Synapsin I is expressed in epithelial cells: localization to a unique trans-Golgi compartment

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SUMMARY

Synapsin I is abundant in neural tissues. Its phosphorylation is thought to regulate synaptic vesicle exocytosis in the pre-synaptic terminal by mediating vesicle tethering to the cytoskeleton. Using anti-synapsin antibodies, we detected an 85 kDa protein in liver cells and identified it as synapsin I. Like brain synapsin I, non-neuronal synapsin I is phosphorylated in vitro by protein kinase A and yields identical ^{32}P-peptide maps after limited proteolysis. We also detected synapsin I mRNA in liver by northern blot analysis. These results indicate that the expression of synapsin I is more widespread than previously thought. Immunofluorescence analysis of several non-neuronal cell lines localizes synapsin I to a vesicular compartment adjacent to trans-elements of the Golgi complex, which is also labeled with antibodies against myosin II; no sub-plasma membrane synapsin I is evident. We conclude that synapsin I is present in epithelial cells and is associated with a trans-Golgi network-derived compartment; this localization suggests that it plays a role in modulating post-TGN trafficking pathways.

Key words: Synapsin I, Vesicular traffic, Epithelial cells, trans-Golgi network, Myosin II

INTRODUCTION

The protein machinery involved in the complex regulation of vesicle trafficking in eukaryotic cells has been intensely studied over the past decades. Studies of vesicle traffic at the nerve synapse have been particularly valuable in elucidating the mechanisms of exocytosis, in part because components of the various protein machines are abundant (Fernández-Chacón and Südhof, 1999). For example, the membrane proteins comprising the so-called SNARE complex were first identified in brain (Sollner et al., 1993). Similarly, virtually all of the proteins comprising exocytic synaptic vesicles (SVs) have been identified and their functions examined. One abundant protein in small SVs is the family of synapsins. They are encoded by three genes, synapsin I, II and III, which give rise to different isoforms: synapsin Ia, 85 kDa; Ib, 80 kDa; IIa, 70 kDa; IIb, 58 kDa; and IIIa-f, 7.9-63 kDa (De Camilli et al., 1990; Porton et al., 1999).

In brain, synapsins associate with the cytoplasmic surface of the small SV membrane where they regulate the interaction of SVs with the cytoskeleton. This regulation is thought to depend on the phosphorylation state of synapsin. All isoforms share a similar N-terminal region with one phosphorylation site for cAMP-dependent protein kinase (PKA) or calcium/calmodulin-dependent protein kinases (CaM kinase) I or IV, but have divergent C-terminal regions, which include sites for proline-directed protein kinases. Unlike other family members, synapsin I contains phosphorylation sites for CaM kinase II (De Camilli et al., 1990; Hilfiker et al., 1999). Early

studies suggested a role of the CaM kinase II phosphorylation sites in the regulation of synapsin I's association with SVs (Schiebler et al., 1986; Ceccaldi et al., 1995; Stefani et al., 1997). More recently, the involvement of the N-terminal phosphorylation site, the PKA/CaM kinase I site, in this regulation has been reported (Hosaka et al., 1999). These studies show that in vitro phosphorylation of synapsin I at several sites decreases its affinity for the SV membrane resulting in dissociation of the protein from the membrane. In vivo, the liberated SVs are thought to then dock and fuse with the plasma membrane. Together, these observations have led to the hypothesis that synapsin I regulates secretion at the nerve terminal by controlling SV availability for release (Greengard et al., 1993).

Using immunohistochemical methods the synapsins were originally found to be widely expressed in almost all types of neurons, but absent from non-neuronal cell types (De Camilli et al., 1983). This and the almost exclusive association of synapsins with small SVs led investigators to conclude that these proteins were neuron-specific. However, later studies identified synapsin I in several cultured cell lines of neural and endocrine origin, among them, PC12, AtT-20, MIN6 insulinoma and βTC3 cells. β cells of pancreatic islets in vivo and cultured astrocytes also express synapsin I. As in neurons, synapsin I in these cells is vesicle-associated (Romano et al., 1987; Tooze et al., 1989; Matsumoto et al., 1999; Krueger et al., 1999; Maienschein et al., 1999).

We report for the first time that synapsin I is present in epithelial cells of non-neuronal origin. Furthermore, it is

concentrated in a vesicular compartment that overlaps with trans elements of the Golgi complex but not with markers of other previously identified membrane compartments located in the Golgi region of cells. This compartment is also enriched in myosin II. Our results, together with the proposed tethering function for neuronal synapsin I, lead us to propose that synapsin I-positive vesicles in epithelial cells represent a novel exocytic compartment. Parts of this work have been presented in abstract form (E.R.K. et al. (1996). *Mol. Biol. Cell* **7**, 454a; E.R.K. et al. (1997). *Mol. Biol. Cell* **8**, 51a; E.R.K. et al. (1997). *Liver Diseases: Single Topic Symposium* **1**, 40a; R.B. et al. (1999). *Mol. Biol. Cell* **10**, 218a; R.B. et al. (2000). *Mol. Biol. Cell* **11**, 136a).

MATERIALS AND METHODS

Reagents and cells

The catalytic subunit of PKA was purchased from Calbiochem (San Diego, CA). *S. aureus* V8 endoprotease was from Boehringer Mannheim (Indianapolis, IN); [γ-^{32}P]ATP with >7000 mCi/mole specific activity was from ICN (Costa Mesa, CA). Chymotrypsin, Protein A-Sepharose beads, nocodazole and cytochalasin D were from Sigma (St Louis, MO). Freshly isolated rat hepatocytes were provided by the Yale Liver Center (Yale Medical School, New Haven, CT) and J. Yager (Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD). Liver and brain total homogenates from wild-type mice and mice lacking synapsins I and II (Rosahl et al., 1995) were provided by T. Südhof (University of Texas-Southwestern Medical School, Dallas, TX). Male Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). NRK cells were grown in DMEM (Life Technologies, Gaithersburg, MD) with 10% fetal bovine serum in a 5% CO_2, 37°C incubator. WIF-B cells were cultured as described (Shanks et al., 1994).

Antibodies

The monoclonal antibodies SNT1 and SNH1 were produced as described (Nicol et al., 1995) and used as hybridoma culture supernates. Antibody SNH1 recognizes an epitope in the first 50 amino acid residues of the N-terminus of synapsins I and II; antibody SNT1 is directed against an epitope in domain D of synapsin I (Nicol et al., 1995). Rabbit polyclonal antibodies (Abs) #212 and #213 were made as described against a peptide comprising one of the CaM kinase II phosphorylation sites on synapsin I (site 2, ATRQASISG) (Matovcik et al., 1994). Rabbit polyclonal antibodies used were anti-TGN38 #1481 (from E. Eipper, Johns Hopkins University, Baltimore, MD), anti-mannosidase II (from C. Machamer, Johns Hopkins University, Baltimore, MD), anti-transferrin receptor (from M. Farquhar, UCSD, San Diego, CA), anti-βCOP (from Y. Ikehara, Fukuoka, Japan), anti-myosin II-A from chicken brush border (from D. Burgess, Boston College, Boston, MA), and anti-SCAMP4 (from D. Castle, University of Virginia, VA). Two mouse monoclonal antibodies, anti-γ adaptin (clone 88) and anti-syntaxin 6, were from Transduction Laboratories (Lexington, KY). Anti-α tubulin (clone DM1A) was from Sigma.

Biochemical fractionation

All procedures were carried out at 4°C unless otherwise noted. Rat brain and liver were homogenized at 8 or 25% (w/v), respectively, in 0.3 M sucrose, 50 mM Tris, pH 7.4, 0.5 M NaCl, 5 mM MgCl$_2$, containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg/ml each of antipain, benzamidine, leupeptin and trasylol) using a Dounce homogenizer and 10 passes of the pestle. Confluent NRK cells were detached by trypsinization and homogenized as described for the brain and liver. The homogenized samples were immediately

mixed with an equal volume of 2× Laemmli electrophoresis sample buffer (Laemmli, 1970), boiled for 3 minutes and aliquots stored at −80°C.

Cytosol containing synapsin I was prepared from rat brain and liver by centrifuging homogenates of each tissue at 600 *g* for 10 minutes, then subjecting the resulting supernate to ultracentrifugation (100,000 *g* for 1 hour) to sediment all membranes. The high-speed supernate constituted the cytosolic fraction.

A crude preparation of synapsin I from NRK cells was used for immunoprecipitation. Cells grown on plastic dishes to 90-100% confluence were trypsinized, sedimented, rinsed twice with PBS and resuspended in ice-cold 5 mM Pipes, pH 6.8, containing the protease inhibitors listed above. After incubation for 10 minutes, sucrose was added to 0.25 M, MgCl$_2$ to 5 mM (from 2× stocks in 5 mM Pipes, pH 6.8) and the cells were homogenized using a Teflon-glass homogenizer with 40 passes of the pestle. Cell lysis was assessed microscopically. The cell homogenate was centrifuged (160,000 *g* for 90 minutes), sedimenting more than 90% of NRK synapsin I (data not shown). The pellet was resuspended in 50 mM Tris, pH 7.4, 1% Triton X-100, 0.5 M NaCl and 1 mg/ml BSA and the insoluble material was removed by centrifugation at 13,600 *g* for 10 minutes. The supernate was used for immunoprecipitation.

Immunochemical procedures

Immunoblot analysis

Solubilized proteins were electrophoretically separated in 7.5% gels (10×20 cm) as described (Maizel, 1971) and transferred to nitrocellulose membranes. Membranes were blocked with phosphate-buffered saline (PBS) containing 10% powdered fat-free milk and 0.1% Tween-20, then incubated with primary antibodies for 1 hour at room temperature (Abs #212 and #213) or 1 hour at room temperature and overnight at 4°C (SNT1 and SNH1 culture supernates). Blots were incubated with secondary antibody conjugated to HRP and processed with the ECL kit (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's instructions.

Immunoprecipitation

Brain or liver cytosol (0.5 or 10 mg total protein) was adjusted to 10 ml with 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, then incubated with 1 ml of SNH1 or SNT1 culture supernate for 2 hours at 4°C on a rotating wheel. Rabbit anti-mouse IgG was added for 1 hour and the immune complexes sedimented after 2 hours incubation with Protein A-Sepharose beads. Following six washes in the above buffer, the beads were boiled in electrophoresis sample buffer, the polypeptides separated by SDS-PAGE and analyzed by immunoblot using Ab #213.

Synapsin I was immunoprecipitated from a high salt extract of NRK membranes, prepared as described above, using SNT1 culture supernate and incubation conditions similar to those for brain and liver samples, but keeping 0.5 M NaCl and 1 mg/ml BSA in the sample throughout the procedure.

In vitro phosphorylation and peptide map analysis

Immunoprecipitated synapsin I was phosphorylated while bound to the Protein A-Sepharose beads in 200 µl of 50 mM Tris-HCl, pH 7.4, 1 mg/ml BSA, 10 mM MgCl$_2$, containing 44U of PKA and 1 mCi/ml [γ-^{32}P]ATP. After incubation for 45 minutes at 30°C, the beads were washed six times with 50 mM Tris, pH 7.4, 1% Triton X-100, 0.5 M NaCl and 1 mg/ml BSA by sedimentation at 2,900 *g* for 3 minutes and resuspension in 1 ml of buffer. The final bead pellet was resuspended in electrophoresis sample buffer and boiled for 5 minutes. Proteins were resolved in 7.5% gels, transferred to nitrocellulose and processed for autoradiography and immunoblot analysis. Peptide mapping after limited proteolysis of PKA-phosphorylated proteins with V8 endoprotease or chymotrypsin was performed as described (Huttner and Greengard, 1979).

Fig. 1. Synapsin I is present in non-neuronal cells. (A,B) Immunoblot analysis of total homogenates of rat brain (1 μg), rat liver (200 μg), rat hepatocytes (Hep, 200 μg) and NRK cells (200 μg) using antibody SNH1 (A) and SNT1 (B). Both antibodies detected an 85 kDa band (*) in non-neuronal samples that co-migrated with rat brain synapsin I. Molecular mass standards (in kDa) are indicated at the left of each panel. (C) Antibodies to synapsin I immunoprecipitate an 85 kDa protein from liver. Proteins from brain (0.5 mg total protein) or liver (10 mg total protein) cytosols were immunoprecipitated with SNH1 (top panel) or SNT1 (bottom panel) and analyzed by immunoblot with Ab#213. Both antibodies immunoprecipitated an 85 kDa protein from either brain (Brain +ab) or liver cytosol (Liver +ab) that was recognized by Ab#213; the protein was not immunoprecipitated from liver cytosol incubated with Protein A-Sepharose beads alone (Liver –ab). (D) The 85 kDa protein is absent from livers of synapsin I- and II-deficient mice. Total homogenates of brain or liver from wild-type mice (wt) or synapsin I and II-deficient mice (syn–/–) were analyzed by immunoblot using Ab#212 (SynI) or antibodies to tubulin (Tub) as a loading control. An 85 kDa protein was detected in both brain and liver of wild-type mice that was absent from brain and liver of synapsin I and II-deficient mice.
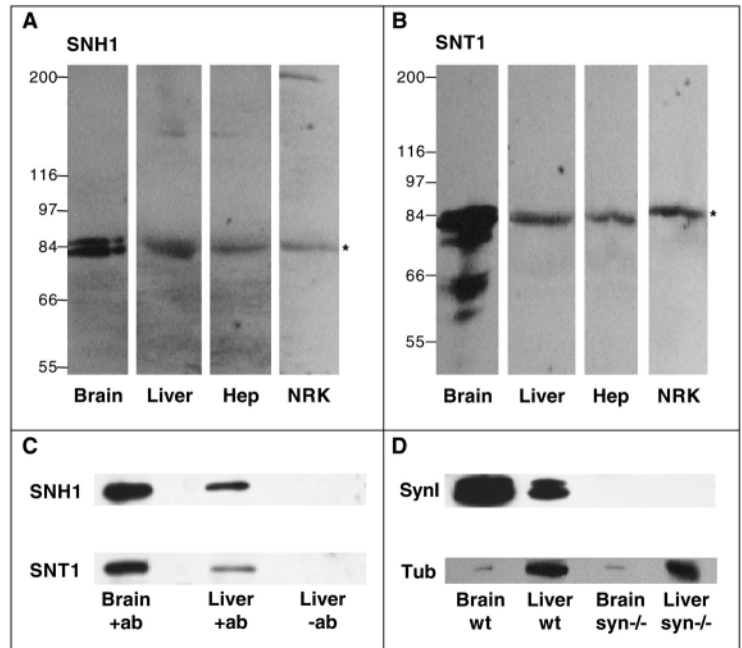
Immunofluorescence and inhibitor treatments

NRK cells were grown to 60-70% confluence on glass coverslips and fixed in 100% methanol at −20°C for 10 minutes or in 4% paraformaldehyde (PFA) in PBS for 30 minutes at room temperature, rinsed with PBS and blocked with 5% FBS, 2% BSA in PBS for 30 minutes at room temperature. PFA-fixed cells were permeabilized with 0.05% saponin, added in all steps. The cells were incubated with undiluted SNT1 culture supernate for 1 hour at room temperature, 12-16 hours at 4°C, then with the polyclonal antibodies for 30 minutes at room temperature. The primary antibodies were detected with Alexa 488- or 568-conjugated secondary antibodies (Molecular Probes, Eugene, OR). To double immunostain NRK cells using two mouse monoclonal antibodies, cells were first blocked, then incubated for 1 hour with primary antibody followed by a FITC-labeled Fab fragment of goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA). Free sites in the mouse IgG were blocked with excess unlabeled Fab fragment of goat anti-mouse IgG (Jackson Immunoresearch) and the cells labeled with SNT1 culture supernate followed by secondary antibody as described above. The labeled cells were examined by epifluorescence (Zeiss Axioplan microscope, Carl Zeiss, Germany) and digital images were collected with a Micromax CCD camera (Princeton Instruments, Trenton, NJ) using IPLab 3.5 software (Scanalytics, Fairfax, VA). Confocal images were acquired on an Olympus IX-70 inverted microscope (Olympus America Inc., Melville, NY) equipped with a Noran OS confocal system (Noran, Middleton, WI) with a Kr/Ar laser for excitation of the Alexa fluorochromes. Confocal Z series were captured throughout the Golgi of stained cells and analyzed using the Intervision software (Noran).

For inhibitor studies, cells were treated with 33 μM nocodazole for 1 hour at 37°C or 5 μM cytochalasin D for 2 hours at 37°C in serum-containing medium. Control cells were treated with the corresponding dilution of DMSO. Treatments were terminated by fixing the cells in cold methanol and immunostaining as described above. To assess the disassembly of the microtubules or actin filaments upon drug treatments, control and treated cells were stained for tubulin with DM1A or for F-actin with FITC-phalloidin (Molecular Probes).

RNA isolation and northern blot

Total RNA was extracted from freshly isolated rat hepatocytes and rat brain using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Poly(A)-selected RNA was prepared from hepatocyte total RNA using either Oligotex mRNA isolation columns (Qiagen, Valencia, CA) or oligo dT Cellulose (Amersham Pharmacia) according to the manufacturer's instructions. Because synapsin I message was detected in some preparations and not others, routinely we isolated total RNA immediately from freshly isolated hepatocytes to avoid degradation of the synapsin I message. Then, twice-poly(A)-selected RNA was separated on a formaldehyde gel as described (Ausubel et al., 1995) and transferred to GeneScreen Plus (NEN Life Sciences, Boston, MA) using a Turbo Blotter (Schleicher and Schuell, Keene, NH). Following crosslinking of the RNA to the membrane with a UV Stratalinker 1800 (Stratagene, La Jolla, CA), the membrane was prehybridized at 42°C in 50% formamide, 10% dextran sulfate, 5× SSPE, 5× Denhardts solution, 1% SDS, 200 μg/ml salmon sperm DNA (Ausubel et al., 1995). Hybridization was performed at 42°C by the addition of the probe purified on a G50 micro column (Amersham Pharmacia). A fragment of the plasmid pLB910 (encoding synapsin I, see below) was labeled with [α-^{32}P]dCTP by random priming, then the probe was preadsorbed 2-3 times overnight at 42°C on a membrane with liver total RNA to remove material that binds nonspecifically to the 28S ribosomal subunit. Following a 16-20 hour hybridization, the probe was removed, and the membrane washed at 45°C twice with 2× SSC, 0.1% SDS for 20 minutes and twice with 0.2× SSC, 0.1% SDS for 20 minutes, then exposed to X-ray film. The film was imaged on a FluorChem 8000 (Alpha Innotech Corporation, San Leandro, CA) for mobility determinations and quantitation.

A cDNA clone encoding Synapsin I 3′ half (pLB910) was obtained using reverse transcription-PCR (enhanced avian RT-PCR kit, Sigma) of adult rat brain total RNA and the product was cloned into pCDNA3.1 (Invitrogen, Carlsbad, CA) with primers specific for Synapsin I (5′ to 3′ orientation): nAH10-GTCCAGAAGATTGGGC and nAH12-GTCGGAGAAGAGGCTGGC. Confirmation of the clone was performed by DNA sequencing at the JHMI DNA Analysis Facility.

Other methods

Protein content of samples was determined using BCA (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions, with BSA (Pierce Chemical) as standard.
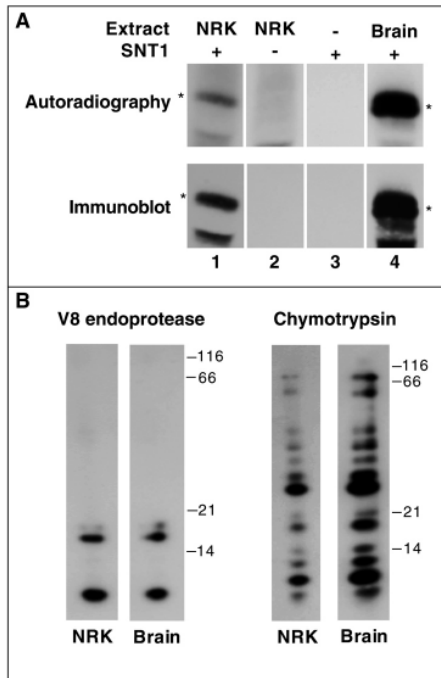
Fig. 2. Synapsin I from NRK cells and rat brain show similar phosphopeptide patterns. (A) Synapsin I immunoprecipitated from NRK cells was phosphorylated in vitro by PKA. By autoradiography, a phosphorylated species was detected that co-migrated with rat brain synapsin I and was labeled by SNT1 in immunoblot (*, lanes 1,4). The signal was absent if the anti-synapsin I antibody or the NRK extract were absent during the immunoprecipitation (lanes 2,3). (B) The PKA-phosphorylated proteins were digested with V8 endoprotease or chymotrypsin, the fragments were resolved on a 15% gel, transferred to nitrocellulose and exposed to autoradiographic film. The phosphopeptide maps for both synapsin I purified from rat brain and synapsin I immunoprecipitated from NRK cells are identical. Position of molecular mass standards (in kDa) is indicated.

RESULTS

Identification of an 85 kDa protein in non-neuronal cells as synapsin I

Using an anti-peptide antibody (Ab#212), we had previously reported detection of an 85 kDa protein (p85) in non-neuronal tissues (Matovcik et al., 1994). The apparent molecular weight of approximately 85 kDa and the presence of an epitope found in synapsin I suggested that synapsin I was present in these cells. To confirm that the p85 polypeptide was synapsin I, we performed a series of analyses, which included use of synapsin I-specific antibodies to immunoblot and immunoprecipitate rat tissues and cells, immunoblotting of liver extracts from synapsin-deficient mice, in vitro phosphorylation by PKA followed by limited proteolysis and phosphopeptide mapping, and northern blot detection of the synapsin I message in freshly isolated hepatocytes. Based on the results of these experiments, which are detailed below, we conclude that non-neuronal p85 is synapsin I.

Monoclonal antibodies SNH1 and SNT1, which were generated against the N-terminal head and C-terminal tail, respectively, of synapsin I, detected the 85 kDa protein in total
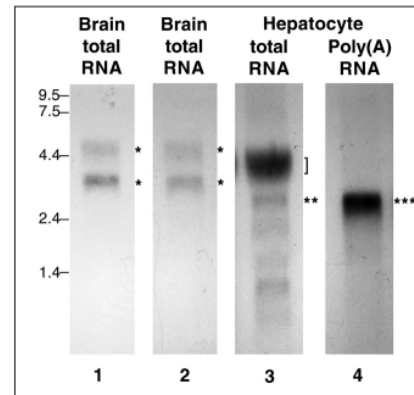

Fig. 3. Detection of synapsin I mRNA from freshly isolated hepatocytes by northern blot. Membrane was probed with a 3′ end probe from rat brain synapsin I. In brain total RNA (2 µg, lanes 1,2), the synapsin I message was detected as two species (*), at 3.3 and 4.3 kb. The synapsin I mRNA was detected in hepatocytes total RNA (22 µg, lane 3) and poly(A) RNA (3.3 µg, lane 4) (** and ***) at 2.5-3.1 kb. Presence of synapsin I message has been confirmed in three independent preparations of hepatocyte poly(A) RNA. Lane 1, 10 day exposure; lanes 2-4, 5 day exposure. Position of size standards is indicated in kb. The square bracket indicates 28S rRNA.

homogenates of rat liver, freshly isolated rat hepatocytes, and NRK cells; this polypeptide co-migrated with rat brain synapsin I (Fig. 1A,B). Furthermore, both SNH1 and SNT1 immunoprecipitated from brain and liver cytosol the 85 kDa protein that was recognized by Ab #213 in blots (Fig. 1C).

The results of the above experiments, showing that p85 and brain synapsin I shared epitopes in three different regions of their amino acid sequences, strongly suggested that p85 was synapsin I. To confirm this, total homogenates of brain and liver from wild-type mice and mice deficient in both synapsin I and II (Rosahl et al., 1995) were analyzed. As shown in Fig. 1D, an 85 kDa protein was detected in brain and liver of wild-type mice, but was absent in brain and liver of synapsin-deficient mice. This meant that either p85 was synapsin I or that p85 expression was dependent on the synapsin I or II genes. Analysis of livers from mice deficient in only synapsin I gave similar results (data not shown).

We next determined that the non-neuronal p85 was a substrate for PKA. This kinase catalyzes the phosphorylation of synapsin I at serine 9 (site 1) (Huttner et al., 1981; Czernik et al., 1987). We used NRK cells as the source of non-neuronal synapsin I for this analysis rather than freshly isolated hepatocytes, since the former are easy to culture, can be obtained in large amounts and cannot contain neuronal contaminants. We immunoprecipitated proteins from NRK and rat brain cytosol with SNT1 antibody and compared their in vitro phosphorylation patterns using PKA by SDS-PAGE and autoradiography. As shown in Fig. 2, there was one major phosphorylated species at 85 kDa in the NRK cell immunoprecipitate (Fig. 2A, top panel, lane 1) and a doublet, most likely corresponding to synapsins Ia and Ib by mobilities, in the rat brain immunoprecipitate (Fig. 2A, top panel, lane 4). These same phosphorylated species were recognized by the SNT1 antibody in the immunoblots (Fig. 2A, bottom panel, lanes 1,4), but not in the controls, which consisted of omission of either the NRK extract or the SNT1 culture supernate during

Fig. 4. Synapsin I localizes to the Golgi area of NRK cells. Cells were double immunostained for synapsin I (SynI) (A,a, B,b) and mannosidase II (mann II) (A',a') or TGN38 (B',b'). Boxed regions in A, A',B,B' are shown enlarged in a,a',b,b', respectively. Asterisks mark cell nuclei. Bar, 10 μm. (C) Confocal sections acquired at different levels of the Golgi of a NRK cell double-stained for synapsin I (green) and TGN38 (red). Consecutive sections are 0.5 μm apart. Bar, 5 μm.
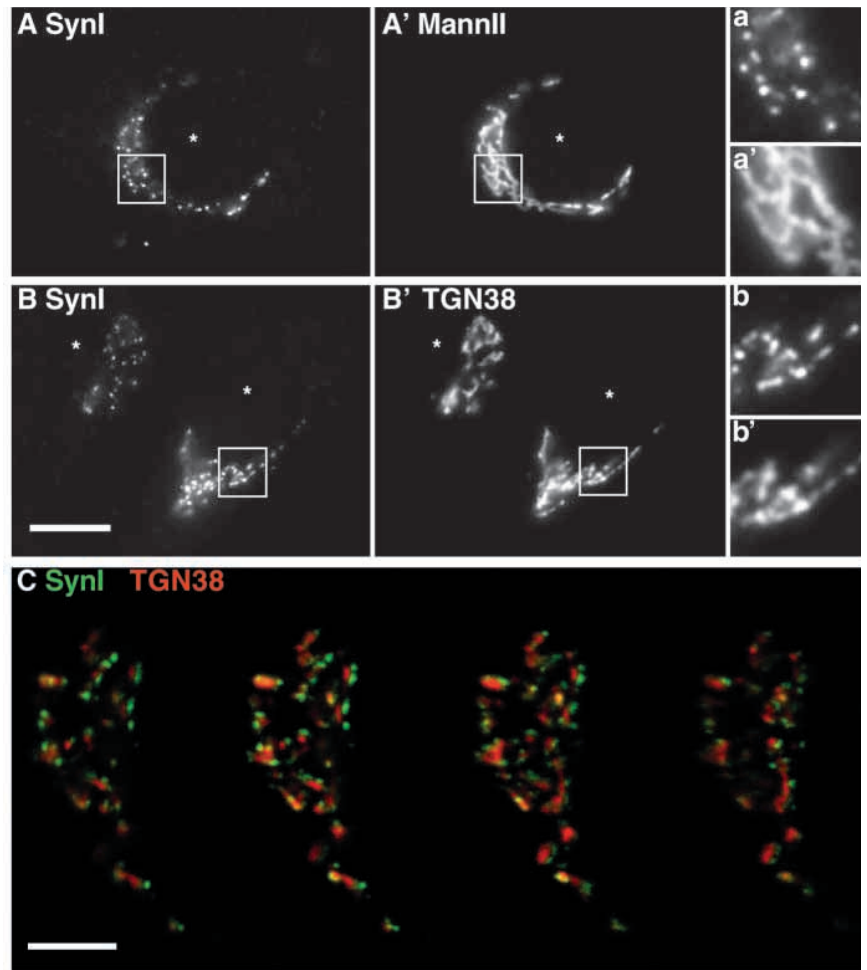
the immunoprecipitation step (Fig. 2A, top and bottom panels, lanes 2,3). Thus, p85 from NRK cells was a substrate for PKA as was the brain synapsin I.

To extend our comparison of the brain and NRK phosphoproteins, we performed one-dimensional peptide map analysis of the ^{32}P-phosphorylated bands. V8 endoprotease has been reported to generate short N-terminal fragments of PKA-labeled synapsin I (Huttner and Greengard, 1979). We confirmed this result from synapsin I purified from rat brain and found that the protein immunoprecipitated from NRK cells gave an identical cleavage profile. Major bands migrated at ~10 and 16 kDa on 15% gels (Fig. 2B, left panels). Higher amounts of V8 protease (up to 1 μg/well) yielded only the faster migrating (~10 kDa) band (data not shown). Using chymotrypsin to digest the PKA-phosphorylated protein, we obtained a more complex ^{32}P-phosphopeptide pattern (Fig. 2B, right panels), a result consistent with the large number of potential chymotryptic cleavage sites located C-terminal to serine 9 in synapsin I. Again, the ^{32}P patterns were identical in the NRK and brain samples. The strong similarity of the peptide maps generated with two different proteases demonstrated that the non-neuronal p85 protein was synapsin I.

Finally, we detected the message for synapsin I by northern blot in RNA obtained from freshly isolated hepatocytes. Using a probe generated from the 3′ end of brain synapsin I, we detected species migrating at 2.5-3.1 kb in both hepatocyte poly(A) RNA (Fig. 3, lane 4) and in total RNA (Fig. 3, lane 3). In brain, we detected 4.3 and 3.3 kb species with this probe (Fig. 3, lanes 1,2), consistent with previous findings (Sauerwald et al., 1990). The amount of synapsin I transcript in liver is roughly 1/50 that of the brain. The different mobilities between the brain and liver species may be explained by the use of a different transcription start site or differences in polyadenylation. Since our peptide maps indicate no extensive splice variation within the coding sequence, splicing outside of the protein coding sequence might occur. The 5′ flanking region of the rat synapsin I gene has been isolated and sequenced (Sauerwald et al., 1990). In addition to a single, brain-specific transcription start site, there is a region that is similar to promoters of many housekeeping genes, supporting our findings of synapsin I expression in liver.

Synapsin I in NRK cells localizes to a trans-Golgi sub-compartment

We examined the subcellular localization of synapsin I in cells by indirect immunofluorescence. In methanol or PFA-fixed NRK cells the SNT1 antibody labeled a juxtanuclear punctate compartment (Fig. 4). This signal was completely abolished by pre-incubation of the SNT1 antibody with pure rat brain synapsin I (not shown). Double labeling revealed that this compartment was located in the Golgi region in close association with a Golgi resident, mannosidase II (Fig. 4A,A') and a TGN resident, TGN38 (Fig. 4B,B'). Comparable localizations were observed in polarized and non-polarized hepatic WIF-B cells, the human osteosarcoma U2OS cells and COS-7 cells (data not shown). No labeled structures were observed in HeLa, MDCK or BHK cells (data not shown).

The similarity of patterns between the synapsin I positive compartment and the TGN, stained for TGN38, suggested that these two compartments were very close. To determine the degree of colocalization and the spatial relationship between the synapsin I compartment and the TGN, we performed confocal imaging of the Golgi region of cells double immunostained for TGN38 and synapsin I. Fig. 4C shows a series of confocal frames acquired through the Golgi stack of a methanol-fixed and double-immunostained NRK cell. These images confirm that the two proteins are in close proximity but nonetheless reside in different compartments that are clearly distinguishable from each other. Similar conclusions were obtained when cells fixed with PFA were examined (data not shown), indicating that the spatial relationship between these compartments is not fixation-dependent.

Fig. 5. Synapsin I remains associated with the Golgi in cells exposed to cytoskeletal-disrupting agents. (A) Nocodazole treatment causes synapsin I to disperse in punctate structures throughout the cytoplasm, in a similar manner to TGN38. NRK cells were treated with 33 μM nocodazole for 1 hour at 37°C, then fixed and stained for tubulin or double stained for synapsin I and TGN38. Tubulin staining shows complete microtubule depolymerization in nocodazole-treated cells. (B) Cytochalasin D treatment has no effect on synapsin I distribution in NRK cells. NRK cells were treated for 2 hours with 5 μM cytochalasin D. FITC-phalloidin staining reveals F-actin disruption in cytochalasin D-treated cells. Staining for synapsin I reveals punctate staining at cell center, similar to untreated cells. Asterisks mark cell nuclei. Bar, 10 μm.

Synapsin I has been reported to bind to microtubules and actin filaments in vitro and, thus, proposed to anchor SVs to the cytoskeleton at the presynaptic terminal (Baines and Bennett, 1986; Petrucci and Morrow, 1987; Bahler and Greengard, 1987; Hirokawa et al., 1989; Valtorta et al., 1992). We examined the effects of the microtubule disrupting agent, nocodazole, and the actin disrupting agent, cytochalasin D, on the localization of synapsin I in NRK cells. When the microtubules were completely depolymerized with nocodazole, synapsin I remained associated with punctate structures, which appeared more dispersed (Fig. 5A). Double labeling for TGN38 showed that the TGN behaved in a similar manner. Remarkably, there was a complete correspondence between the synapsin I-labeled structures and a number of TGN38 labeled puncta. However, TGN38-labeled structures were more numerous (Fig. 5A). Treatment of NRK cells with cytochalasin D disrupted the normal cell morphology, causing cell rounding and retraction of the cytoplasm towards the cell center. Nonetheless, the synapsin I staining remained punctate and juxtanuclear, and continued to overlap with the TGN as in non-treated cells (Fig. 5B). These experiments showed that synapsin I in non-neuronal cells was tightly associated with a trans-Golgi membrane compartment and that this interaction did not change following disruption of the microtubule or actin cytoskeleton.

Next we sought to determine the identity of the synapsin I compartment. Owing to its proximity to the TGN, we reasoned that this compartment could be involved in post-TGN traffic. Therefore, we looked at markers from the known trafficking pathways emerging from the TGN, for colocalization with synapsin I. Among these, we examined γ adaptin, βCOP, the transferrin receptor, SCAMP4 and syntaxin 6 in double immunofluorescence experiments with synapsin I. None of these markers showed clear localization to the synapsin I compartment (Fig. 6), and neither did liver-specific markers,
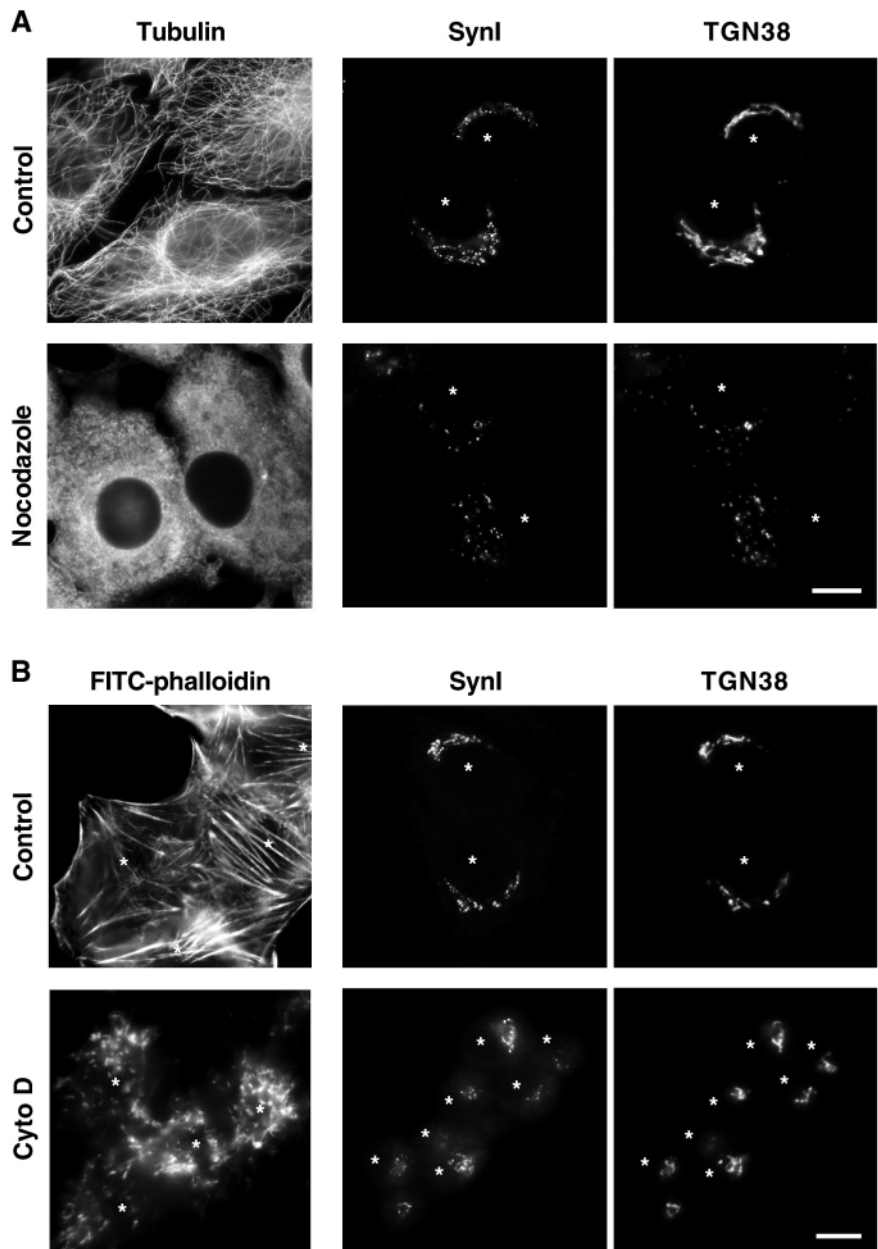
such as the copper transporter, Wilson protein, and albumin in WIF-B cells, (data not shown). However, in NRK cells, antibodies against myosin II stained structures in the Golgi region that almost exactly colocalized with the synapsin I compartment (Fig. 7A). This motor protein has been implicated in the in vitro production of vesicles from Golgi preparations (Müsch et al., 1997). Interestingly, in the WIF-B cell line, myosin II antibodies stained a more complex pattern than the synapsin I antibodies, but colocalization was also observed, suggesting that, in this cell type, myosin II might be present in other Golgi subdomains as well (Fig. 7B).

DISCUSSION

Synapsin I is present in epithelial cells

We have shown that synapsin I, previously identified as a

Fig. 6. Colocalization studies between synapsin I and different markers in NRK cells. NRK cells were double stained with SNT1 (A,a,B,b,C,c,D,d,E,e) and antibodies against γ adaptin (A',a'); the transferrin receptor (Tf R) (B',b'); βCOP (C',c'); syntaxin 6 (D',d'); and SCAMP4 (E',e'). Boxed regions in A,A',B,B',C,C',D,D',E,E' are shown enlarged in a,a',b,b',c,c',d,d',e,e', respectively. Synapsin I staining localizes to the juxtanuclear region of the cells as well as staining for the other markers. However, in all cases, the synapsin I compartment does not appear to be stained by the second antibody. Asterisks mark cell nuclei. Bar, 10 μm.
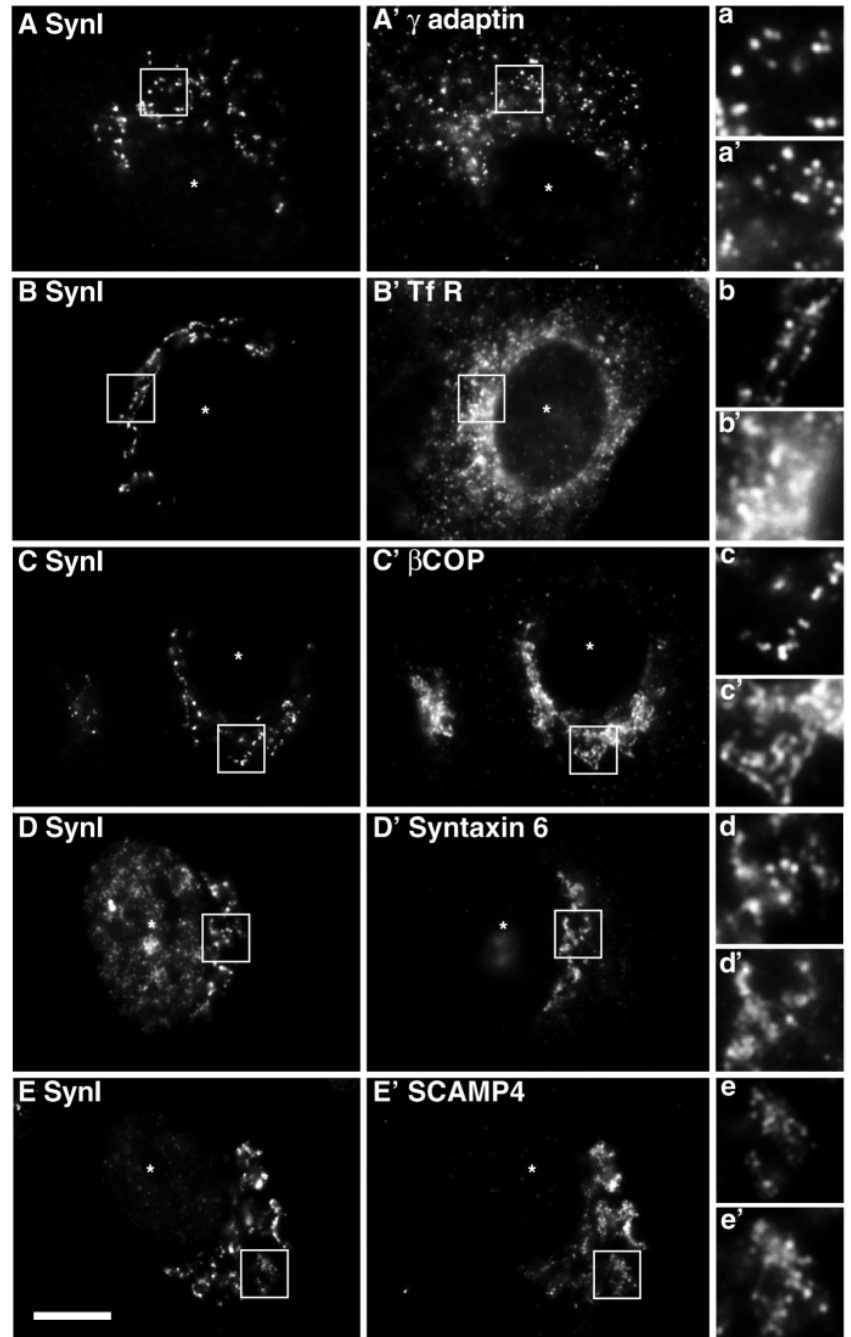
neural protein, is present in epithelial cells. Four different antibodies against synapsin I detected an 85 kDa polypeptide in liver, NRK cells and hepatocytes, but not in liver from synapsin I and II-deficient mice. The phosphopeptide maps of the non-neuronal protein were identical to those from brain synapsin I. Finally, the message for synapsin I was detected by northern blot in RNA obtained from freshly isolated hepatocytes, indicating that synapsin I is neither induced as a consequence of cell culture nor derived from innervating neurons.

Based on the amount of total protein required to detect synapsin I in non-neuronal tissues, we conclude that this protein is present at much lower levels in non-neuronal tissues than in brain. However, since synapsin I is such an abundant protein in brain, representing ~0.4% of the total protein of the cerebral cortex and ~6% of total synaptic vesicle protein (Huttner et al., 1983), the relatively low levels in peripheral tissue still represent a reasonable amount of cellular protein. A rough calculation suggests that hepatocytes could express ~10,000-50,000 copies of synapsin I per cell[1]. The expression levels of other molecules participating in exocytosis, including isoforms of syntaxin 1 (Jagadish et al., 1997), Munc 18 (Hata and Südhof, 1995), synaptotagmin (Li et al., 1995) and rab3 (Weber et al., 1994) are also comparably lower in peripheral tissues than in brain. This is not surprising, since the brain's requirement for rapid neurotransmission is accomplished, in part, by high levels of the relevant exocytic machinery in each synapse. Alternatively, the synapsins may regulate distinct exocytic processes that are less frequently used in non-neural tissues.

Our findings suggest that this protein may be more widely expressed and not neuron- or neuroendocrine-specific as was originally proposed (De Camilli et al., 1983). Perhaps the expression of the protein was missed in earlier studies due to its protease-sensitivity and relatively low abundance outside of the brain.

We investigated the expression of other SV proteins in liver and NRK cells, namely, synaptophysin and the enzyme glutamic acid decarboxylase (GAD). These two proteins localize to synaptic-like vesicles in the pancreas β cell (Thomas-Reetz and De Camilli, 1994). By immunoblot analysis, we did not detect synaptophysin in liver or NRK cell extracts but we did detect GAD in both cell systems. By immunofluorescence, NRK GAD showed a Golgi pattern, but it was not similar to that of synapsin I. Although undetectable in liver by immunofluorescence, results of subcellular fractionation experiments indicated that GAD was not in the Golgi (data not shown).

Our present analysis didn't allow us to decipher whether one or both isoforms of synapsin I are expressed in liver or any of the cell lines we examined, since the antibodies we used

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[1]The estimated number of synapsin I copies per hepatocyte is based on the following assumptions: 100 μg liver protein is equivalent to ~2×10^5 hepatocytes; brain has ~500-times more synapsin than liver, as judged by the relative intensities of the immunoblots in Fig. 1, and brain synapsin is 0.4% of total protein.

recognized both synapsin Ia and Ib. Both isoforms are present in brain, but their distributions and relative levels in individual neurons differ (Südhof et al., 1989). The functional significance is presently unclear. The major difference between these isoforms rests in a C-terminal E domain in synapsin Ia, which is absent in Ib. The presence of this domain in synapsin IIa and IIIa suggests a unique role for them all in regulating vesicle traffic (see below).

In epithelial cells, synapsin I is localized to a trans-Golgi compartment containing myosin II

In neurons, synapsin I localizes primarily to SVs. Recently, astrocytes in culture were reported to express several presynaptic proteins, including synapsin I, which associated with ATP storage compartments (Maienschein et al., 1999). Synapsin I is also vesicle-associated in endocrine cells. For example, in PC12 cells it associates with synaptic-like vesicles, and its expression increases upon NGF-induced differentiation (Tao-Cheng et al., 1995). In the MIN6 insulinoma, synapsin I reportedly codistributes with insulin-containing secretory granules in sucrose density gradients (Matsumoto et al., 1999), whereas in βTC3 cells it has been shown by immunoelectron microscopy to associate with small vesicles and not insulin-containing granules (Krueger et al., 1999). In NRK cells, we also find synapsin I associated with a

Fig. 7. Synapsin I and myosin II colocalize at the Golgi region in NRK and WIF-B cells. Methanol-fixed NRK (A,A',a,a') and WIF-B (B,B',b,b') cells were double-stained for synapsin I (A,a,B,B) and myosin II (A',a',B',b'). Both antigens localize to the same juxtanuclear structures in these cells. Boxed regions in A,A',B,B' are shown enlarged in a,a',b,b', respectively. Non-Golgi staining of myosin II antibody is due to labeling of stress fibers. Asterisks mark cell nuclei. Stars mark an apical cyst enclosed by two polarized WIF-B cells. Bar, 10 μm.

vesicular compartment but, surprisingly, it is located in the Golgi region, not at the cell periphery. Another rat epithelial cell line, the hepatic WIF-B cells, shows a similar Golgi pattern; significantly, this pattern is expressed in both polarized and non-polarized WIF-B cells. Although MDCK cells, the well-studied model of polarized epithelial cells, did not express a synapsin I that was recognized by our antibodies, we predict that different types of epithelial cells, including this dog kidney-derived line, have the protein. Finally, a subset of cells in rat pancreatic islets of Langerhans also expressed synapsin I in a Golgi-like pattern, whereas pancreatic acinar cells were negative (data not shown).

What might the synapsin I-positive compartment be? The vesicles with which synapsin I associates in NRK cells are probably a post-TGN compartment, given their close relationship with the Golgi complex and TGN-associated molecules. However, most markers of known post-TGN trafficking pathways do not colocalize with synapsin I. The one exception is myosin II-A, which is reportedly present on vesicles budded from Golgi in vitro (Narula et al., 1992; Ikonen et al., 1997). Both myosin II and actin's associations with Golgi membranes are sensitive to brefeldin A, suggesting that they bind via an ARF-regulated mechanism (Narula et al., 1992; Fucini et al., 2000). We found that the immunofluorescence signals of both myosin II and synapsin I were rapidly lost upon exposure of NRK cells to brefeldin A (10 minutes; data not shown). Thus, synapsin I might associate with its post-Golgi compartment in an ARF-dependent manner, as does myosin II.

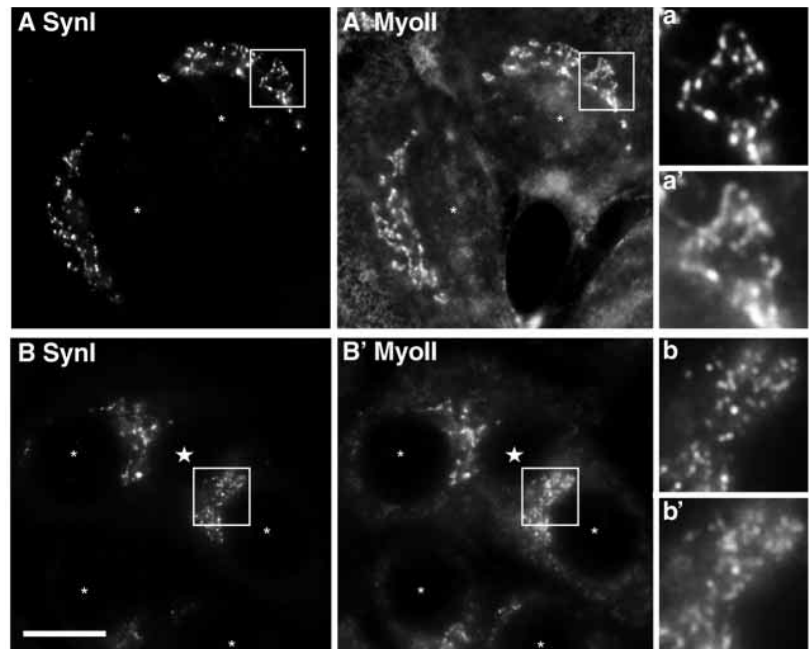A recent study reported the presence of different actin binding proteins on distinct populations of vesicles budding from isolated liver Golgi membranes in vitro. Myosin II-A and -B were detected on separate vesicle populations, which were also different from vesicles carrying β COP or γ adaptin (Heimann et al., 1999). At present, the role of these motor proteins in post-TGN trafficking remains controversial (Ikonen et al., 1996; Müsch et al., 1997; Simon et al., 1998). Müsch et al. reported that myosin II-A was involved in the in vitro formation of Golgi-derived vesicles containing newly synthesized VSV-G but not HA (Müsch et al., 1997). These viral membrane glycoproteins are targeted to the basolateral (VSV-G) and apical (HA) surfaces of transfected MDCK cells (Rodriguez-Boulan and Pendergast, 1980). We have conducted temperature-shift experiments in NRK cells expressing one or the other of these viral proteins. Our preliminary observations show minimal overlap of HA or VSV-G with either synapsin I or myosin II-positive structures. When the pIgA receptor, another marker for basolaterally targeted vesicle carriers, was exogenously expressed in NRK cells, the overlap with synapsin I or myosin II was also minimal (data not shown). Thus, our challenge will be to identify the cargo contained in this TGN compartment.

What is the function of synapsin I in epithelial cells?

Drawing on results from extensive in vitro and in vivo studies carried out on brain synapsin I, the most obvious role for synapsin I in epithelial cells is that of a tether linking a population of exocytic vesicles to the cytoskeleton. If this view is correct, it is intriguing that the location of such a pool is in the Golgi region and not beneath the plasma membrane domain with which these vesicles would putatively dock and fuse.

Perhaps there is not the same requirement for rapid secretion of the epithelial vesicle contents, as is the case at the synapse. An additional puzzle is the failure of depolymerization of either actin filaments or microtubules to release the synapsin I-positive vesicles from their association with the Golgi. It is possible that synapsin I is binding to membrane buds not vesicles. Interestingly, myosin II also remained vesicle-associated after depolymerization of actin filaments and overlapped with synapsin I as in untreated cells (data not shown). This result suggests that myosin II might associate with the compartment in an actin-independent manner. Alternatively, the cytoskeletal elements with which synapsin I and myosin II associate may be resistant to the pharmacological agents and conditions we used. The presence of Golgi-specific cytoskeletal elements is well documented, with the actin/myosin and spectrin/ankyrin cytoskeletal systems being the most actively studied (Beck and Nelson, 1998; De Matteis and Morrow, 1998; De Matteis and Morrow, 2000; Heimann et al., 1999; Fucini et al., 2000). The extensive data on the ability of synapsin I to interact with actin (Petrucci and Morrow, 1987; Bahler and Greengard, 1987; Ceccaldi et al., 1995) and spectrin (Baines and Bennett, 1985; Iga et al., 1997) in vitro suggest a possible role for this protein in the organization of cytoskeletal systems on Golgi membranes. We have not been able to detect spectrin or ankyrin isoforms on the synapsin I compartment by immunocytochemistry (data not shown), but the presence of a myosin isoform associated with it supports a role for the actin cytoskeleton in events related to this compartment's function.

It is possible that synapsin I's role in epithelial cells is not as a tether. Certainly, the mild phenotypes observed of synapsin I- and II-knockout mice have necessitated a re-examination of the essential nature of synapsins as tethers in the brain. Mice lacking synapsin I are viable and fertile, and have a normal synaptic architecture (Rosahl et al., 1993; Rosahl et al., 1995). However, the number of SVs at the pre-synaptic terminal is reduced compared with wild-type neurons, and the reservoir pool of SVs is more severely affected than the population at the active zones. This latter finding is thought to contribute to a decreased ability of the synapse to respond normally to sustained stimulation (Takei et al., 1995; Li et al., 1995). While these experiments place synapsins as important regulators of the availability of SVs at the nerve terminal, they show that they are dispensable, at least in mice.

Finally, recent studies have led to new views of synapsins. Studies in neurons isolated from synapsin I- and II-deficient mice have implicated these proteins in neuronal development, since in vitro synaptogenesis and axonal extension were delayed in the deficient compared with wild-type neurons (Chin et al., 1995; Ferreira et al., 1998). Experiments in which peptides from the E domain of squid synapsins were injected into the giant squid axon, reportedly inhibited neurotransmitter release without affecting the reservoir pool of SVs. This suggested a role for synapsins in the priming and docking events in the SV cycle (Hilfiker et al., 1998). In separate studies, the proline-rich D domain of synapsin I has been shown to bind specifically to a subset of SH3 domain-containing proteins such as c-Src and Grb2 (Onofri et al., 1997; Onofri et al., 2000). Moreover, synapsin I was shown to be the major binding partner for c-Src on purified SVs, and the interaction with synapsin I increased the tyrosine kinase activity of c-Src (Onofri et al., 1997). These studies suggest the involvement of synapsins in the regulation of signaling pathways and protein-protein interactions at the synaptic terminal. Together, these experimental data have broadened our view of synapsin function and place these molecules as important regulators of vesicle traffic. Therefore, it would not be unexpected if non-neuronal cells used these molecules in the regulation of vesicle trafficking. The challenge is to elucidate the mechanisms by which synapsins are operating.

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