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Expression and Allocation of Proteins of the Exo-Endocytotic Machinery in U373 Glioma Cells: Similarities to Long-Term Cultured Astrocytes

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SUMMARY

1. Cultured astrocytes cells release a variety of low and high molecular weight messenger substances and express proteins of the exocytotic pathway including synaptic SNARE proteins. For analyzing the molecular mechanisms of astrocytic messenger release, permanent cell lines with astrocytic properties would provide useful tools.

2. We analyzed the potential of the human malignant astrocytoma-derived cell line U373 MG to express proteins involved in regulated exo- and endocytosis. An immunoblot analysis identified the astrocyte marker glial fibrillary acidic protein, microtubule-associated protein 2, the v-SNAREs VAMP I, VAMP II, and cellubrevin and the t-SNAREs syntaxin I, SNAP-23, and SNAP-25.

3. The cells also express the secretory granule protein secretogranin II. Although secretogranin II immunofluorescence reveals larger fluorescence spots, the majority of the SNARE proteins is associated with smaller organelles. The immunofluorescence is distributed throughout the cytoplasm and accumulates at processes and the growing edges of cells.

4. The organellar association of SNARE proteins was confirmed by heterologous expression of recombinant fusion proteins. Following subcellular fractionation organelles of lower buoyant density carried the majority of VAMP II. Secretogranin II was associated with organelles of high buoyant density containing a small contribution of VAMP II.

5. The results suggest that U373 MG cells have in common a considerable number of properties with long-term cultured astrocytes rather than with cultured oligodendrocytes or neurons. They contain two types of organelles that can be physically separated and may be employed in the differential release of messengers.

KEY WORDS: astrocyte; glioma; granule; SNARE; U373.

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INTRODUCTION

Tumor-derived cell lines have been successfully employed as models for studying the molecular mechanisms underlying regulated exo- end endocytosis in nerve cells. These include the rat pheochromocytoma-derived PC12 cell line (Chen *et al.*, 2001) and the human neuroblastoma SH-SY5Y cell line (Goodall *et al.*, 1997). The recent observation that glia intensely participates in intercellular signaling processes within the nervous system (Araque *et al.*, 2001; Bezzi and Volterra, 2001; Haydon, 2001; Vesce *et al.*, 1999) calls for glial model systems in which functional properties of cultured glia cells can be investigated. This applies in particular to astrocytes. Cultured astrocytes respond to various neurotransmitters with elevations in $[Ca^{2+}]_i$, and astrocytic excitation can be propagated to adjacent astrocytes in the form of Ca^{2+} waves also in acutely isolated preparations (Haydon, 2001). Astrocytes directly respond to synaptically released messengers and communicate, via signaling substances, with neurons in a reciprocal manner (Araque *et al.*, 2001; Deitmer *et al.*, 1998; Grosche *et al.*, 1999; Vesce *et al.*, 1999). Peptide and nonpeptide signaling substances synthesized and released by astrocytes include glutamate, L-serine, D-serine ATP, GABA, and a number of neuropeptides and growth factors (Baranano *et al.*, 2001; Calegari *et al.*, 1999; Hide *et al.*, 2000; Queiroz *et al.*, 1999; Verleysdonk and Hamprecht, 2000). Interestingly, receptor-mediated cellular release from cultured astrocytes of glutamate (Bezzi *et al.*, 1998; Parpura *et al.*, 1994) or of the secretory granule protein secretogranin II (Calegari *et al.*, 1999) was found to be dependent on an increase in intracellular Ca^{2+}. Astrocytes may, therefore, contain a dual secretory pathway for the release of low molecular substances such as glutamate and for secretory granule constituents such as secretogranin II.

Similar to neurotransmitter release astrocytic release was found to be reduced by clostridial neurotoxins. Botulinum neurotoxin A and botulinum neurotoxin C (which cleave the t-SNAREs SNAP-25 and syntaxin I, respectively) diminished bradykinin-evoked glutamate release of cultured astrocytes (Jeftinija *et al.*, 1997). Botulinum neurotoxin B (that cleaves the v-SNARE VAMP II) inhibited an astrocyte-induced glutamate response in cocultured neurons (Araque *et al.*, 2000). Cultured astrocytes also exhibit a developed endocytic system mainly composed of caveolae, clathrin-coated pits and vesicles, and a variety of endosomal structures (Megías *et al.*, 2000). Moreover, a variety of proteins that are typically associated with synaptic vesicles or are involved in synaptic transmission were found to be expressed in cultured astrocytes. These include the synaptic vesicle proteins VAMP II (a v-SNARE), synaptotagmin I, synaptophysin, rab3, rab5, or synapsin I, the two t-SNAREs SNAP-25 and syntaxin I, or also the more ubiquitous SNARE proteins cellubrevin and SNAP-23 (Hepp *et al.*, 1999; Madison *et al.*, 1996; Maienschein *et al.*, 1999; Megías *et al.*, 2000; Parpura *et al.*, 1995). It is noteworthy that the expression of individual synaptic proteins in astrocytes varies with culture time (Maienschein *et al.*, 1999). Although all these data imply the presence of astrocytic vesicular release mechanisms in vitro, the significance of this release pathway in situ is still debated (Fields and Stevens, 2001; Kimelberg, 2001).

For analyzing the molecular mechanisms of astrocytic messenger release permanent cell lines with astrocytic properties would provide useful tools. A variety of

glioma cell lines is presently available but to date these have only been purely characterized regarding their molecular characteristics and their relation to individual glial subtypes (Dai and Holland, 2001; Tohyama *et al.*, 1993). Most glioma cell lines do not express the astrocytic marker glial fibrillary acidic protein (GFAP) (Bigner *et al.*, 1982). The human malignant astrocytoma-derived cell line U373 MG was shown to expresses the astrocytic intermediate filament protein glial fibrillary acidic protein (GFAP), the light chain of neurofilament proteins (NF-L, but not NF-M and NF-H), and to contain mRNA for isoforms of the microtubule associated protein 2 (MAP2) (Tohyama *et al.*, 1993). It was shown to release glutamate (Ye and Sontheimer, 1999) and nerve growth factor (NGF) (Emmett *et al.*, 1997). In order to further characterize the molecular phenotype of U373 MG cells and to compare their properties with those of cultured glial cells and neurons we characterized the expression of proteins potentially involved in membrane fusion and regulated exo- and endocytosis. We identify both v-SNAREs and t-SNAREs and the secretory granule protein secretogranin II in U373 MG cells and reveal their subcellular distribution by fluorescence microscopy and sucrose density centrifugation. Our results suggest that the molecular outfit of U373 MG cells with SNARE proteins resembles that of long-term cultured astrocytes rather than that of oligodendrocytes or of neural cells.

MATERIALS AND METHODS

Cell Culture

The human glioma cell line U373 MG (gift of Frank Fahrenholz, Mainz, Germany) was kept at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) plus GlutaMax-1 (4 mM L-alanyl-L-glutamine) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Medium and reagents were from Gibco BRL (Eggenstein, Germany). After 2–3 days in culture, cells were split following incubation in trypsin-EDTA solution (phosphate buffered saline (PBS) containing 0.05% trypsin, 0.53 mM EDTA) for 5 min at 37°C in a shaking water bath. Detached cells were washed twice in trypsin-free medium (5 min, 200g_{av}) and replated.

Antibodies

Antibodies against the following proteins were applied: synaptobrevin I/VAMP I, clone SP10, recognizes in addition VAMP II and cellubrevin (Boehringer Ingelheim Bioproducts, Heidelberg, Germany); synaptobrevin II/VAMP II, clone 69.1; rab3a, clone 42.2; rab5, clone 621.3; syntaxin I, clone 78.2; SNAP-25, clone 71.1; SNAP-23, polyclonal antibody; synaptophysin, clone 7.2; synaptotagmin I, polyclonal antibody, and clone 41.1 (all Synaptic Systems, Göttingen, Germany); synaptophysin, rabbit serum G63, provided by Reinhard Jahn (Göttingen, Germany); synapsin I, affinity-purified polyclonal antibody (#574777, Calbiochem, San Diego, USA), and clone A10C (Chemicon, Temecula, USA); SV2, clone 10H4 (hybridoma supernatant), provided by Regis Kelly (San Francisco, USA); secretoneurin provided by Reiner Fischer-Colbrie (Innsbruck, Austria); secretogranin II, provided by Hans-Hermann

Gerdes (Heidelberg, Germany); cellubrevin, affinity purified polyclonal antibody (McMahon *et al.*, 1993), provided by Harvey McMahon (Cambridge, UK); myc-epitope, clone 9E10 (hybridoma supernatant); green fluorescent protein (GFP, purified monoclonal antibody #8120863, Clontech, Heidelberg, Germany); VSV glycoprotein-epitope, clone P5D4; glial fibrillary acidic protein (GFAP), clone G-A-5 IgG1 isotype; MAP2, clone HM-2*AS (all Sigma, Deisenhofen, Germany). Secondary antibodies against mouse and rabbit IgGs conjugated with fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), or horseradish peroxidase (for immunoblotting) were obtained from Sigma. Gold conjugates (for cryo-immunoelectron microscopy) were from BioCell(Cardiff, UK).

Genetic Engineering of Tagged cDNAs

PCR-reactions with primers containing the sequences of tags were used to construct modified cDNAs of synaptic proteins (VSVG-syntaxin I, myc-VAMP II). Tags were inserted between ATG and the second codon of the open reading frame. PCR products were subcloned into a mammalian expression vector (pcDNA3, Invitrogen, Groningen, The Netherlands). Construction of red fluorescent protein (RFP)-fusion proteins (RFP-syntaxin I, RFP-VAMP II) was carried out using single strand overlap extension (SOE)-PCR and the pDsRed1-N1 vector (Clontech, Heidelberg). Human rab4a, rab5a, or rab11 contained in the pEGFP C3 vector were kindly provided by Dr Zerial (Dresden). The vectors were applied for transient transfection by electroporation.

Transfection

For transient transfection a suspension of U373 MG cells and of DNA (50 μg/800 μL) were electroporated in electroporation buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose, 20 mM HEPES, pH 7) using a E600 electroporator (BTX-Genotronics, San Diego, USA) with the following settings: mode, 500 V; capacitance and resistance 2250 μF, 186 Ω, and 240 V.

Subcellular Fractionation

The protocol for subcellular fractionation of U373 MG cells was previously described for cultured astrocytes (Maienschein *et al.*, 1999). In brief, approximately 2×10^6 cells were mechanically detached from plastic dishes using a cell scraper in buffer A (150 mM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$, adjusted to pH 7.4 with 10 mM HEPES/NaOH) containing a cocktail of the protease inhibitors antipain, leupeptin, chymostatin (2 μg/mL each), pepstatin (1 μg/mL), and benzamidine (1 mM). Cell suspensions were first centrifuged for 5 min at $375g_{av}$ yielding a pellet fraction P1 and a supernatant fraction S1. Fraction P1 was resuspended, homogenized in a glass-teflon homogenizer (12 up-and-down strokes), and recentrifuged for 5 min at $1000g_{av}$ to yield a supernatant fraction S2. The supernatant fraction S1 was subjected to high speed centrifugation (60 min at $180{,}000g_{av}$). The pellet fraction obtained was resuspended and combined with the supernatant fraction S2. The pooled fractions

(crude postnuclear supernatant fraction) were layered onto linear sucrose gradients (4.8 mL, ranging from 0.2 to 1.6 M sucrose) and centrifuged for 60 min at 150,000g_{av} in a swinging bucket rotor (Kontron, Neufahrn, Germany). In a second series of experiments a microsomal fraction was prepared. In this protocol, the supernatant fraction S1 (as described above) was stored on ice and the pellet fraction P1 was resuspended in 800 μL of buffer A containing the protease inhibitors and thoroughly homogenized by 12 up-and-down strokes using a 0.5 mL glass-Teflon homogenizer. The homogenate was centrifuged for 5 min at 1000$g_{av}$to yield the supernatant fraction S2. Fraction S2 was combined with fraction S1 and centrifuged at 180,000g_{av} for 60 min. The resulting pellet fraction (microsomal fraction) was homogenized in 650 μL of Buffer A and further fractionated on sucrose density gradients as described above. Fractions of 300 μL were collected starting from the top of the gradient. The sucrose density of gradient fractions was determined using an Abbé refractometer. Gradient fractions were precipitated with 10% trichloroacetic acid and further processed for SDS-PAGE and immunoblotting.

Protein Determination and Immunoblotting

Protein was determined by the method of Spector (1978) using bovine serum albumin as a standard. Proteins of total cell homogenates or gradient fractions were precipitated with 10% trichloroacetic acid at 10,000g_{av} for 60 min. The pellet was washed in 1 mL acetone (−20°C) containing 200 mM HCl, recentrifuged and resuspended in sample buffer and subjected to SDS/PAGE and western blotting. Polyacrylamide gel electrophoresis was carried out in the presence of SDS on minigels (10, 15, or 20% acrylamide). Protein corresponding to 50 μL of gradient fraction was loaded per lane. Immunodetection was performed after transfer of polypeptides onto nitrocellulose using the enhanced chemiluminescence method according to the manufacturer's instruction (Amersham, Braunschweig, Germany).

Immunocytochemistry

For indirect immunocytochemistry cells were plated on poly-D-lysine (MW >300,000, 5 μg/cm^2; Sigma) coated cover slips in 24-well tissue culture plates (Dunn, Asbach, Germany). After washing for 5 min at 37°C in physiological saline solution (in mM: 120 NaCl, 4.8 KCl, 1.22 KH_2PO4, 25.5 $NaHCO_3$, 14.3 glucose, 1.5 $MgSO_4$, 2 $CaCl_2$, adjusted to pH 7.4 with HCl) to remove excess medium, cells were fixed in absolute methanol. Nonspecific binding sites were blocked with 5% bovine serum albumin in PBS, followed by application of the primary antibody. After several washing steps the respective secondary antibodies were applied. In control experiments only the secondary antibodies were applied. Immunofluorescence was visualized using a Zeiss Axiophot I (excitation 450–490 nm, emission 515–565) equipped with an MCID 4 imaging analysis system (Imaging Research, St. Catharines, Canada) or a laser-assisted Leica TCS4D true confocal scanning microscope. In some of the images (indicated in the figure legends) the fluorescence signal was intensified using the Adobe Photoshop 5.0 software.

Immunoelectron Microscopy

Cryo-immunoelectron microscopy employing the colloidal gold technique was performed similar to the method previously described (Zhang *et al.*, 2000). Briefly, after two 5 min washes in physiological saline at 37°C, cells were fixed for 1 h with 4% paraformaldehyde (PFA) and 0.05% glutaraldehyde in PBS. Cells were mechanically detached using a cell scraper and centrifuged for 15 min at $1000g_{av}$. To remove excess of PFA cells were resuspended in PBS and recentrifuged. This step was repeated once. To optimize infiltration of cryoprotectant the pellet was cut into pieces and transferred to a mixture of 1.8 M sucrose and 20% polyvinyl-pyrrolidone (PVP). This procedure was repeated every 30 min for at least 3 h using fresh sucrose/PVP solution. The cryo-protected cell pellet was mounted to a holder and dropped into liquid nitrogen. Ninety-five-nanometer thick sections of cells were cut at −110°C. Sections were transferred to pioloform and carbon coated Ni-grids. After washing in double distilled H_2O and blocking of nonspecific binding sites the primary antibody was applied for 2 h. After six washes for 5 min in PBS, sections were incubated with the secondary antibody (conjugated to 10 nm gold particles, British BioCell International, Cardiff, UK) for 2 h. Fixed and labeled cells were washed five times in double distilled water and postfixed with 2.5% glutaraldehyde. After five washes cells were stained for 20 min with 2% uranyl acetate containing 1.5% methylcellulose. Excess staining solution was removed and the sections were air-dried.

RESULTS

Expression of Endogenous Proteins

Total homogenates were subjected to SDS-Page and immunoblotting (Fig. 1) to document the inventory of U373 MG cells in proteins potentially involved in membrane fusion processes. The cells express both GFAP and MAP2. In addition we identified all three known members of the synaptic SNARE complex, the v-SNARE VAMP II, and the two t-SNAREs syntaxin I and SNAP-25. However, the immunosignal for the ubiquitous SNAP-23 was considerably stronger than that for the neuronal SNARE protein SNAP-25. U373 MG cells also expressed the v-SNAREs VAMP I and cellubrevin. The endosome-associated small GTP-binding protein rab5 revealed a strong immunosignal. An antibody against secretoneurin was used to detect the secretory granule constituent protein secretogranin II. Secretoneurin represents a 33 aa peptide derived by proteolysis from the 87-kDa protein secretogranin II. The identification of a single protein band of 63 kDa suggests the presence of a processed form of secretogranin II. An antibody against total secretogranin II revealed an identical immunoreactive band (not shown). It is noteworthy that the following synaptic proteins could not be detected by immunoblotting in U373 MG cells: synaptic vesicle protein 2 (SV2), synapsin I, synaptophysin, synaptotagmin I, and rab3a. In order to verify that the antibodies applied bind to human proteins we performed immunoblots also with the human neuroblastoma SH-SY5Y cell line (not shown). Positive results were obtained for all proteins investigated with the exception of rab3a. Its presence in U373 MG cells may have remained undetected due to lack of immunoreactivity with the human protein.

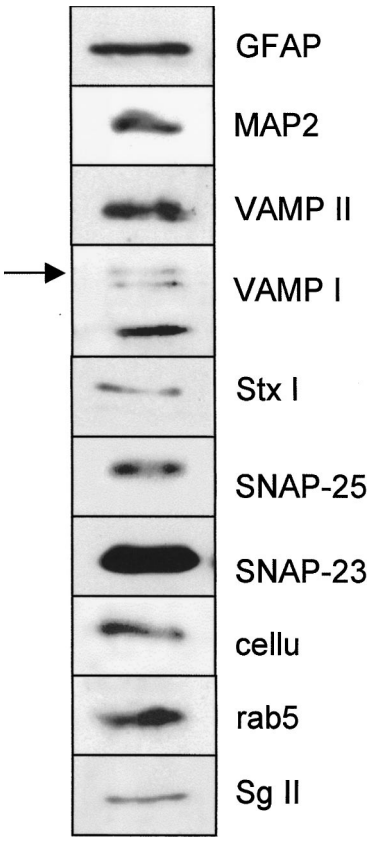


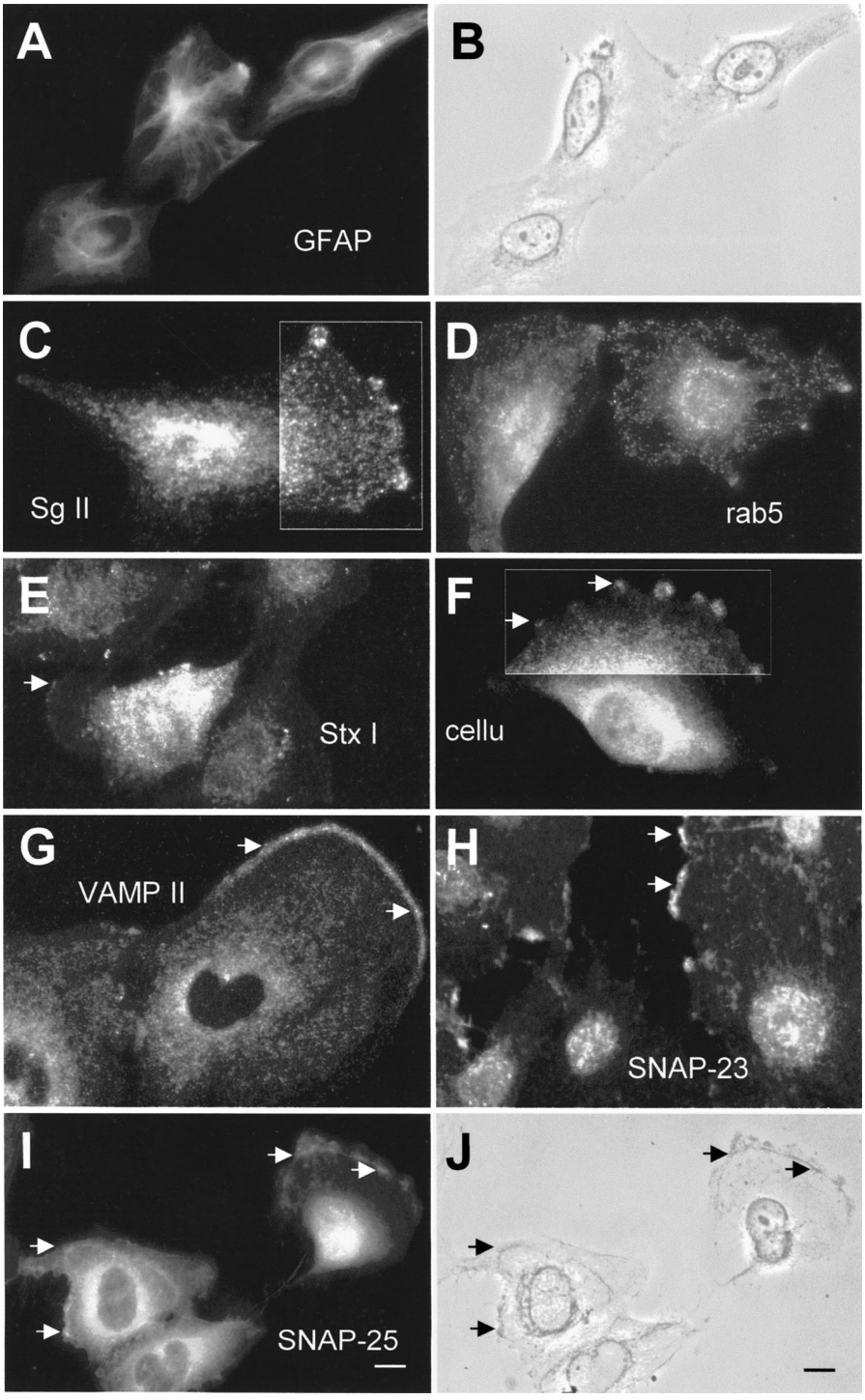
Fig. 1. Expression of synaptic proteins in the U373 MG cells. Cell homogenates were analyzed by immunoblotting. Apparent M_r (kDa, in parentheses): GFAP (52); MAP2, microtubule-associated protein 2 (70); VAMP II/synaptobrevin II (18); VAMP I (19, arrow); Stx I, syntaxin I (34); SNAP-25 (25); SNAP-23 (23); cellu, cellubrevin (14); rab5 (25); Sg II, secretogranin II (63). Note that the anti-VAMP I antibody also binds to VAMP II and cellubrevin (lower bands). Equal amounts of total protein (22 μg) were applied per lane.

Immunolocalization of Endogenous Proteins

The cellular distribution of endogenous proteins was analyzed by immunocytochemistry (Fig. 2). U373 MG cells revealed the expected filamentous pattern of GFAP (Fig. 2(A) and (B)). The punctuate distribution of secretogranin II (Fig. 2(C)) corresponded to that expected for a secretory granule protein. Similarly the endosome-associated small GTP-binding protein rab5 was associated with large punctuate structures throughout the cytoplasm (Fig. 2(D)). The v-SNAREs cellubrevin (Fig. 2(F)) and VAMP II (Fig. 2(G)) and the t-SNAREs syntaxin I (Fig. 2(E)), SNAP-23 (Fig. 2(H)), and SNAP-25 (Fig. 2(I) and (J)) were distributed throughout the entire cell with an enhanced perinuclear staining. The SNARE-proteins accumulated at ruffling edges at the growing end and at cellular processes. This also applied to secretogranin II (Fig. 2(C)).

Localization of Recombinant Proteins

The immunofluorescence analyses suggested that U373 MG cells may contain larger organelles expressing secretogranin II and smaller organelles expressing the


A
GFAP
B
C
Sg II
D
rab5
E
Stx I
F
cellu
G
VAMP II
H
SNAP-23
I
SNAP-25
J

majority of SNARE proteins. We, therefore, aimed to verify this observation by subcellular fractionation experiments. Since only very weak immunosignals could be obtained in immunoblots of subcellular fractions for the endogenous proteins VAMP II, syntaxin I, SNAP-25, and rab5 the experiments were performed following transient transfection with tagged proteins. This was supplemented by the expression of small GTP-binding proteins that are associated with various endosomal compartments.

To probe for proper sorting and cellular localization, the heterologously expressed proteins were first visualized by fluorescence staining. Similar to the endogenous proteins the recombinant proteins revealed a punctuate cellular distribution. The two SNAREs myc-VAMP II and RFP-syntaxin I were colocalized on vesicular organelles (Fig. 3(A) and (B)). GFP-rab4 and RFP-VAMP II as well as GFP-rab5 and RFP-VAMP II were partially colocalized suggesting that part of the v-SNARE VAMP II is associated with endosomal organelles (Fig. 3(C)–(H)). GFP-Rab11 was colocalized with RFP-VAMP II or with RFP-syntaxin I only to a small extent (shown for RFP-syntaxin I, Fig. 3(G) and (H)). Whereas GFP-rab4 and GFP-rab11 were associated with very fine organellar structures, GFP-rab5 immunoreactivity revealed larger punctae (see insets in Fig. 3(C), (E) and (G)).

The organellar association of the expressed proteins was further substantiated by immunoelectron microscopy using the cryo-immunogold technique. Proteins were immunolocalized via monoclonal antibodies directed either against a myc-tag or a VSVG-tag. Myc-VAMP II was detected at numerous vesicular organelles of varying shape including vesicles at the Golgi apparatus and presumptive endosomal structures. Occasionally a high density of gold particles was found in association with subplasmalemmal organelles (Fig. 4(A)) or also directly with the plasma membrane including gold-labeling of omega-shaped structures (Fig. 4(B)). Immunolabeling was high at the Golgi apparatus, particularly at the trans-Golgi network (TGN) (Fig. 4(C)). A similar distribution was obtained for VSVG-syntaxin I (Fig. 4(D)). Rare immunogold labeling was observed when only the secondary antibody was applied (Fig. 4(E)).

Organellar Association of Proteins Following Subcellular Fractionation

Transfected and nontransfected U373 MG cells were homogenized and subjected to sucrose density centrifugation (Fig. 5). Immunosignals for endogenous SNAP-23 and secretogranin II could readily be identified in subcellular fractions. In order to identify organelles containing the v-SNARE VAMP-II cells were transfected with myc-VAMP II (comp. Fig. 3(A)). For comparison with the sedimentation

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Fig. 2. Distribution of endogenous proteins in U373 MG as revealed by immunofluorescence microscopy. (A) Filamentous immunostaining for GFAP with corresponding phase contrast image. (B) An intense perinuclear fluorescence and a punctuate fluorescence in the cell periphery is apparent after immunostaining for secretogranin II (C), rab5 (D), syntaxin I (E), cellubrevin (F), VAMP II (G), SNAP-23 (H), and SNAP-25 (I). (J) Phase contrast image corresponding to (I). The immunofluorescence for syntaxin I, cellubrevin, VAMP II, SNAP-23, and SNAP-25 is enhanced at ruffling surfaces (arrows) of U373 MG cells. In the insets of (D) and (F) the immunofluorescence signal for secretogranin II and cellubrevin in the cell periphery was intensified. cellu, cellubrevin; Sg II, secretogranin II; Stx I, syntaxin I. Scale bars = 10 μm.

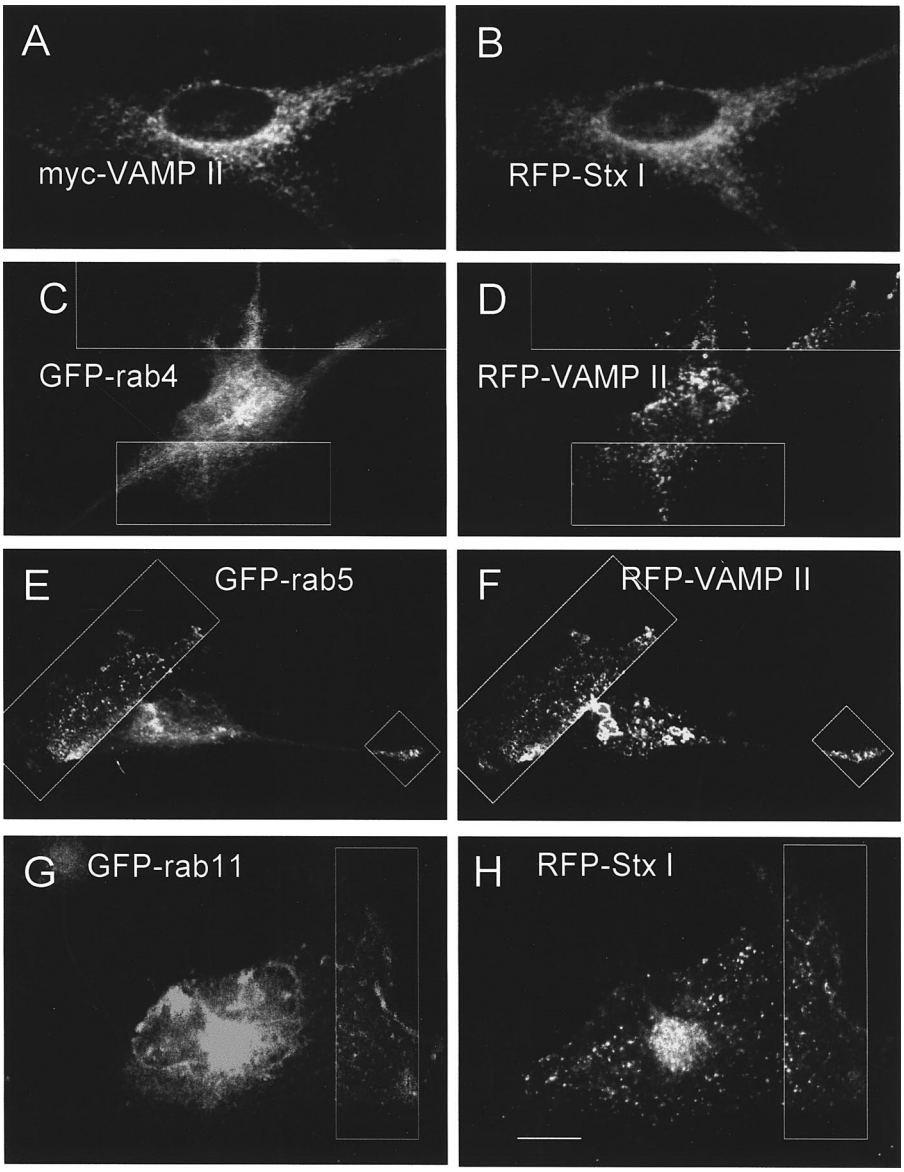

Fig. 3. Localization of recombinant proteins in U373 MG cells using confocal scanning microscopy. (A, B) Distribution of recombinant myc-VAMP II (A) and RFP-syntaxin I (B) in double transfected cells. (C–H) Comparison of the cellular localization of the recombinant rab fusion protein GFP-rab4 (C), GFP-rab5 (E), and GFP-rab11 (G) with that of the recombinant SNARE fusion proteins RFP-VAMP II (D, F) and RFP-syntaxin I (H) (double transfected cells, confocal laser scanning images of green and red fluorescent fusion proteins). Insets depict areas with intensified fluorescence in order to visualize the distribution of proteins in the cell periphery. cellu, cellubrevin; Sg II, secretogranin II; Stx I, syntaxin I. Scale bar = 10 μm.

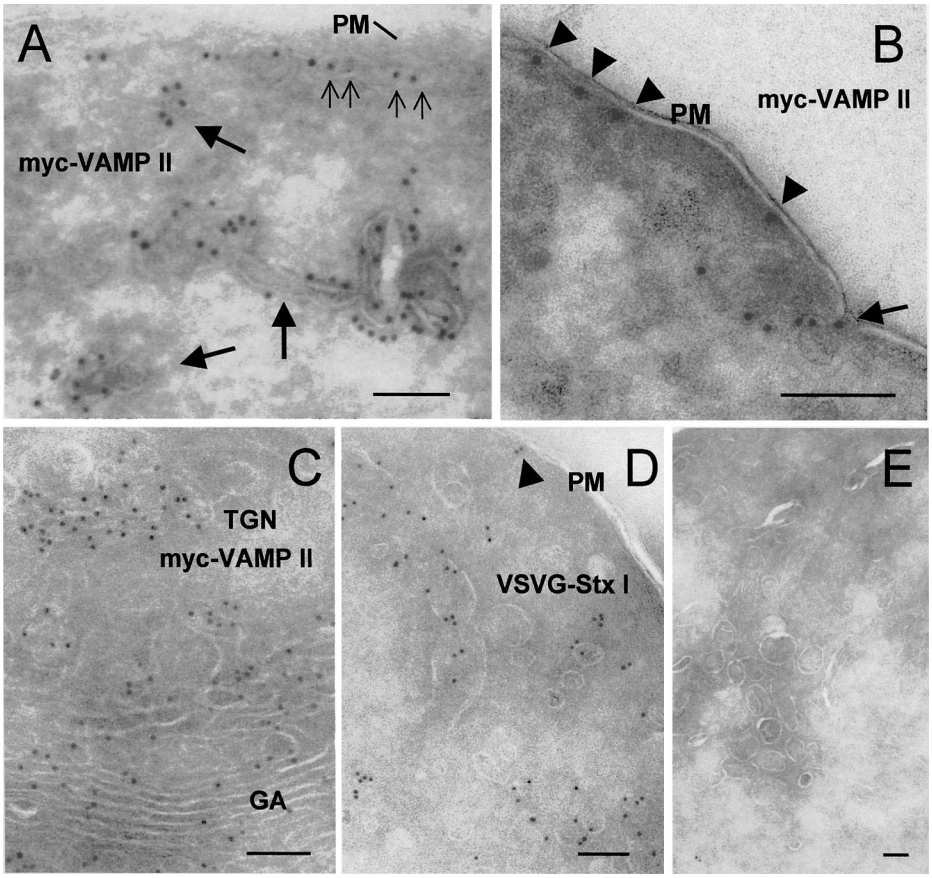

Fig. 4. Association of recombinant proteins with vesicular structures in transiently transfected U373 MG cells as revealed by cryo-immunoelectron microscopy. (A–C) Localization of recombinant myc-VAMP II detected by a monoclonal antibody recognizing the myc-tag. (D) Distribution of VSVG-syntaxin I detected by a monoclonal antibody against the VSVG-tag. (E) Control section where only the 10-nm gold conjugate secondary antimouse antibody was applied. Small arrows in (A) depict labeled vesicular organelles in close proximity to the plasma membrane. Large arrows point to labeled larger organelles presumably representing endosomes. Arrow heads in (B) and (D) depict myc-VAMP II or VSVG-syntaxin I, respectively at the plasma membrane. The arrow in (B) points to an omega-shaped profile protruding into the cell. GA, Golgi apparatus; PM, position of plasma membrane; Stx I, syntaxin I; TGN, *trans*-Golgi-network. Scale bars = 150 nm.

properties of endosomal vesicles, U373 MG cells were also transfected with the GFP-tagged endosomal marker rab5 (GFP-rab5) (comp. Fig. 3(E)). When a crude postnuclear supernatant fraction was layered onto the sucrose gradient, most of the protein sedimented in the top fraction of the gradient, suggesting that this fraction contained mainly cytosolic proteins. SNAP-23 was recovered to a large extent in the upper fractions of the gradient, implicating that part of the protein was in a soluble form, and in addition in the bottom fraction. Secretogranin II sedimented at the top of the gradient suggesting the presence of a soluble protein pool generated by cell disruption, and between 1.0 and 1.2 M sucrose implicating

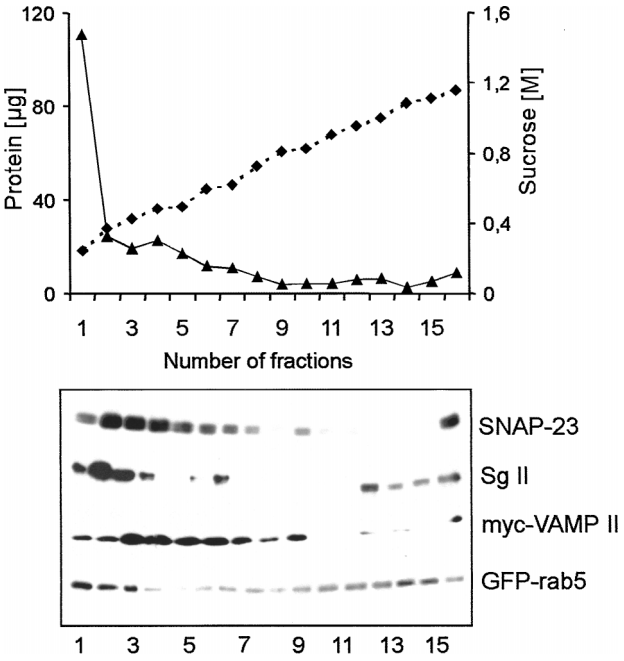

Fig. 5. Subcellular fractionation of U373 MG cells and immunodetection of endogenous and recombinant proteins. Postnuclear supernatant fractions were loaded onto sucrose gradients. Fractions of 300 μL were collected from sucrose gradients (◆) and each fraction was analyzed by immunoblotting. Protein contents (▲) were highest in the top fractions of the gradients. The lanes of the immunoblots correspond to the fractions in the upper graph. Material corresponding to 50 μL of gradient fraction was loaded per lane. The blots show the distribution of endogenous SNAP-23 and secretogranin II (Sg II) and of recombinant myc-VAMP II (monoclonal antibody against the myc-tag) and GFP-rab5 (monoclonal antibody against the green fluorescent protein). The data shown are representative of four experiments.

an association with organelles of high density. Transfected myc-VAMP II sedimented in the upper part of the gradient between 0.4 and 0.6 M sucrose with a small fraction of the protein reaching the bottom of the gradient and overlapping the distribution of secretogranin II. Transfected GFP-rab5 was enriched in the top fractions of the gradient but also distributed in denser, organellar fractions.

In order to remove the majority of soluble proteins prior to sucrose density gradient fractionation, a microsomal fraction of U373 MG cells was used in a second series of experiments (Fig. 6). Under these experimental conditions the majority of protein enters the density gradient forming a broad peak between 0.7 and 1.1 M sucrose. SNAP-23 immunoreactivity revealed a broad distribution over the entire density gradient, suggesting that SNAP-23 is associated with organelles of a very broad density range. Secretogranin II was now associated mainly with particles sedimenting at

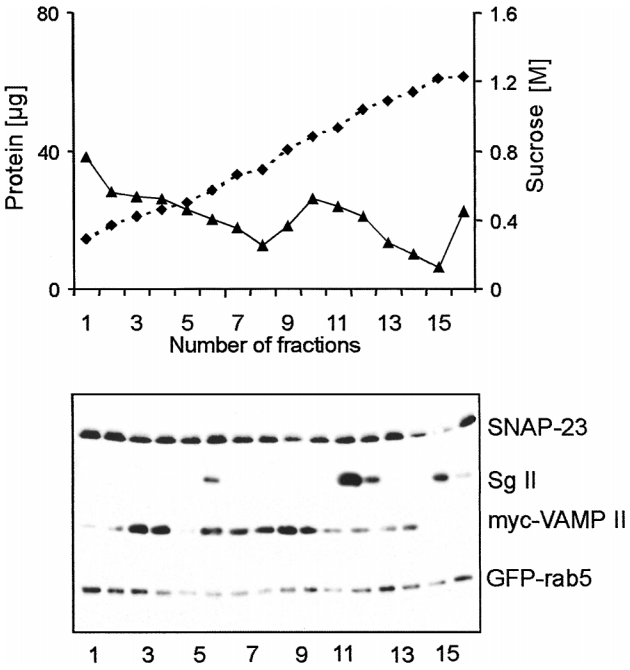

Fig. 6. Differential organellar association of secretogranin II and myc-VAMP II as revealed by subcellular fractionation. Sucrose gradients were loaded with microsomal fractions derived from U373 MG cells. Fractions of 300 μL were collected from the sucrose gradient (◆) and every fraction was analyzed by immunoblotting; protein contents(▲). The lanes of the immunoblots correspond to the fractions in the upper graph. Material corresponding to 50 μL of gradient fraction was loaded per lane. The blots show the distribution of endogenous SNAP-23 and secretogranin II (Sg II) and of recombinant myc-VAMP II, and GFP-rab5 (antibodies as for Fig. 5). Note that the bulk of secretogranin II immunoreactivity sediments around 1.0 M sucrose, whereas the majority of myc-VAMP II immunoreactivity is contained in less dense gradient fractions. The data shown are representative of four experiments.

1.0 and 1.2 M sucrose. Myc-Vamp II immunoreactivity was strongest between 0.4 and 0.7 M sucrose. Organelles containing the majority of myc-VAMP II clearly differed in density from those containing secretogranin II suggesting the presence of a dual vesicular pool of organelles with low and high density, respectively. Nevertheless a small amount of myc-VAMP II sedimented in the secretogranin II-containing fractions at 1.0 M sucrose, implicating the association of a minor pool of this v-SNARE with secretory granules. The broad distribution of GFP-rab5 largely corresponded to that of fractionation experiments in which the postnuclear supernatant fraction was loaded onto the sucrose gradient (comp. Fig. 5). No difference was observed between transfected and nontransfected cells in the overall protein pattern of the gradients and in the distribution of the endogenous proteins SNAP-23 and secretogranin II (not shown).

DISCUSSION

U373 MG Cells Resemble Astrocytes in Long-Term Culture

The human malignant astrocytoma-derived cell line U373 MG expresses not only GFAP but also three additional intermediate filament proteins (vimentin, neurofilament light chain, and nestin) and MAP2. Therefore, it has been suggested that U373 MG cells resemble bipotential neuronal and glial precursors (Tohyama *et al.*, 1993). Our results demonstrate in addition that U373 MG cells express multiple members of SNARE proteins (VAMP II, cellubrevin, SNAP-23, and to a lesser extent VAMP I, syntaxin I, and SNAP-25). We also identified syntaxin 4 by RT-PCR (our unpublished observations). VAMP II, syntaxin I, and SNAP-25 constitute the SNARE complex involved in regulated exocytosis at nerve terminals (Rizo and Südhof, 1998). Both VAMP I and VAMP II are associated with synaptic vesicles in the nervous system but are also expressed in a variety of other tissues (Elferink *et al.*, 1989; Rossetto *et al.*, 1996). Cellubrevin is a ubiquitous v-SNARE that is associated with a variety of intracellular organelles and functions in constitutive exocytosis (Annaert *et al.*, 1997; McMahon *et al.*, 1993). Syntaxin 4, occurs in a variety of nonneural tissues, is sorted to the plasma membrane after heterologous expression (Bennett *et al.*, 1993), and participates in SNARE assembly with the ubiquitous SNAP-23 (ST-Denis *et al.*, 1999). U373 also express rab5 that is known to function in endosomes (Nielsen *et al.*, 1999) but which is also contained in synaptic vesicles (Jahn and Südhof, 1999).

The protein expression pattern of U373 MG cells differs from that previously obtained for freshly platet astrocytes prepared from cerebral cortices of newborn rats. These cells expressed a considerable number of synaptic vesicle-associated proteins (Maienschein *et al.*, 1999). In contrast to short-term cultured astrocytes (and to neurons), U373 MG cells reveal no immunoreactivity for synapsin I, synaptophysin, or synaptotagmin I. The expression pattern of U373 MG cells rather resembles that of long-term cultured astrocytes. These were found to express SNAP-23 (Hepp *et al.*, 1999), VAMP II, cellubrevin, and syntaxin I (Jeftinija *et al.*, 1997; Parpura *et al.*, 1995). We have previously observed that the astrocytic expression of synaptotagmin, synapsin I, syntaxin I, and particularly of synaptophysin and SNAP-25 decreases within the first 2 weeks of culture whereas that of VAMP II and rab3a remains constant. In contrast, the immunosignal for cellubrevin and for SNAP-23 increases (Maienschein *et al.*, 1999; Volknandt, in press). Interestingly, a considerable variety of SNARE proteins and additional presynaptic proteins were also detected in cultured oligodendrocytes. These include VAMP II, SNAP-25, syntaxin-2 and syntaxin-4 (but not syntaxin I), munc-18–1 and the synaptic vesicle proteins synaptophysin, rab3a/c, and synapsin Ia/b. Similar to astrocytes cultured from neonatal rodent brain these cells revealed changes in the protein expression profile during differentiation from an early (O2A) progenitor form to maturing oligodendrocytes (Madison *et al.*, 1999). Altogether these data reveal that the protein profiling obtained positions U373 closer to long-term cultured astrocytes than to cultured oligodendrocytes. They clearly differ from neurons. It should be noted that U373 MG cells express only the NF-L subunit and may thus not be able to produce functional neurofilaments. Also the expression

of MAP2 does not necessarily indicate a neuronal phenotype since it can also be expressed in astrocytes (Tohyama *et al.*, 1993).

Organellar Distribution of Proteins

Immunocytochemistry and subcellular fractionation experiments suggest that the proteins identified in U373 MG cells by immunoblot are organelle-associated. This applies to the endogenous v-SNAREs VAMP II and cellubrevin and to the t-SNAREs SNAP-25, SNAP-23, and syntaxin I equally well as to the tagged recombinant proteins. Heterologous expression revealed complete or partial colocalization: Myc-VAMP II and RFP-syntaxin I exhibited nearly equal immunofluorescence profiles suggesting that they are sorted into identical organelles. In contrast GFP-rab5 only partially colocalized with RFP-Vamp II suggesting that the endosomal rab5 protein is associated with VAMP II-containing organelles only during part of their life cycle. Similarly, the fusion proteins of the endosomal markers rab4 (early endosomes) and rab11 (trans-Golgi-network, recycling endosomes) (Martinez and Goud, 1998) showed only a limited colocalization with RFP-VAMP II- or RFP-Syntaxin I-containing organelles. This implies that VAMP II and syntaxin I are associated with vesicular organelles in U373 MG cells throughout their life cycle, from biogenesis to endosomal recycling and possibly lysosomal degradation.

The subcellular fractionation experiments suggest that U373 cells contain organelles of lower density that carry the majority of the v-SNARE VAMP II as well as granules of high buoyant density. The majority of myc-VAMP II sedimented between 0.4 and 0.7 M sucrose, similar to the sedimentation behavior of VAMP II after subcellular fractionation of cultured astrocytes (Maienschein *et al.*, 1999). The majority of secretogranin II sedimented at a considerably higher sucrose density (1.0–1.2 M), in accordance with a granular localization.

At present it is not known whether U373 cells employ the v-SNAREs VAMP I, VAMP II, and cellubrevin and the t-SNAREs SNAP-23 and SNAP-25 in identical fusion processes. In nerve cells SNAP-25 and syntaxin I reveal a primarily plasma membrane-associated distribution (Jahn and Südhof, 1999), but the two proteins were also found in association with synaptic vesicles or granules (Ref. in Kretzschmar *et al.*, 1996). In accordance with results obtained with cultured astrocytes (Hepp *et al.*, 1999; Maienschein *et al.*, 1999) none of the four SNARE proteins revealed a significant association with the plasma membrane in U373 MG cells, except at ruffling cell surfaces, indicative of sites of cell growth.

Taken together our results demonstrate that U373 cells share many properties with long-term but not with short-term cultured astrocytes. This includes the SNARE proteins and their organellar association. Except of VAMP II U373 cells do not express typical synaptic vesicle proteins and also the prevalence of their SNARE-proteins differs from that of cultured oligodendrocytes and from neurons.

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