combined with mass spectrometry to identify mVti1b, Syntaxin 6, and α-SNAP as Syntaxin 7-binding proteins. In addition, we find that Syntaxin 7 forms a complex with both VAMP7 and VAMP8, suggesting that the t-SNARE Syntaxin 7 may regulate distinct fusion events within the endocytic pathway by associating with distinct subsets of partner SNARE proteins.

MATERIALS AND METHODS

Cells and Membrane Preparation—B16 Melanoma cells were kindly provided by Dr. Peter Parsons (QIMR, Brisbane, Australia) and were cultured in RPMI medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.). For immunoblot analysis, total membranes were prepared as follows. Cells were lysed by passage through a 27-gauge needle in HES buffer (20 mM Hepes, 0.5 mM EDTA, 250 mM sucrose) containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 250 μM phenylmethylsulfonyl fluoride) before centrifugation at 150,000 $\times$ *g* for 30 min. The membrane pellet was resuspended in HES buffer containing 1% Triton X-100 and incubated on ice for 1 h, after which insoluble material was removed by centrifugation at 17,500 $\times$ *g* for 10 min. Triton X-100-soluble extracts of rat tissues, obtained from male Wistar rats, were prepared by homogenization of the relevant tissues in HES buffer containing protease inhibitors. Homogenates were incubated for 30 min at 4 °C in the presence of 1% Triton X-100, after which insoluble material was removed by centrifugation at 17,500 $\times$ *g* for 10 min.

Antibodies, Plasmids, and Immunoblot Analysis—Polyclonal antiserum against mouse Syntaxin 7 was obtained by immunizing rabbits with a chimeric protein consisting of the cytosolic domain of Syntaxin 7 fused to GST (pGST-Syn7a) (14). Antibodies raised against the GST portion of the antigen were removed by passing the antiserum over a column of GST linked to Affi-Gel (Bio-Rad). The flow-through from this column was subsequently passed over a second column made by coupling the GST-Syn7antigen to Affi-Gel. Syntaxin 7-specific antibodies were eluted from this column with 100 mM glycine, 150 mM NaCl (pH 2.8). These affinity-purified antibodies were then titrated to pH 7.5 using Tris-HCl (pH 8). The VAMP8 antibodies have been described previously (14). Antibodies to VAMP7 were raised against GST fusion protein containing the entire cytosolic portion of VAMP7, which was expressed from pPL815. Similarly, antibodies against Syntaxin 6 were raised by immunizing rabbits with a GST protein containing the entire cytosolic tail of this protein (pGST-Syn6; 14) or were purchased from Transduction Laboratories. A plasmid encoding the cytosolic tail of human Syntaxin 13 fused to GST and an antibody produced against this fusion protein were kindly provided by Dr. R. Teasdale (Institute for Molecular Bioscience, University of Queensland). The EEA1 antibody was the generous gift of Dr. Ban Hock Toh (Monash Medical School, University of Melbourne, Australia). Antibodies against mVti1b (19) were kindly provided by Dr. G. Fischer von Mollard (University of Gottingen, Germany). Antibodies against cytosolic domains of Syntaxin 4 and VAMP3 have been described previously (20). Membrane samples were subjected to SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes. Membranes were blocked in 1% (w/v) non-fat dried milk and then incubated with primary antibodies in phosphate-buffered saline containing 0.1% (v/v) Tween 20 and 1% (w/v) non-fat dried milk for 1 h at room temperature at dilutions that were optimized for each antibody. Visualization of antibody-labeled bands was achieved with the use of horseradish peroxidase-labeled secondary antibodies purchased from Amersham Pharmacia Biotech, and Supersignal Dura chemiluminescent substrate was from Pierce. Immunoblotting competition assays were performed by including the relevant GST fusion protein antigen (100 μg/ml) during incubation with the primary antibody. The GFP-Syn6 plasmid that encodes the full-length murine Syntaxin 6 fused to the C terminus of enhanced GFP (eGFP) was a kind gift from Dr. Jeffrey Pessin (University of Iowa) (21).

Immunofluorescence Microscopy—Cells were grown to 70% confluence on glass coverslips and fixed in 2% paraformaldehyde for 30 min. In some cases B16 cells were transiently transfected with the GFP-Syntaxin 6 vector using the LipofectAMINE reagent according to the manufacturer's instructions (Life Technologies, Inc.). This plasmid generated a protein of the expected molecular mass (~60 kDa) which could be specifically immunoblotted with antibodies specific for either Syntaxin 6 or GFP (data not shown). After fixation, cells were quenched for 5 min in 150 mM glycine, washed in phosphate-buffered saline, and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 15 min. After blocking coverslips with 2.5% normal swine serum for 30 min at room temperature primary antibody incubations were performed in 1% normal swine serum in phosphate-buffered saline for 1 h at room temperature, followed by an appropriate fluorescein isothiocyanate- or Texas Red-labeled secondary antibody (Molecular Probes, Eugene, OR) for 30 min at room temperature. Cells were viewed using a 63×/1.4 Ziess oil immersion objective on a Zeiss Axiovert fluorescence microscope, equipped with a Bio-Rad MRC-600 laser confocal imaging system. Images were imported into Adobe Photoshop™ (Adobe Systems Inc.) and assembled as indicated.

Immunoprecipitation—Affinity matrices were prepared by first binding antibodies (affinity-purified anti-Syn7 antibodies with or without an excess of GST-Syn7 fusion protein and purified rabbit IgG antibodies) to protein A-Sepharose beads (Amersham Pharmacia Biotech). Antibodies were covalently cross-linked to protein A beads using dimethyl pimelimidate (Pierce). Triton X-100-soluble membrane extracts prepared from B16 cells, which had been cultured for 7 days, were prepared as outlined above and incubated with antibody-coated beads for 1 h in the presence of 150 mM NaCl, 20 mM Hepes, and 0.5 mM EDTA. The beads were washed in the same buffer containing 1% Nonidet P-40. Elution of bound proteins was achieved by boiling for 10 min in Laemmli sample buffer containing 4% SDS. Proteins were separated on a 5–15% SDS-polyacrylamide gradient gel and visualized by staining with either silver or Coomassie Brilliant Blue. Individual protein bands were excised and subjected to sequence analysis.

Protein Sequencing and Mass Spectrometer Analysis—In-gel proteolytic digestion (using 0.5 μg of trypsin) of resolved proteins was performed as described previously (22). An electrospray ionization ion trap mass spectrometer (LCQ Finnigan MAT, San Jose, CA) coupled on-line with a capillary HPLC (Hewlett-Packard model 1090A) modified for capillary chromatography was used for peptide sequencing. The column used in this study was a 150 $\times$ 0.20-mm (inner diameter) capillary column (Brownlee RP-300, 7 μm C8) manufactured using a polyvinylidene difluoride end frit A 60-min linear gradient (flow rate 1.7 μl/min) was used from 0–100% B, where solvent A was 0.1% v/v aqueous trifluoroacetic acid, and solvent B was 0.1% aqueous trifluoroacetic acid in 60% acetonitrile. The electrospray ionization parameters were as follows: spray voltage, 4.5 kV; sheath gas and auxiliary gas flow rates, 5 and 30 (arbitrary value), respectively; capillary temperature, 150 °C; capillary voltage, 20 V; and tube lens offset, 16 V. The sheath liquid used was 2-methoxyethanol (99.9% HPLC grade) delivered at a flow rate of 3 μl/min. The electron multiplier was set to −860 V. In both MS and MS/MS modes, the trap was allowed a maximum injection time of up to 200 ms. The automatic gain control parameter was turned on for all experiments, ensuring that the number of ions in the trap was automatically kept to a constant preset value. The range scanned in MS mode was 350–2,000 kDa, and in MS/MS the range varied according to the mass of the ion selected for MS/MS. After acquiring one scan in MS, the most intense ion in that spectrum above a threshold of 1×10^5 was isolated for subsequent zoom scan (to determine charge state), then collision-induced dissociation or MS/MS in the following scans. The dissociation energy for MS/MS was set to 55%. All spectra were recorded in centroid mode. The sequences of individual peptides were identified using the SEQUEST algorithm (incorporated into the Finnigan Xcalibur-Biomass software) (23). Spectra not identified by SEQUEST were interpreted manually using *de novo* methods.

RESULTS

Specificity of the Syntaxin 7 Antibody—A Syntaxin 7-specific antibody was raised against a bacterial fusion protein contain-

ing the cytosolic tail (residues 1–234) of Syntaxin 7 fused to GST. Antibodies that specifically recognize the Syntaxin 7 portion of this fusion protein were affinity purified as described under "Materials and Methods." This antibody recognizes one band of molecular mass 40 kDa (Fig. 1*A*, *lane 1*). Immunolabeling of this band was completely inhibited in the presence of an excess of the GST-Syn7 antigen (*lane 2*). In contrast, a fusion protein consisting of the analogous region of Syntaxin 13 fused to GST had no significant effect on the recognition of this band (*lane 3*). In addition, the mobility of the polypeptide recognized by the Syntaxin 7 antibody was significantly different from the bands labeled with antibodies specific for either Syntaxin 13 or Syntaxin 6 (Fig. 1*A*, *lanes 4* and *5*). To validate further the specificity of the Syntaxin 7 antibody we performed immunofluorescence microscopy using B16 melanoma cells. The antibody labeled a tubulovesicular compartment that was concentrated in the perinuclear region of the cell (Fig. 1*B*). This labeling was not significantly different upon inclusion of excess GST-Syn13 fusion protein. However, in the presence of excess GST-Syn7 the immunolabeling was almost completely absent, consistent with the immunoblotting data (Fig. 1*A*). Collectively, these data show that our Syntaxin 7 antibody is specific and does not recognize other closely related Syntaxin isoforms.

Syntaxin 7 Is Expressed at High Levels in B16 Cells—We have shown previously that Syntaxin 7 is widely expressed in rodent tissues and that it regulates a membrane transport step within the late endosome/lysosomal system of mammalian cells (14). In view of this function we reasoned that cell types that are specialized for late endosomal/lysosomal biogenesis might up-regulate the machinery that is involved in these transport steps. Melanocytes are a specialized cell type whose primary function is the biogenesis of melanosomes, which represent lysosome-like organelles (24). Consistent with this notion is our finding that the levels of the Syntaxin 7 protein in the B16 melanosome cell line is ~10-fold higher than in all other tissues and cell lines tested (Fig. 2). In contrast, the level of Syntaxin 4, which localizes to the plasma membrane, was much more uniform across all tissues and cell types, including B16 cells. A similar uniformity was observed for Syntaxin 6 (Fig. 2) and Syntaxin 13 (data not shown) across these tissues and cell types. Intriguingly, VAMP8, a v-SNARE that has also been implicated in late endosomal transport (17), is also expressed at very high levels in B16 cells compared with other tissues and cell lines (Fig. 2).

Immunoprecipitation of Syntaxin 7-interacting Proteins—The high levels of Syntaxin 7 which we observed in B16 cells gave us a good opportunity to find interacting proteins using a coimmunoprecipitation approach. The Syntaxin 7-specific antibody (Fig. 1) was covalently linked to protein A-Sepharose, forming an affinity matrix. This was used to immunoprecipitate Syntaxin 7-containing protein complexes from a B16 cell extract. Using a non-ionic detergent solubilized extract of B16 cells we were able to immunoprecipitate Syntaxin 7 with high efficiency and specificity (Fig. 3). Initially, to identify proteins that interact with Syntaxin 7, immunoprecipitates were subjected to SDS-PAGE followed by silver staining (Fig. 3*B*). We were able to detect a number of proteins that coprecipitated with Syntaxin 7 under these experimental conditions (see *first* and *fourth lanes*). Most of these protein bands could not be detected when the immunoprecipitation was performed using a control IgG (*second lane*) or when the incubation with the Syntaxin 7 antibody was performed in the presence of excess recombinant GST-Syn7 (*fifth lane*). The most prominent bands detected were of average molecular mass 230, 130, 120, 90, 35, and 30 kDa. To identify these proteins we increased the scale of the immunoprecipitation procedure so that bands could be visualized by staining with Coomassie Brilliant Blue (see Fig. 3*C*). These bands were excised, and tryptic peptide products were sequenced using electrospray mass spectrometry. The

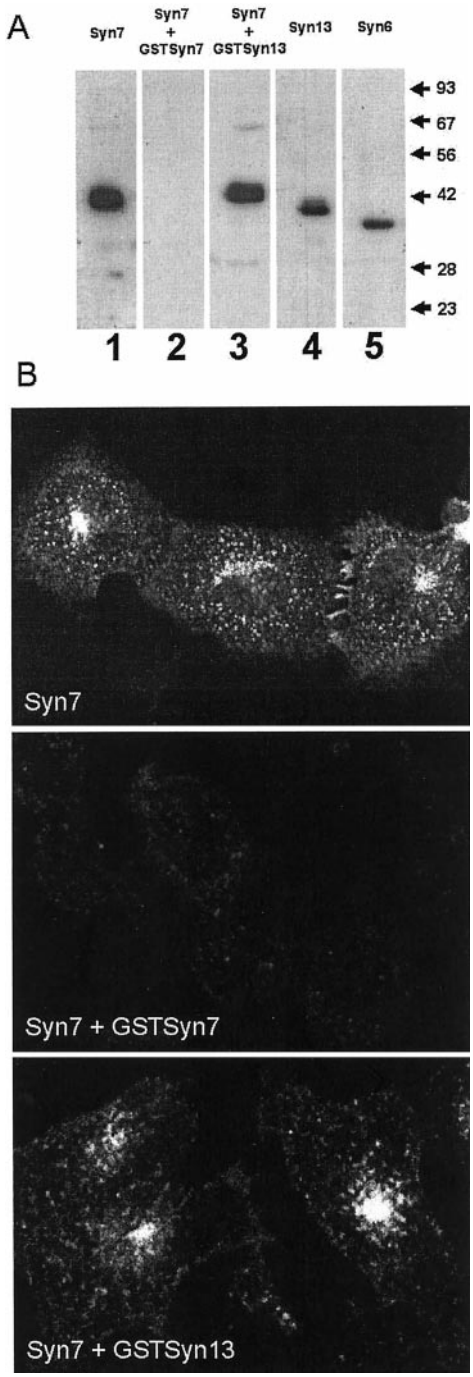

FIG. 1. **Specificity of the Syntaxin 7 antibody.** A rabbit polyclonal antibody was raised against a GST fusion protein containing the cytosolic tail of Syntaxin 7. The antibody was affinity purified and used either for immunoblotting (*panel A*) or immunofluorescence microscopy (*B*). Equal amounts of protein (10 μg) from a B16 mouse melanoma extract were subjected to SDS-PAGE followed by immunoblotting using either the Syntaxin 7 antibody (*lanes 1–3*) or antibodies against Syntaxin 13 (*lane 4*) or Syntaxin 6 (*lane 5*). Immunoblotting using the Syntaxin 7 antibody was also performed in the absence or presence of either excess GST-Syn7 protein (100 μg/ml, *lane 2*) or excess GST-Syn13 protein (100 μg/ml, *lane 3*). The relative position of molecular weight markers is shown at the *right*. In *panel B*, B16 cells were plated on glass coverslips, fixed, permeabilized, and immunolabeled with the Syntaxin 7 antibody alone or in the presence of either excess GST-Syntaxin 7 (100 μg/ml) or excess GST-Syntaxin 13 (100 μg/ml). Specific labeling was visualized using a Texas Red-conjugated secondary antibody.

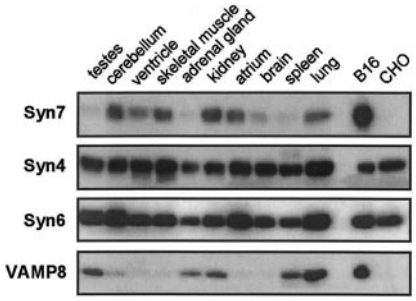

FIG. 2. **Expression of different SNAREs among rat tissues and cell lines.** Rat tissues were prepared as described under "Materials and Methods" to obtain a Triton X-100-soluble total membrane extract. Extracts from either Chinese hamster ovary (*CHO*) or B16 cells were similarly obtained. Equal amounts of lysate protein from each sample (10 μg) were subjected to SDS-PAGE and immunoblotting with antibodies specific for the various SNARE proteins as indicated. Each antibody immunolabeled one major band of the appropriate molecular mass.

sequences obtained are shown in Table I. Several of the bands yielded very clear sequence data and gave positive identities when these sequences were used for BLAST searching of the protein data base. The doublet at 130/120 kDa contained sequences corresponding to the protein phosphatase 110-kDa regulatory subunit (25) and a tyrosine kinase associated protein known as BAP-135 (26). In an effort to verify the presence of BAP-135 in the Syntaxin 7 immunoprecipitate B16 cells were incubated with pervanadate because this causes a marked stimulation of BAP-135 tyrosine phosphorylation in B cells (26). However, immunoblotting of the Syntaxin 7 immunoprecipitate, obtained from these cells, using a phosphotyrosine antibody failed to reveal any detectable product corresponding to BAP-135 (data not shown). Of particular interest, we were able to detect a number of peptide sequences in the Syntaxin 7 immunoprecipitate corresponding to Syntaxin 7, α-SNAP (27), mVti-1b (GenBank NP058080), and VAMP8 (28). The t-SNARE, Syntaxin 6, was also identified in the same band that contained α-SNAP, migrating with an average molecular mass of 32 kDa. The abundant band at 55 kDa corresponded to IgG. None of the other bands that were identified using this approach yielded any detectable peptide sequences.

To verify the identity of these proteins as Syntaxin 7-interacting partners we immunoblotted immunoprecipitates obtained from B16 cells using antibodies specific for a variety of these proteins. As shown in Fig. 4, Syntaxin 6, VAMP8, and mVti1b were all enriched in the Syntaxin 7 immunoprecipitate to almost the same extent as Syntaxin 7. None of these proteins was present when the immunoprecipitation was performed using preimmune serum-coated protein A beads (Fig. 4, *second lane*). The immunoprecipitation efficiency of Syntaxin 7 was ~50% (Fig. 4, compare *first* and *third lanes*). In the case of Syntaxin 6, VAMP8, and mVti1b ~20%, 20, and 10% of each protein was coprecipitated with Syntaxin 7, indicating that each of these proteins was highly enriched in the Syntaxin 7 complex suggesting that they likely form stable SNARE complexes *in vivo*. It has been reported recently that another v-SNARE, VAMP7, can play a role in the delivery of epidermal growth factor to a degradative compartment, most likely the late endosomal/lysosomal system, where VAMP7 is localized (18). Although we did not detect this protein in the Syntaxin 7 immunoprecipitate by MS/MS we were able to detect coprecipitation of VAMP7 with Syntaxin 7 by immunoblotting (Fig. 4). VAMP7 was enriched in the Syntaxin 7 immunoprecipitate to the same extent as VAMP8 (Fig. 4). Although a significant proportion of VAMP7 was found in the Syntaxin 7 immunoprecipitate, our inability to detect it by MS/MS raised the possibility that it may be expressed at lower abundance than other proteins. We also immunoblotted these fractions with antibodies specific for Syntaxin 4 and VAMP3 to investigate the specificity of the immunoprecipitation. Fig. 4 shows that neither Syntaxin 4 nor VAMP3 was found in the Syntaxin 7 immunoprecipitate to any significant extent. Although we did detect a faint band for both proteins in the Syntaxin 7 immunoprecipitate upon overexposure, this represents a very minor enrichment (~1%) compared with other SNARE proteins (see above).

Immunolocalization of Syntaxin 7 and VAMP8 in B16 Cells—The identification of Syntaxin 6 as a Syntaxin 7-binding partner was somewhat surprising because this t-SNARE has been immunolocalized previously to the *trans*-Golgi network in PC12 cells (13). However, it was clear from the immunoblotting data (Fig. 4) that a significant amount of Syntaxin 6 coprecipitates with Syntaxin 7 in B16 cells. Hence, one interpretation of these data is that Syntaxin 6 participates in Syntaxin 7-mediated fusion events as a t-SNARE light chain. To verify further that Syntaxin 7 and Syntaxin 6 might form a complex *in vivo* we conducted a series of double labeling immunolocalization experiments (Fig. 5). First, consistent with our previous data (14) there was very little overlap between Syntaxin 7 and the early endosomal protein EEA1 (Fig. 5, *A and B*). Syntaxin 7 was localized to large vesicles scattered throughout the cytoplasm and clustered in the perinuclear region of the cell. Based on our previous studies these likely correspond to late endosomes (14). We next compared the distribution of GFP-Syn6 with mVti1b, Syntaxin 7, and Syntaxin 13 in B16 cells. As shown in Fig. 5, *E* and *F*, there was substantial overlap between Syntaxin 6 and Syntaxin 7, particularly in the dispersed vesicles. There was an additional pool of Syntaxin 6 in the perinuclear region, which did not appear to contain Syntaxin 7. This pool presumably corresponds to the *trans*-Golgi network (13). Similarly, there was also a high degree of overlap between mVti1b and Syntaxin 6 in cytosolic vesicles. In contrast, we observed very little if any overlap between Syntaxin 6 and Syntaxin 13. Intriguingly, the distribution of Syntaxin 13 was also quite distinct from EEA1 in these cells being enriched in a very fine vesicular tubular network (Fig. 5*G*).

Expression of Different SNARE Proteins during Melanogenesis—Our earlier finding that production of Syntaxin 7 is upregulated upon induction of melanogenesis raised the possibility that this expression would be part of a developmental program that would drive the coordinate expression of other proteins that functionally interact with Syntaxin 7. Thus, to determine if Syntaxin 7 expression correlated with the expression of the proteins we identified as interacting with Syntaxin 7, we examined lysates prepared from B16 cells at different stages of melanosome development. The synthesis and storage of melanin in intracellular organelles can be observed readily as an increased deposition of dense black melanin granules or melanosomes that are scattered throughout the cytoplasm of the cell. As is shown in Fig. 6, a lysate prepared from B16 cells at different stages of melanogenesis demonstrated an increased deposition of melanin with increased time in culture. From this time course we observed a parallel increase in the levels of Syntaxin 7, Syntaxin 13, and the v-SNAREs, VAMP8 and VAMP7. In contrast, the levels of Syntaxin 4 and Syntaxin 6 remained constant throughout this time course (Fig. 6). We did note an increase in the expression of two other v-SNARE proteins, VAMP2 and VAMP3. However, the time course of these changes did not parallel the increase in melanin production. Because of our experience with different sublines of B16 cells behaving slightly differently, we also analyzed this phenomenon in a B16 cell line that only underwent melanogenesis after stimulation by α-melanocyte-stimulating hormone. Similar results were obtained in that Syntaxin 7, VAMP7, and VAMP8

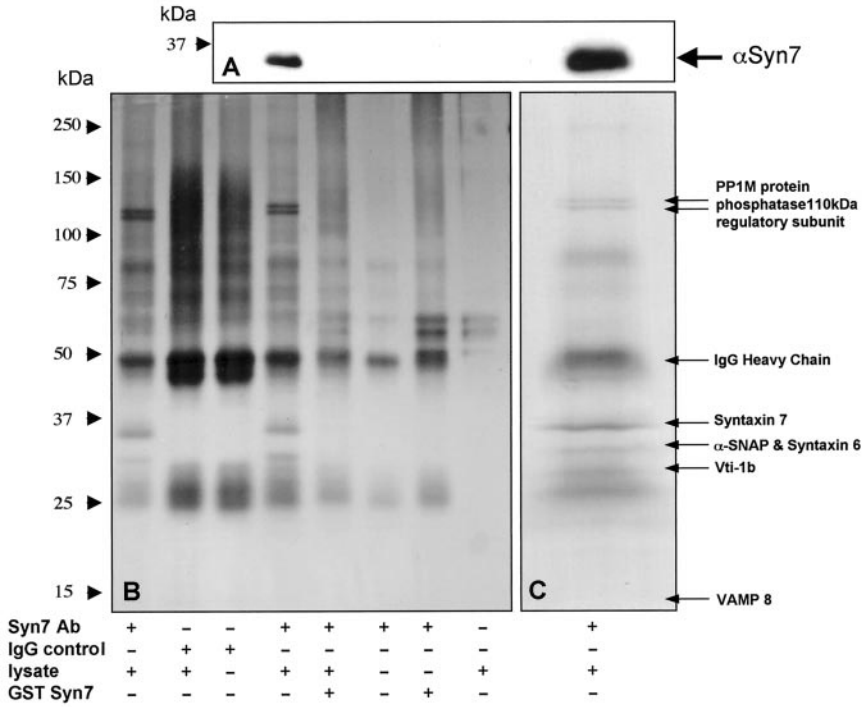

FIG. 3. **Immunoprecipitation of Syntaxin 7-containing polypeptide complexes.** *Panels A* and *B*, affinity-purified Syntaxin 7 antibodies or an IgG control antibody was covalently linked to protein A-Sepharose beads and incubated with a detergent-solubilized B16 cell lysate in the absence or presence of excess GST-Syn7 protein. In some cases the cell lysate was omitted from the incubation to determine which bands were arising from beads alone. Where indicated in *panel B*, 500 μg of B16 lysate was included in each incubation. Samples were then subjected to SDS-PAGE followed by either immunoblotting using the Syntaxin 7 antibody (*panel A*) or silver staining (*panel B*) to visualize protein complexes. The relative position of molecular mass standards in the gel, which were run in parallel, is indicated at the *left* in *panel B*. *Panel C*, to identify some of the proteins indicated in the *first lane* of *panel B*, the incubation of B16 extract with Syntaxin 7 antibodies was scaled up such that 400 μl of antibody-coupled beads was incubated with 4 mg of lysate protein. Samples were subjected to SDS-PAGE followed by Coomassie Blue staining. The bands indicated by the *arrows* were excised from the gel, digested with trypsin, and sequenced by electrospray ionization MS. Bands that gave positive identifications are shown at the *right* of the gel, and the corresponding peptide sequences are shown in Table I. It is noteworthy that the VAMP8 band, although not visible on this reproduction, was evident in gels freshly stained with Coomassie Blue.

were profoundly increased, whereas levels of Syntaxin 6 and Syntaxin 4 were unchanged.[2]

DISCUSSION

The conventional view is that melanosomes represent a specialized type of lysosome (24). As such, melanosomes are likely to be derivatives of the late endosomal pathway. Previously it has been found that the induction of melanogenesis results in the coordinate expression of tyrosinase and lysosome-associated membrane protein 1 (29), implying that these proteins are expressed as part of a developmental program that facilitates the biogenesis of melanosomes. Consistent with this idea we have found that expression of Syntaxin 7 is also induced during melanogenesis in B16 cells (Fig. 6). Thus, it is likely that elevated levels of Syntaxin 7 facilitate melanogenesis given the established role of Syntaxin 7 in lysosomal biogenesis (14, 16). We also find that the levels of the v-SNARE proteins that interact with Syntaxin 7 are also increased upon melanocyte differentiation, suggesting that the biochemical interactions we observe between these proteins have significance *in vivo*.

Among the cohort of proteins we found associated with Syntaxin 7 were the Q-SNAREs, mVti1b and Syntaxin 6, and the R-SNARE, VAMP7 (Fig. 3). Furthermore, we also found VAMP8 associated with Syntaxin 7 in B16 cells, consistent with our previous findings in Madin-Darby canine kidney cells (14). The identification of these novel Syntaxin 7-binding partners will permit a more detailed analysis of the composition of the various SNARE complexes that control fusion events within the late endocytic pathway. These interactions are best interpreted in light of recent experiments from Rothman and colleagues (3, 4, 30). The core SNARE complex comprises a four-helix bundle, and this may be formed by either three or four different proteins. One helix is contributed by the v- or R-SNARE, which resides in the "vesicle" membrane, and the remaining three helices are contributed by the t- or Q-SNAREs, residing in the "target" membrane. Thus, Syntaxin 7 is predicted to regulate vesicle transport events in the late endosomal system in tandem with other Q-SNAREs.

Our studies strongly suggest that mVti1b is at least one of the relevant Q-SNARE partners for Syntaxin 7. Based on both sequence homology and functional studies in yeast, both mVti1a and mVti1b appear to be orthologs of the yeast Vti1p (31). Yeast Vti1p is best characterized as a generic Q-SNARE because it interacts with the endoplasmic reticulum to Golgi t-SNARE, Sed5p, with the endosomal t-SNAREs, Pep12p, Tlg1p, and Tlg2p; and with the vacuolar t-SNARE, Vam3p (5, 32–34). The identification of mVti1p as a Syntaxin 7-interacting protein is consistent with the notion that Syntaxin 7 is functionally equivalent to Vam3p in yeast (14, 15) because Vam3p and Vti1p form a functional SNARE complex to regulate vacuolar transport. Thus, these results bolster the concept that the molecular regulation of vacuolar fusion and lysosomal fusion is highly conserved between these distinct organisms.

Consistent with previous results (14), our studies substantiate an interaction between VAMP8 and Syntaxin 7. In addition, antibodies against VAMP8 that block its ability to participate in SNARE core complex formation block homotypic fusion of both early and late endosomes *in vitro* (17). These data, combined with observations that homotypic fusion between late endosomes is blocked by reagents designed to disrupt

[2] S. Richardson and R. C. Piper., unpublished data.

TABLE I

Peptide sequences predicted from mass spectrometer analysis

Immunoprecipitated proteins visualized in Fig. 3 were subjected to MS analysis as described under "Materials and Methods." Sequence matches for the tryptic peptides obtained. Also shown is the predicted molecular mass of the complete matched protein and the apparent molecular mass by SDS-PAGE of the excised band from which those peptide matches were derived.

Protein	Sequence	Molecular mass *kDa*	
		Predicted	Measured
Protein phosphatase 1 M 110-kDa regulatory subunit, *Rattus* sp. aorta	AQLHDTNmELTDLK YDSSSTSSSDRYDSLLGR RSTQGVTLTDLQEAEK VGQTAFDVADEDILGYLEELQKK	109.6	120
BAP-135 homolog, *Mus musculus*	TPTQTNGSNVPFKPR	110.3	115
Syntaxin 7, *M. musculus*	ETDKYIKEFGSLPTTPSEQR EKNLVSWESQTQPQVQVQDEEITEDDLR DRLVAEFTTSLTNFQK QLEADIMDINEIFK DLGMMIHEQGDmIDSIEANVESAEVHVQQANQQLSR ITQcSVEIQR EFGSLPTTPSEQR TLNQLGTPQDSPELR ac-SYTPGIGGDSAQLAQR YIKEFGSLPTTPSEQR	29.8	35
A-soluble SNAP, *M. musculus*	AIAHYEQSADYYKGEESNSSANK LLEAHEEQNVDSYTEAVK HDAATcFVDAGNAFK KADPQEAINcLMR AIDIYEQVGTSAmDSPLLK LLEAHEEQNVDSYTEAVKEYDSISR NSQSFFSGLFGGSSK LAVQKYEELFPAFSDSR HHISIAEIYETELVDVEK LDQWLTTMLLR	33.9	33
Syntaxin 6, *M. musculus* (low level product)	IGGELEEQAVmLDDFSHELESTQSR	29.0	32
Putative v-SNARE Vtilb, *M. musculus*	EFGSLPTTPSEQR TLNQLGTPQDSPELR LVAEFTTSLTNFQK QLEADImDINEIFK	29.8	30
VAMP8, *M. musculus*	NKTEDLEATSEHFK	11.4	15

Syntaxin 7 function, suggest that Syntaxin 7 and VAMP8 are the relevant Q- and R-SNAREs for homotypic fusion reactions in the late endosomal pathway. However, we also find that Syntaxin 7 is in a complex with VAMP7. The structural and experimental models to date indicate that the three-helix Q-SNARE subcomplex interacts with a single v-(R)-SNARE to complete the four-helix bundle core complex (2–4, 30). Based on these experiments it is unlikely that one SNARE complex will contain two different R-SNARE helices. This raises the possibility that Syntaxin 7 can form two distinct SNARE complexes, one with VAMP7 and one with VAMP8. Whether a Syntaxin 7·VAMP8 complex catalyzes fusion events that are distinct from those catalyzed by a Syntaxin 7·VAMP7 complex remains to be definitively determined. Like VAMP8, VAMP7 has been localized to late endosomal compartments in some cell types and is implicated in the control of fusion events within the late endocytic pathway (16, 18). Thus, these separate complexes may control fusion of late endosomes with endosomes derived from different origins. Alternatively, a Syntaxin 7·VAMP8·mVti1b complex may regulate homotypic fusion of late endosomes, whereas a Sytnaxin 7·VAMP7·mVti1b complex may facilitate fusion of late endosomes with lysosomes. Interestingly, our VAMP8 antibody has no detectable effect in an *in vitro* late endosome/lysosome fusion assay derived from rat liver fractions (14), whereas VAMP7 antibodies are inhibitory in this assay.[3] To distinguish between these possibilities it will be important to discern carefully which type of fusion events are under investigation in these types of assay.

Based on previous structural studies and on the interaction of Syntaxin 7 with two different v-(R)-SNAREs and the Q-

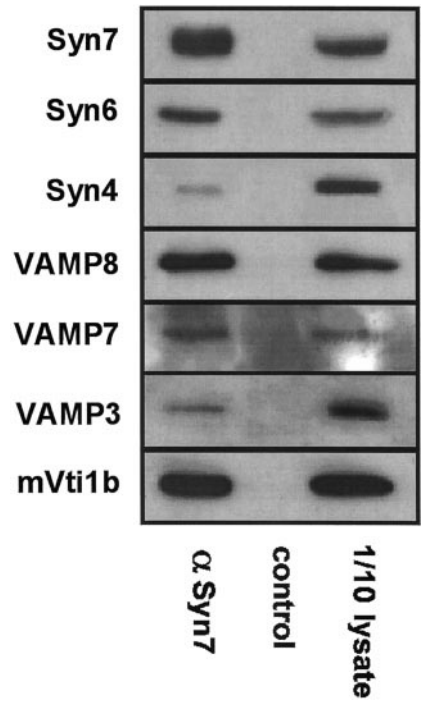

FIG. 4. **Detection of SNARE proteins in the Syntaxin 7 complex by immunoblotting.** B16 lysates were incubated with protein A beads that had been precoated with Syntaxin 7 antibodies in the absence (*αSyn7*) or presence of excess recombinant GST-Syn7 (*control*). These samples together with an aliquot of B16 extract (1/10 of the starting material used for the immunoprecipitation) were immunoblotted with antibodies specific for Syntaxin 7, Syntaxin 6, mVti1b, VAMP3, VAMP8, VAMP7, and Syntaxin 4.

[3] B. Mullock and J. P. Luzio, unpublished data.

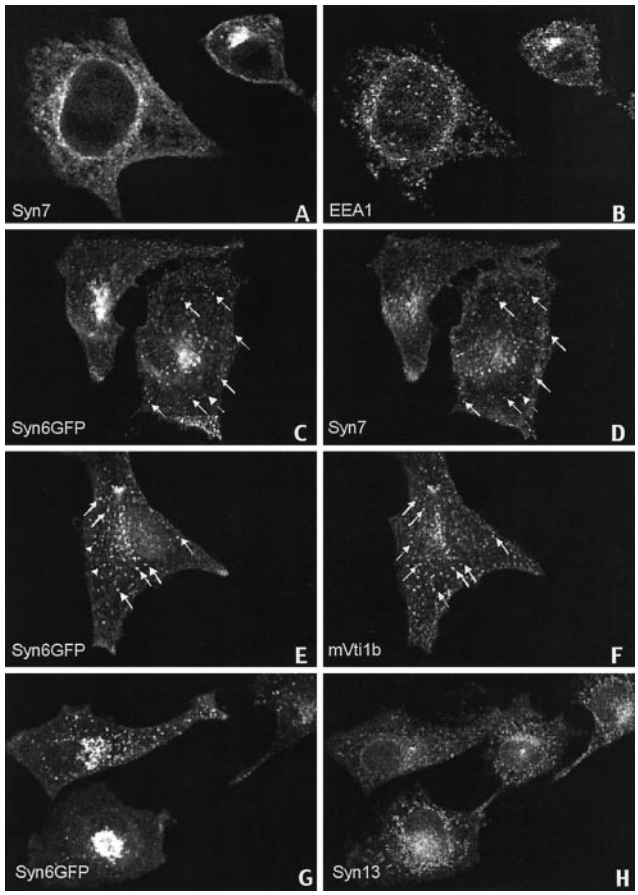

FIG. 5. **Immunolocalization of Syntaxin 7 and VAMP8 in B16 cells.** B16 cells, cultured on glass coverslips, were double labeled with antibodies specific for Syntaxin 7 and EEA1 (*panels A* and *B*). B16 cells were also transiently transfected with pEGFP-Syn6. At 24 h after transfection, cells were fixed, permeabilized, and immunolabeled. *Panels C* and *D* show GFP-Syn6 and anti-Syntaxin 7 labeling, *panels E* and *F* show GFP-Syn6 and anti-mVti1b labeling, and *panels G* and *H* show GFP-Syn6 and Syntaxin 13 labeling. Images were obtained using a confocal immunofluorescence microscope.

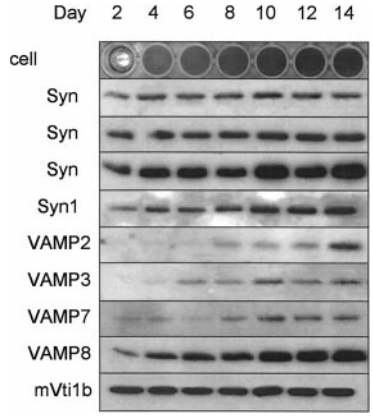

FIG. 6. **Increased expression of various SNARE proteins during B16 cell differentiation.** B16 cells were maintained as confluent monolayers for different times in culture between 2 and 14 days. Cells were prepared as described under "Materials and Methods" to obtain a Triton X-100-soluble total membrane extract. Equal amounts of cell extract (10 μg of protein) were subjected to SDS-PAGE followed by immunoblotting with antibodies specific for proteins as shown. The increase in melanogenesis with time in culture is indicated in the *top panel* by the progressive darkening of the cell extracts.

SNARE Vti1p we would predict that at least one other protein would be required in this complex to complete the four-helix bundle requisite within a core complex (2–3, 30). Although this could be accomplished by the presence of two mVti1b subunits, we were surprised to find that in B16 cells, the most likely protein to fulfill this role is Syntaxin 6. Syntaxin 6 was originally described as a Golgi t-SNARE that could interact with the v-SNAREs, cellubrevin and VAMP2 (13). However, more recently it has been shown that Syntaxin 6 interacts with other SNAREs including SNAP23 (36), Syntaxin 13, and the Q-SNARE Syntaxin 16.[4] What emerges from these data is the view that within a particular specialized cell type, SNARE proteins may be recruited selectively to new locations to fulfill distinct and selective functions. One particularly informative example is the observation that in neutrophils, Syntaxin 6 is targeted to the plasma membrane where it mediates regulated secretion (36). Furthermore, VAMP7 also appears to be localized differentially in specialized cell types such as neurons, polarized epithelial cells, and basophils (37–39). Thus, the localization of Syntaxin 6 to late endosomal compartments in B16 cells is consistent with this view (Fig. 5). These observations indicate that the specialized nature of different cells may dictate the steady-state localization of SNARE proteins, thus highlighting the potential limitation in using members of the SNARE family as generic compartment-specific markers. Furthermore, these observations require that understanding the compartmentation and function of the endocytic system in terms of particular SNARE proteins must be undertaken on a cell-by-cell basis.

The significance of the association between Syntaxin 7 and PP1M (a particular regulatory subunit of protein phosphatase I) remains an area for future investigation. Given the abundance and specificity of this protein in our immunoprecipitates it seems likely that this protein indeed associates with Syntaxin 7 *in vivo*. Because Syntaxin 7 mediates lysosomal fusion, a possible role for PP1M could be in the fusion process. Interestingly, *in vitro* homotypic fusion of yeast vacuoles requires a protein phosphatase I that is activated by calcium and calmodulin (40). Although a functional role has not yet been demonstrated for a protein phosphatase I in lysosomal fusion, there is a requirement for calcium and calmodulin in lysosomal fusion which mirrors the requirement for calcium and calmodulin in homotypic vacuolar fusion (41). Intriguingly, melanosomes are transported to the cell periphery in a manner that is dependent on myosin Va (42) and Rab27a (43). Mouse mutations (*dilute* and *ashen,* respectively) in these proteins result in coat color abnormalities. Furthermore, changes in the phosphorylation state of myosin V are correlated with its activity (44, 45). Thus PP1M could regulate the activity of myosin V and via its interaction with Syntaxin 7, or PP1M could regulate interaction between the melanosome and myosin V. Interestingly, movement of pigment granules in angelfish melanophores is also blocked by inhibitors of protein phosphatase I (35).

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[4] S. Martin and D. James, unpublished data.

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Syntaxin 7 Complexes with Mouse Vps10p Tail Interactor 1b, Syntaxin 6, Vesicle-associated Membrane Protein (VAMP)8, and VAMP7 in B16 Melanoma Cells

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