Endogenous syntaxins 2, 3 and 4 exhibit distinct but overlapping patterns of expression at the hepatocyte plasma membrane

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To investigate the mechanisms regulating polarized vesicle delivery to the cell surface in hepatocytes, we have characterized the endogenous plasma membrane (PM)-associated syntaxins. These integral membrane proteins are components of the membrane docking/fusion apparatus and are thought to function as vesicle receptors at the PM. In hepatocytes, the PM is divided into two domains, the apical and basolateral. If syntaxins are mediating the specific recognition of vesicles delivered to either membrane surface, the simple prediction is that each domain expresses one syntaxin isoform. However, we report that rat hepatocytes express three endogenous PM-associated syntaxin isoforms, syntaxins 2, 3 and 4. By biochemical subfractionation, we determined that the syntaxins exhibit distinct, but overlapping patterns of expression among the PM domains. Syntaxin 4 is primarily expressed at the basolateral surface while syntaxins 2

and 3 are enriched at the apical PM. The immunolocalization of syntaxins 2 and 4 in rat hepatocytes and PM sheets revealed similarly complex patterns of PM expression with enhanced apical staining for both. A significant proportion of syntaxin 3 (25%) was detected in subcellular fractions containing transport vesicles. We have used quantitative immunoblotting to determine that the syntaxins are relatively abundant PM molecules (11–260 nM) in rat liver, spleen and kidney. Also, we determined that the syntaxin binding protein, Munc-18, is present at concentrations from 1.5–20 nM in the same tissues. Although this fundamental quantitative and morphological information is lacking in other systems, it is critical not only for defining syntaxin function, but also for predicting the specific mechanisms that regulate vesicle targeting in hepatocytes and other tissues.

INTRODUCTION

Epithelial cells line all body organs and serve as selective barriers between two different worlds (the internal and external). They accomplish this essential function by cementing themselves together through intercellular junctions, which in turn restrict distinct activities to specific plasma membrane (PM) domains (either basolateral or apical). The functional asymmetry, or polarity, of the two surface domains is mirrored by the asymmetrical distribution of PM proteins. The intracellular compartments and pathways in these cells are also characterized by functional and compositional polarity. How is this polarity established and maintained, given the dynamic nature of cellular processes? Answers come, in part, from an understanding of vesicle traffic in polarized epithelial cells.

Our laboratory studies vesicle traffic in hepatocytes, the major epithelial cell of the liver [1]. Hepatocytes are strategically positioned between the blood and bile and thereby perform many polarized functions that make this organ the biochemical centre of the body [2]. For example, at the sinusoidal (basolateral) pole, there is exchange of metabolites with the blood, including transport of small molecules across the basal membrane, secretion of plasma proteins and lipoproteins via fusion of secretory vesicles with the basal membrane, and internalization of circulating macromolecules via clathrin-coated pits and vesicles. The specialized exocrine functions at the apical or bile canalicular membrane include: transport of bile acids and products of detoxification across the membrane bilayer into bile, release/

transport of lipids at the canalicular front, and delivery of immunoglobulin A to bile by fusion of transport vesicles with the apical membrane. These functions all rely on the continuous operation of two pathways, the biosynthetic (exocytic or secretory) and endocytic pathways. While most of bile acid transport itself is probably not vesicular [3–5], many of the proteins that mediate this vectorial process are integral membrane proteins that certainly traverse the biosynthetic and endocytic pathways during their lifetimes. The organelles comprising these two pathways serve as the hepatocyte's system for exchanging macromolecules with its surroundings and for replenishing, removing and recycling components that function in the participating compartments [1,6].

How do cargo-bearing vesicles deliver their contents to the correct target domain? Morphological, biochemical and genetic approaches have been successfully used to identify many molecules thought to be involved in vesicle formation, transport, targeting, docking and consumption [7]. The results from these approaches indicate that the basic mechanisms may be conserved among the membrane compartments throughout the biosynthetic and endocytic pathways and in organisms ranging from yeast to humans [8–9]. The players so far identified fall into two broad categories: some are used repeatedly throughout both pathways [10], others belong to discrete protein families, where one or a few members each act at only a few transport sites [9]. One current hypothesis {the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) hypothesis, also see [11]} maintains that vesicles recognize their appropriate target com-

Abbreviations used: DPPIV, dipeptidyl peptidase IV; GST, glutathione *S*-transferase; IPTG, isopropyl- β -thiogalactopyranoside; MDCK, Madin–Darby canine kidney; Munc-18, non-neuronal syntaxin binding proteins; 5'-NT, 5'-nucleotidase; PM, plasma membrane; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; t-SNARE, target membrane SNAP receptor; v-SNARE, vesicle SNAP receptor; TxR, Texas Red.

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partment by the pairwise coupling of integral vesicle membrane proteins (the v-SNAREs) with corresponding integral target membrane proteins (the t-SNAREs). Binding of the respective SNARE molecules (docking) is followed by the recruitment of other cytosolic factors, including the soluble *N*-ethylmaleimide-sensitive factor attachment protein (α -SNAP) and the ATPase, *N*-ethylmaleimide-sensitive fusion protein. Upon hydrolysis of ATP by the *N*-ethylmaleimide-sensitive fusion protein, the complex disassembles. Whether this step occurs before or during vesicle fusion is currently under considerable debate [12]. According to the SNARE hypothesis, each transport step is regulated by its own specific set of v- and t-SNAREs, while the *N*-ethylmaleimide-sensitive fusion protein and α -SNAP are common to all types. Other mechanisms that do not require the activities of the SNARE molecules are probably also mediating vesicle delivery [13,14]. However, the molecular players involved have not been clearly identified.

Since the PM of polarized epithelial cells is separated into two distinct domains, it is of particular interest to understand how the targeting of either apical or basolateral-specific vesicles is regulated. The t-SNAREs, which belong to the syntaxin family, are likely candidates for regulators of transport to the cell surface. To date, seven syntaxin family members (syntaxins 1A, 1B, 2–6) have been identified in mammalian cells of which five (syntaxins 1A, 1B, 2–4) are PM-specific isoforms [15,16]. Analysis of related proteins in both yeast (*Sso1p* and *Sso2p*) and *Drosophila* (*Dsynt1*) have provided compelling evidence that syntaxins are involved in membrane transport to the cell surface. Yeast strains that were depleted of both *Sso1p* and *Sso2p* expression were observed to accumulate transport vesicles destined for the PM and they were unable to secrete invertase [17]. In *Drosophila*, neurotransmitter release was completely abolished in *Dsynt1* deletion mutants [18,19]. The results of studies in which the mechanism of inhibition of neurotransmitter release by bacterial neurotoxins was investigated have further suggested that syntaxins are involved in PM vesicle delivery. In neurons and isolated synaptosomes, the decrease in neurotransmitter release observed in the presence of botulinum C1 neurotoxin was found to correlate with the specific cleavage of syntaxin isoforms 1A, 1B, 2 and 3 [20–23]. Similar results were obtained in intact or permeabilized chromaffin cells where decreases in catecholamine release were accompanied by specific cleavage of syntaxins 1A and 1B [24]. The results of a number of studies examining the effects of microinjected anti-syntaxin antibodies or soluble syntaxin fragments on secretion also strongly supported the proposed role of syntaxins in vesicle targeting to the PM [15,25–27].

Based on these observations, we chose to investigate the role(s) syntaxins play in targeting vesicles to the PM in hepatocytes. Since the PM is divided into two domains, we predicted that two syntaxin isoforms would be expressed in hepatocytes, one at each domain. Consistent with this prediction was the detection of two PM syntaxin transcripts (encoding syntaxins 2 and 4) in liver [15]. However, using quantitative biochemistry and morphology, we report that rat hepatocytes express three endogenous PM syntaxins (syntaxins 2, 3 and 4) that are all relatively abundant PM-associated molecules. Biochemically, all are highly enriched in the hepatocyte PM and show distinct but overlapping distributions in the PM subdomains. However, none appears to be exclusively present in a single PM domain as predicted. Localization of syntaxins 2 and 4 by indirect immunofluorescence in intact hepatocytes and purified PM sheets reveals a similarly complex distribution with enhanced apical staining for both. Only now, armed with these fundamental quantitative and morphological data, are we poised to examine the specific and complex mechanisms regulating vesicle targeting and docking in

polarized hepatocytes. Part of this work has already been published in abstract form [27a].

EXPERIMENTAL

Materials

Male Sprague–Dawley rats (CD strain; 125–150 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.). Syntaxin 2 and 4 cDNAs and the pGEX-KG vector were provided by R. H. Scheller (Howard Hughes Medical Institute, Stanford University Medical Center, Stanford, CA, U.S.A.). All the molecular biology reagents, restriction enzymes, DNA ligase (New England Biolabs Inc.), PCR reagents (Perkin-Elmer) and Sequenase (USB) were purchased from the Johns Hopkins University School of Medicine Core Facility, Baltimore, MD, U.S.A. Thrombin, glutathione and glutathione agarose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Isopropyl-D-thiogalactopyranoside (IPTG) was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). FITC–goat anti-rabbit IgG, Texas Red (TxR)–goat anti-mouse IgG and FITC–donkey anti-mouse IgG secondary antibodies were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, U.S.A.). FITC–goat anti-rat IgG was from Cappel (Durham, NC, U.S.A.). All other reagents and compounds were of the highest purity available and were purchased from Sigma Chemical Co. Affinity-purified syntaxin 3 antibodies and the cDNA encoding glutathione *S*-transferase (GST)–syntaxin 3 fusion protein were provided by M. K. Bennett (University of California, Berkeley, CA, U.S.A.). The antibodies against Munc-18 isoforms were provided by J. Pevsner (Johns Hopkins University, School of Medicine, Baltimore, MD, U.S.A.). The rat monoclonal ZO-1 antibodies were from ascites produced by hybridoma cells provided by B. Stevenson (University of Alberta, Edmonton, Canada). Sialyl transferase antibodies were provided by G. Hart (Johns Hopkins University, School of Medicine, Baltimore, MD, U.S.A.). The antibodies against CE9, HA4, dipeptidyl peptidase IV (DPPIV) and HA321 have been described elsewhere [28–32].

Plasmid construction

The constructs encoding the cytoplasmic domains of syntaxin 2 (amino acid residues 1–266) and syntaxin 4 (amino acid residues 1–273) were generated by PCR with oligonucleotides corresponding to the N- and C-terminal regions of the cytoplasmic domains. For syntaxin 2, primers were synthesized corresponding to an *EcoRI* site (ACACGAATTCTAATGCGGGACCGGCTGCCG) at the 5' end and a *HindIII* site (ACACAAGCTTT-CACCGTCTGGCCTTGCTCT) at the 3' end. For syntaxin 4, the primers corresponded to an *XbaI* site (ACACTCTAGACATGCGCGACAG GACCCATG) at the 5' end and an *XhoI* site (ACACCTCGAGTCACTTTTCTTCCTCGCCTTCTTC) at the 3' end. The resulting PCR fragments were directionally cloned into the *EcoRI/HindIII* and *XbaI/XhoI* sites of the pGEX-KG vector respectively. PCR-amplified DNA was verified by dideoxynucleotide sequencing using Sequenase according to the instructions of the manufacturer.

Expression and purification of GST-syntaxins

Fusion proteins were expressed in the *Escherichia coli* strains TG1 (syntaxins 2 and 4) and DH5 (syntaxin 3) using standard methods of growth and IPTG induction [33]. Cells were harvested by centrifugation (12000 *g* for 20 min at 4 °C) and resuspended in PBS containing 1% (v/v) Triton X-100, 5 mM benzamidine, 2 mM EDTA, 0.2 mM PMSF and 0.1% (v/v) 2-mercapto-

ethanol. After sonication and centrifugation (12000 *g* for 10 min at 4 °C) to remove the insoluble fraction, the supernatant was mixed with an equal volume of a 50 % (v/v) slurry of glutathione-agarose equilibrated in PBS containing 1 % (v/v) Triton X-100. The mixture was incubated for 2 h to overnight at 4 °C with gentle rotation. The agarose with bound fusion proteins was washed 4–6 times by resuspension in PBS containing 1 % (v/v) Triton X-100 followed by sedimentation at 1000 *g* for 5 min at 4 °C. To elute the bound fusion proteins, the agarose was incubated in an equal volume of 50 mM Tris/HCl (pH 8.0)/10 mM glutathione for 20 min. This elution step was repeated twice. Free glutathione was removed by dialysis against TBS [50 mM Tris/HCl (pH 8.0)/150 mM NaCl] containing 0.1 % (v/v) 2-mercaptoethanol. To prepare thrombin-cleaved syntaxin proteins, the agarose with bound fusion proteins was equilibrated in dialysis buffer and subsequently eluted with dialysis buffer containing 10 units/ml of thrombin for 2 h at room temperature. The beads were sedimented and the supernatants containing the cleaved syntaxins were recovered. The additional sequences, GSPGISGGGGGIL and GSPGISGGGGGILD, are located at the N-termini of thrombin-cleaved syntaxins 2 and 4 respectively. The purified proteins were divided into aliquots and stored at –80 °C.

Antibody production

Both GST-fusion and thrombin-cleaved forms of syntaxins 2 and 4 were used to prepare rabbit polyclonal antibodies. The soluble native proteins and SDS/PAGE gel slices were sent to a commercial producer (Covance, Denver, PA, U.S.A.). Antibodies were affinity-purified using antigen-Sepharose 4B columns.

Subcellular fractionation

PM sheets were isolated from rat liver according to previously published methods [34]. Vesicles from the membranes were prepared by sonication of PM sheets, applied to continuous 0.46–1.42 M sucrose gradients, centrifuged to equilibrium (72000 *g* for 16–20 h at 4 °C) and fractions were collected from the top [35]. A pellet was also obtained, which was resuspended to the same volume as the other fractions. This procedure separates vesicles derived from bile canalicular (apical) and basolateral membranes, and has been used extensively in our laboratory. The one-step Golgi flotation fractionation was performed according to Bergeron et al. [36], with minor modifications [37].

Quantitative analysis of syntaxin protein isoforms

Liver homogenates [20 % (w/v)] were prepared from fasted rats as described [34]. For the quantitative data shown in Table 1, rat liver, spleen and kidney were homogenized in a Dounce homogenizer in one volume of lysis buffer [100 mM NaCl/4 mM Hepes (pH 7.4)/3.5 mM CaCl₂/3.5 mM MgCl₂/1 mM EDTA/1 % (v/v) NP-40]. The homogenate samples were mixed with Laemmli sample buffer and subjected to SDS/PAGE. The polypeptides were electrophoretically transferred on to nitrocellulose membranes for analysis by Western blotting [38]. The amount of the syntaxin species in rat hepatocytes was determined by the densitometric analysis (Microcomputer Imaging Device, Imaging Research Inc., Ontario, Canada) of immunoreactive species relative to a standard curve prepared from purified protein. Calculations were based on 1.38×10^8 hepatocytes/g wet weight of liver. The amounts of syntaxin isoforms present in kidney and spleen were determined by densitometric comparison of their immunoreactive bands with those present in liver. Data were generated from 4–6 different preparations.

Isolation of syntaxin binding proteins

Syntaxin binding proteins were isolated by affinity chromatography as described [39]. Briefly, homogenates from one rat liver, spleen or kidney were prepared in lysis buffer as described above and extracted on ice for 2 h, mixing occasionally by inverting. To preclear the tissue homogenates of endogenous GST, each sample was incubated with 1/4 volume of a 50 % (v/v) glutathione-agarose slurry for 2 h at 4 °C, with gentle mixing. The extracts were recovered by sedimentation and the preclearing step was repeated. The precleared extracts were subsequently mixed with 0.1 ml of a 50 % (v/v) slurry of glutathione-agarose to which either GST-syntaxin 2 or 4 (approx. 100 µg) was bound. The mixtures were incubated overnight at 4 °C while gently mixing on a rotating wheel. The beads were recovered by sedimentation (1000 *g* for 5 min at 4 °C), were washed 4–6 times by resuspension in TBS containing 1 % Nonidet P-40 and were recentrifuged. The final bead pellet was eluted with Laemmli sample buffer (50 µl) and the eluent was subjected to gel electrophoresis. To determine the concentration of the Munc-18 isoforms recovered, densitometric analysis of Coomassie-Blue-stained gels, with BSA as the standard, was performed.

Preabsorption of anti-syntaxin 3 antibodies

Affinity purified anti-syntaxin 3 antibodies (15 µl) were combined with 125 µg of purified GST-syntaxin 2 and/or 4 in a final volume of 0.2 ml PBS. The mixtures were incubated on ice for 2–4 h, with occasional mixing by inversion. The mixture was further diluted in 5.0 ml of PBS containing 1 % (w/v) BSA before Western blotting.

Other methods

Western blots were assayed for immunoreactivity with the following primary antibodies: anti-HA321, anti-DPPIV and anti-CE9 (rabbit polyclonal antibodies, 1:2000, 1:2500 and 1:10000 dilution respectively), affinity-purified anti-syntaxin 2 and 4 antibodies (rabbit polyclonal antibodies, 0.5 µg/ml), affinity-purified and preabsorbed anti-syntaxin 3 antibodies (rabbit polyclonal antibodies, 1:333 dilution). Immunoreactivity was assayed by enhanced chemiluminescence detection of horseradish-peroxidase-conjugated secondary antibodies (Amersham, Arlington Heights, IL, U.S.A.), according to the instructions of the manufacturer. 5'-Nucleotidase (5'-NT) activity was assayed by the method of [40]. Total protein content was determined using bicinchoninic acid reagent (BCA) (Pierce, Rockford, IL, U.S.A.). Total IgG was recovered from mouse ascites cells by using EZ Sep, according to the instructions from the manufacturer (Pharmacia, Piscataway, NJ, U.S.A.).

Immunofluorescence analysis

Isolated PM sheets (~0.1 mg) were allowed to settle by gravity on to glass coverslips for 20–30 min at room temperature. The coverslips were rinsed briefly with PBS and the adsorbed PM sheets were fixed with methanol at –20 °C for 5 min followed by rehydration with PBS. Fixed PM sheets were blocked with PBS containing 1 % (w/v) BSA and processed for indirect immunofluorescence as described [41], using the following primary antibodies: affinity-purified syntaxin antibodies (rabbit polyclonal, 50 µg/ml), anti-HA4 and anti-HA321 mouse monoclonal ascites (1:100 dilution) or rat monoclonal antibodies against ZO-1 (purified IgG fraction, 50 µg/ml). All the secondary antibodies (FITC-goat anti-rabbit, TxR-goat anti-rabbit and TxR-goat anti-mouse) were used at 10–15 µg/ml, except for FITC-goat anti-rat which was used at 50 µg/ml.

The liver was extracted by perfusion via the portal vein with PHEM buffer (60 mM Pipes/25 mM Hepes/10 mM EGTA/2 mM MgCl_2 , pH 6.8) containing 0.025 % (w/v) saponin as described [42]. The livers were fixed in periodate, lysine and paraformaldehyde as described [43] and semi-thin sections (0.5 μm) cut with a Ultracut E microtome (Reichert Jung). The liver sections were processed for immunofluorescence according to methods published previously [29], using the primary and secondary antibodies as described above.

Imaging

PM sheet preparations and extracted liver sections were examined by epifluorescence (Axioplan universal microscope, Carl Zeiss Inc., Thornwood, NY, U.S.A.). Images of the isolated PM sheets were analysed using the Microcomputer Imaging Device image-analysis system. All images were subsequently assembled and labelled using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA, U.S.A.).

RESULTS

Antibody specificity

Since the amino acid sequences of syntaxins 2, 3 and 4 are highly conserved (41–63 % sequence identity and 62–79 % sequence homology), we examined the specificity of the antibodies used in this study. Cross-reactivity with non-PM-associated syntaxins was not evaluated since only 21–26 % and 44–48 % respectively of the amino acids are shared or conserved among the syntaxin 2, 3 and 4 sequences and those of syntaxins 5 and 6 [15,16]. Figure 1(A) demonstrates the specificity of the generated syntaxin 2 and 4 antibodies. Affinity-purified syntaxin 2 and 4 antibodies reacted strongly with their cognate antigens in both the GST- and thrombin-cleaved forms (60 and 30 kDa respectively); neither antibody cross-reacted with the reciprocal antigen, free GST (Figure 1A) nor GST-syntaxin 3 (results not shown). The affinity-purified syntaxin 3 antibodies cross-reacted with GST-syntaxin 2; this was overcome by preadsorption against this protein before use. As shown in Figure 1(B), the preabsorption step virtually eliminated immunoreactivity against 500 ng of GST-syntaxin 2. We further confirmed the specificity of these antibodies in immunodepletion experiments. Preadsorption of the affinity-purified antibodies with a 10-fold molar excess of their cognate antigens abolished immunodetection on Western blots of either purified rat liver PM fractions or the purified recombinant proteins (results not shown). Preincubation with mismatched antibodies and antigen (e.g., anti-syntaxin 2 antibodies and purified syntaxin 4) had no effect on immunoreactivity (results not shown).

Quantitative analysis

Although syntaxin isoforms have been identified in a number of different cell types and tissues, little is known about their relative abundance. Such information is important in defining the potential functions of these proteins (e.g., whether they act stoichiometrically or catalytically) and their interactions with other members of the vesicle targeting machinery. Previous examination of the tissue distributions of these non-neuronal, PM-associated syntaxin isoforms by Northern blotting indicated that syntaxins 2, 3 and 4 were all relatively abundant in rat spleen and kidney, whereas only syntaxins 2 and 4 were detected in rat liver [15]. Using specific antibodies, we examined the tissue distributions and expression levels of the syntaxin proteins.

Figure 1 Antibodies directed against syntaxins 2, 3 and 4 are specific

Western blots that were probed with either affinity purified (A) syntaxin 2 or 4 antibodies or (B) syntaxin 3 antibodies are shown. (A) Western blots of 1 μg purified PM (lane 1; lanes are numbered from the left in each panel), 4 ng of recombinant GST, GST-syntaxin 2, GST-syntaxin 4 (lanes 2, 3, and 5 respectively) and 4 ng of thrombin-cleaved syntaxin 2 and 4 (lanes 4 and 6 respectively) were probed with anti-syntaxin 2 and 4 antibodies. The antibodies each recognized polypeptides of ~ 30 kDa in the PM sheet preparations as indicated. Neither showed cross-reactivity with the related syntaxin species nor with purified GST. (B) Affinity purified or affinity purified and preabsorbed anti-syntaxin 3 antibodies were immunoreacted against Western blots containing 500 ng GST-syntaxin 2, 3 or 4 (lanes 1–3 respectively). Before preabsorption, the syntaxin 3 antibodies cross-reacted significantly with syntaxin 2 (– Immunodepletion). The cross-reactivity was virtually abolished by a simple incubation step (+ Immunodepletion) as described in the Experimental section.

Table 1 Quantitative analysis of Munc-18 and syntaxin isoforms in rat liver, spleen and kidney

The quantitative analysis was performed as described in the Experimental section. Concentrations are expressed as the mean $\pm$ S.D. for three to four determinations. The syntaxin 3 and Munc-18 concentrations in spleen and kidney are the means of two determinations. The level of Munc-18 species in rat liver was determined from one preparation. The values in square brackets indicate the number of molecules/cell ($\times 10^{-5}$). For comparison, the PM proteins HA321, DPPIV and CE9 are present in hepatocytes at $13 \pm 4 \times 10^5$, $55 \pm 16 \times 10^5$ and $78 \pm 16 \times 10^5$ molecules/cell respectively [32].

Tissue	Concentration (nM)			
	Syntaxin 2	Syntaxin 3	Syntaxin 4	Munc-18
Liver	11.4 $\pm$ 3.4 [0.5 $\pm$ 0.2]	56.7 $\pm$ 43.3 [2.6 $\pm$ 2.0]	28.2 $\pm$ 14.1 [1.2 $\pm$ 0.6]	1.5
Spleen	21.3 $\pm$ 15.6	228.8	50.5 $\pm$ 23.7	20
Kidney	16.1 $\pm$ 12.2	259.8	39.2 $\pm$ 29.7	5.5

Consistent with the Northern-blot analysis, the syntaxin 2 and 4 proteins were detected in rat liver, spleen and kidney at similar concentrations (Table 1). Syntaxin 2 was expressed at

Table 2 Distributions of syntaxins 2, 3 and 4 in the one-step Golgi flotation

Fractions from the one-step Golgi flotation were assayed by quantitative immunoblotting. Percentages are expressed as the means $\pm$ S.D. from three or four separate fractionations. Fractions were separated and collected from the top of the tube as described in the Experimental section. The fractions were designated as follows: A, top of the tube to the floated band; B, the floated band enriched in Golgi membranes; C, between the floated band and the load fraction; D, the load fraction; E, the pellet.

Fraction	Percentage distribution						
	Protein	Sialyltransferase	DPPIV	CE9	Syntaxin 2	Syntaxin 3	Syntaxin 4
A	0.4 $\pm$ 0.3	5.0 $\pm$ 6.1	0.9 $\pm$ 0.6	0.9 $\pm$ 0.9	0.6 $\pm$ 0.5	2.0 $\pm$ 1.9	0.2 $\pm$ 0.2
B	0.9 $\pm$ 0.1	58.5 $\pm$ 9.2	3.0 $\pm$ 3.1	1.0 $\pm$ 0.7	0.9 $\pm$ 0.8	2.3 $\pm$ 0.9	1.1 $\pm$ 0.5
C	3.1 $\pm$ 2.2	21.9 $\pm$ 8.6	1.9 $\pm$ 0.7	0.6 $\pm$ 0.3	1.0 $\pm$ 0.7	3.5 $\pm$ 0.4	0.5 $\pm$ 0.4
D	41.3 $\pm$ 1.1	10.8 $\pm$ 14.2	3.8 $\pm$ 4.6	1.7 $\pm$ 1.1	3.5 $\pm$ 2.9	25.2 $\pm$ 11.7	0.7 $\pm$ 0.4
E	54.3 $\pm$ 1.0	3.8 $\pm$ 6.5	90.5 $\pm$ 3.9	95.8 $\pm$ 2.0	94.2 $\pm$ 4.1	67.3 $\pm$ 10.8	97.4 $\pm$ 0.7

11–21 nM while syntaxin 4 was present at twice that level, ranging from 28–50 nM. Also in agreement with the Northern-blot analysis was the detection of high levels of syntaxin 3 protein in spleen and kidney. However, substantial syntaxin 3 protein was unexpectedly detected in rat liver, although at 4-fold lower levels than in spleen and kidney ($\sim$ 60 nM versus 230 and 260 nM respectively). The significantly lower concentration of syntaxin 3 in liver may have precluded its detection previously. Interestingly, in liver, the syntaxin 3 concentration was about two-fold more than either syntaxin 2 or 4. Although relatively abundant, other hepatocyte PM proteins are expressed at 25- to 150-fold greater levels. Syntaxins 2, 3 and 4 were expressed at 0.5×10^5 , 2.6×10^5 and 1.2×10^5 molecules/cell respectively, whereas HA321, DPPIV and CE9 have been shown to be expressed at 13, 55 and 78×10^5 molecules/cell [32]. At present it is unclear why the levels of syntaxin expression vary so much among tissue types. It is compelling to speculate that syntaxin expression correlates with the rates, amounts or kinds of vesicle targeting to the PM in these different cell types.

We also examined the tissue concentrations of the known non-neuronal syntaxin binding proteins, the Munc-18 species [44–46]. At present, the role that these proteins play in vesicle targeting to the PM is not clear. Knowledge of the concentrations of Munc-proteins, however, is essential in order to test the models describing their associations. We purified the Munc-18 isoforms from rat liver, spleen and kidney by affinity chromatography according to previously published procedures [39], and their identity was confirmed by Western blotting using Munc-18 antibodies (results not shown). As shown in Table 1, the concentrations of Munc-18 proteins in rat liver and kidney were nearly equal (1.5 and 5.5 nM respectively), whereas somewhat higher concentrations (20 nM) were expressed in spleen. These concentrations were much lower than those of either syntaxin 2 or 3 in all cases (syntaxin 4 does not bind Munc-18 isoforms [45,46], and results not shown). Interestingly, the proportions of syntaxins 2 and 3 relative to Munc-18 proteins were roughly equivalent among the three tissues. The ratio of expression of syntaxin 2 and syntaxin 3 to Munc-18 molecules in liver was 45, while the ratio was 32 in spleen and 50 in kidney. This suggests that the roles these molecules play in PM vesicle targeting may be conserved among cell types.

Biochemical subfractionation

The next stage of our analysis was aimed at determining the subcellular locations of the syntaxin isoforms in rat liver. Results from others indicate that syntaxins 2, 3 and 4 are predominantly expressed at the PM in a variety of cell types [15,47–54]. To

determine whether these syntaxin isoforms share similar distributions in rat liver, two distinct subcellular fractionation schemes were used: a one-step Golgi flotation and the isolation of PM sheets. We further examined the PM distributions of the syntaxin isoforms by analysing preparations of apically and basolaterally derived PM vesicles. All three methods have been used extensively in our laboratory and are designed specifically for use with rat liver.

One-step Golgi membrane flotation

Although the Golgi flotation was devised for the separation of rat liver Golgi from other membrane fractions, this scheme provides a rapid and efficient method for assessing the approximate location of proteins among various membrane compartments. In these experiments, a linear sucrose gradient was poured on top of rat liver homogenate and the whole was centrifuged at 82000 *g* for 3.5 h. The pelleted fraction (Table 2, fraction E) and the four fractions collected from the top of the gradient (Table 2, fractions A–D) were assayed for the presence of the syntaxin isoforms and specific organelle-marker proteins by quantitative immunoblotting. In these preparations, the Golgi membranes were distributed predominantly in fraction B. Almost 60% of total sialyltransferase (a *trans*-Golgi marker) was recovered in this fraction, which represented a 50-fold enrichment of Golgi membranes. In contrast, only approx. 1–2% of total syntaxin 2, 3 or 4 was found in this fraction. More than 90% of syntaxins 2 and 4 and the PM-marker proteins (CE9 and DPPIV) were recovered in fraction E (Table 2). Interestingly, syntaxin 3 was not as highly enriched in fraction E as the other syntaxin isoforms and only 67% of total syntaxin 3 was detected; a significant proportion (25%) was found in fraction D, the 'load' fraction. Although this fraction contained cytosolic proteins, membrane vesicles of unknown origin were also present ([37], and the Discussion section).

PM sheet purification

The results described above combined with those of other workers [15,47–54] provide strong evidence that the majority of syntaxin 2 and 4 expression is at the PM in liver at steady state. However, fraction E contained other membrane populations, most notably the ER [36]. To ensure that the enrichment of the syntaxin isoforms in this fraction was not due to their association with the ER or other membranes, we prepared highly purified PM sheets [34] and assayed them for syntaxin immunoreactivity. PM enrichment was determined by assaying fractions for 5'-NT activity, as described previously [40]. As shown in Table 3, the PM sheets

Table 3 Plasma membrane enrichment of syntaxins 2 and 4

Hepatocyte PM sheets were purified from rat liver and assayed for total protein content, 5'-NT activity, or by quantitative immunoblotting. Assay values are expressed as the mean $\pm$ S.D. from three separate preparations. Values in parentheses represent the fold enrichment of 5'-NT activity or the syntaxins in the purified PM sheets relative to whole homogenate. The percentages of total protein, 5'-NT activity, syntaxin 2 and syntaxin 4 recovered in the PM preparations were 1.11 ± 0.32 , 16.1 ± 2.4 , 37.0 ± 15.3 and 26.2 ± 1.8 respectively.

Fraction	Total protein (mg)	5'-NT activity (units/mg)	Syntaxin 2 (ng/mg)	Syntaxin 4 (ng/mg)
Homogenate	574.8 $\pm$ 155.4	23.8 $\pm$ 10.6	5.04 $\pm$ 1.34	9.46 $\pm$ 2.02
PM	6.07 $\pm$ 1.7	332.9 $\pm$ 116.0 (14.4 $\pm$ 1.8)	162.5 $\pm$ 13.1 (33.8 $\pm$ 8.9)	237.5 $\pm$ 55.4 (25.3 $\pm$ 4.6)

were significantly enriched in these preparations (14-fold relative to the liver homogenate) and syntaxins 2 and 4 were enriched to an even greater extent (approx. 34- and 25-fold relative to the homogenate respectively). Similar values were observed when the total recovery of these proteins in PM sheets was calculated. The recovery of total 5'-NT activity in this fraction was 16% compared with 37 and 26% of total syntaxins 2 and 4 respectively. From these results, we concluded that nearly all of the detectable syntaxin 2 and 4 reside at the PM in rat liver.

PM domains purification

We next determined the distribution of the syntaxin isoforms among the PM domains of hepatocytes. This was achieved by assaying preparations of vesicles derived from the apical or basolateral PM domains for syntaxin immunoreactivity. For this procedure, PM sheets from rat liver and PM vesicles were prepared as described in the Experimental section. Since the densities of the two vesicle populations are different, they were easily separated by equilibrium centrifugation on linear sucrose gradients and were easily identified by immunodetection of domain-specific antigens [35]. Figure 2 (upper panel) shows the distribution of three of these antigens in the collected fractions. The majority of DPPiV (a resident apical PM protein) distributed to fractions 3–6 while CE9 and HA321 (resident basolateral PM proteins) were enriched in heavier fractions (8–10). Significant amounts of each of these markers were also detected in the pelleted fraction (ppt) which contained PM sheets that were not completely vesiculated. The distributions of the syntaxin isoforms in the same preparation are shown in Figure 2 (lower panel). Of the three syntaxin isoforms assayed, only syntaxin 4 expression was restricted to a single PM domain. Peak syntaxin 4 immunoreactivity was detected in fractions 8–10 coinciding with the distribution of the basolateral PM resident proteins. In contrast, maximal syntaxin 2 immunoreactivity was detected in a broad peak spanning fractions 4–10 suggesting its expression in both PM domains. However, syntaxin 2 is more highly expressed in the apical PM relative to total protein since the heavier (basolateral) fractions contain 4-fold greater total protein. The distribution of syntaxin 3 was more restricted than that of syntaxin 2 and was enriched in fractions 5–9. Although this distribution does not strictly coincide with that of DPPiV (the apical PM protein), it is highly suggestive of an apical PM enrichment of syntaxin 3. The percentage of DPPiV, CE9 and HA321 in fractions 3–6 (the apical PM vesicle peak fractions) were 67, 9 and 7% respectively. Syntaxins 2, 3 and 4 were present at 24, 46 and 10% respectively in the same fractions. Each syntaxin isoform, therefore, has a distinct pattern of expression at the hepatocyte PM. Syntaxin 4 most likely resides at the basolateral PM, and syntaxins 2 and 3 are expressed in both PM domains with an enrichment at the apical PM. The enrichment of syntaxin

Figure 2 Hepatocyte syntaxins differentially distribute among the PM domains

Isolated hepatocyte PM sheets were sonicated and the resulting vesicles were separated on linear sucrose gradients (see the Experimental section). Collected fractions were analysed by SDS/PAGE and quantitative immunoblotting with antibodies against known PM marker proteins (upper panel) or the three syntaxin isoforms (lower panel). The data obtained from densitometric analyses of the immunoreactive species detected in each fraction relative to the total recovered were plotted and indicated as percentage distribution. The blotting profile for each of the proteins is shown in the insets. Upper panel: the majority of the apical PM marker, DPPiV (●), distributed to fractions 4–6 and the basolateral markers, HA321 (○) and CE9 (■), were detected in fractions 8–10. Lower panel: the syntaxin 4 distributions (■) mirrored those obtained for the basolateral markers; however, the profiles obtained for syntaxins 2 (●) and 3 (○) were distinct from the PM markers as well as from each other. Syntaxin 2 distributed partially in the lighter (apical) fractions while syntaxin 3 showed more apical immunoreactivity. The data shown are representative of three to ten experiments.

3 at the apical PM is greater than that observed for syntaxin 2.

Immunolocalization

To confirm and extend the results obtained from biochemical subfractionation, we analysed the distributions of the syntaxin

Figure 3 Syntaxin 2 is localized to both the apical and basolateral domains in purified PM sheets

Isolated PM sheets (~ 0.1 mg) were absorbed on to glass coverslips, fixed with methanol for 5 min at -20°C and processed for indirect immunofluorescence. Panels a, b and c are phase images of the PM sheet preparations. Panels a', b' and c', show the syntaxin 2 staining patterns. The PM sheets were double labelled for HA4 (a''), HA321 (b'') or ZO-1 (c''). HA4, an apical PM protein, was localized strictly to the bile canalicular area, whereas HA321 (a basolateral PM marker) staining was excluded from these membranes. The tight junctions, indicated by ZO-1 staining, were detected in narrow regions adjacent to the canalicular membranes. The syntaxin 2 staining patterns were partially coincident with all three marker proteins; the most intense fluorescence was observed at the bile canalicular domain.

isoforms in rat liver PM sheets and semi-thin cryosections by indirect immunofluorescence. Only the anti-syntaxin 2 and 4 antibodies proved useful for immunodetection at this level and so syntaxin 3 was omitted from this analysis.

Isolated PM sheets

The data presented in Figure 2 indicated that each syntaxin isoform has a distinct pattern of expression among the hepatocyte

Figure 4 Syntaxin 4 is expressed predominantly at the basolateral domain in isolated PM sheets

The PM sheets were prepared and stained as described in the legend for Figure 3 except that antibodies to syntaxin 4 were used. Syntaxin 4 staining was detected mainly at the basolateral PM domain (compare a', b' and c' with HA321 staining in panel b'').

PM domains. To define these patterns morphologically, we examined the distributions of syntaxin 2 and 4 in isolated PM sheets by indirect immunofluorescence. PM sheets retain both the apical and basolateral domains in near-normal surface area ratios as well as a full complement of intercellular junctions and subplasmalemmal cytoskeleton network [34], providing a useful

preparation to examine PM-associated molecules. In addition, apical and basolateral PM antigens as well as tight junctional components exhibit distinct staining patterns in the PM sheets. As shown in Figures 3(a'') and 4(a''), the relatively intense staining for HA4 (an apical PM protein) was restricted to the bile canaliculi membranes in an evenly distributed pattern.

Figure 5 Syntaxins 2 and 4 are expressed at both PM domains in intact hepatocytes

Extracted rat liver semi-thin cryosections were processed for indirect immunofluorescent detection of syntaxin 2 (a' syn 2), syntaxin 4 (b' syn 4) and HA4 (a'' and b''). The phase images of the liver sections are shown in panels a and b. Syntaxin 2 staining was observed mainly at the bile canaliculi as indicated by colocalization with HA4 (compare a' and a''). Syntaxin 2 was also detected at the basolateral surfaces, but to a lesser extent. Syntaxin 4 was detected in both PM domains. However, in this case, the basolateral staining was more intense than the staining at the apical domain. BC, bile canaliculus; N, nucleus.

In contrast, staining for HA321 (a basolateral PM protein) was excluded from the canicular structures and was detected in the surrounding membranes as a more diffuse and less intense signal (Figures 3b'' and 4b''). The tight junction protein, ZO-1, was

detected in regions immediately adjacent to (outside) the apical PM in a ring-like pattern, indicating the location of the junctional complexes that form the barrier between the PM domains (Figures 3c'' and 4c''). The staining patterns observed for syntaxin

4 confirmed its basolateral PM localization (Figure 4a'–c'). As for HA321 (Figure 4b''), syntaxin 4 staining was excluded from the bile canaliculi and was detected only in the surrounding membranes. The syntaxin 2 expression patterns were also confirmed by immunofluorescent detection (Figures 3a'–c'). Staining was observed in both the canalicular structures as well as the surrounding PM, mirroring the combined patterns of HA4 and HA321. The relative intensity of staining in the apical PM was greater than that of the surrounding membranes, consistent with its enrichment in the lighter fractions of the PM domain gradients. When compared with the ZO-1 staining patterns, it appeared that syntaxin 2 was also present in the regions that contain tight junctions (compare Figures 3c' and c''). Whether syntaxin 2 is a component of tight junctions is unknown (see the Discussion section).

Rat liver sections

The next stage of our analysis was to examine the distributions of syntaxins 2 and 4 in intact cells. Since syntaxin immunofluorescence is not detectable in conventionally fixed liver sections, we used extracted liver preparations. As shown in Figure 5, both syntaxins appeared to be expressed predominantly in hepatocytes, with little or no staining observed in sinusoidal lining cells. Consistent with the results described above, syntaxin 2 was detected at the hepatocyte PM and in both domains (Figure 5a'). As observed in isolated PM sheets, the signal was much more pronounced at the apical PM (compare syntaxin 2 with HA4 staining in Figures 5a' and a'' respectively). As expected, syntaxin 4 was detected at the basolateral PM (Figure 5b') but, surprisingly, staining was also detected in regions that were positive for HA4 (compare Figures 5b' and b''). The relative intensity of syntaxin 4 staining at the apical PM was highly dependent on the degree of extraction with the more highly extracted regions yielding a more intense signal (C. Finnegan and H. Fujita, unpublished work). The basolateral PM signal, however, remained consistently bright. Given the disparity of the syntaxin 4 distributions in isolated PM sheets versus liver sections, we examined the specificity of the staining patterns observed in the rat liver sections. When the anti-syntaxin 4 antibodies were preabsorbed with the cognate antigen in its native form (as described for Figure 1), or when denatured and transferred to nitrocellulose, the staining at both domains was abolished (results not shown). The use of mismatched antibody and antigen did not alter the staining pattern of either domain (results not shown). The results presented suggest that the antibodies are specifically recognizing syntaxin 4 in the liver sections. However, the reason(s) for the altered staining patterns is presently unclear (see the Discussion section).

DISCUSSION

Vesicle targeting to the PM in hepatocytes: one possible model

Much attention has been directed lately to the involvement of the SNARE family of proteins in conferring specificity to vesicle targeting events. We are interested in applying this model to PM targeting in polarized epithelial cells, and to hepatocytes in particular. In this context, the target membrane (the PM) is divided into two distinct domains, the apical and basolateral surfaces. How are vesicles targeted specifically to these domains? If the t-SNAREs (syntaxins) are conferring specificity to the targeting event, one would predict that hepatocytes encode two PM-associated syntaxins, whose expression patterns are restricted to a single PM domain. In the present study, we have examined this possibility and report that rat hepatocytes express three endogenous PM-associated syntaxin isoforms (syntaxins 2,

3 and 4). Quantitative immunoblotting revealed that all three syntaxin species are relatively abundant in rat liver. Biochemically, each of the isoforms was observed predominantly at the hepatocyte PM with overlapping but distinct patterns of expression among the PM domains. When examined morphologically, syntaxins 2 and 4 also exhibited complex expression patterns at the hepatocyte PM. With this information, we were able to test proposed models for the events that confer specificity to vesicle targeting and further predict possible mechanisms.

Quantitative analysis

As shown in Table 1, syntaxins 2, 3 and 4 are fairly abundant molecules in rat hepatocytes, suggesting they may be functioning stoichiometrically in the putative SNARE complex, i.e., each vesicle docking event requires interactions with a syntaxin isoform. Furthermore, from measurements of bulk fluid movement from the basolateral to apical environments in hepatocytes, it has been estimated that approx. 600–850 vesicles (~100 nm diam.)/min are delivered to the apical PM [5]. High concentrations of target membrane receptors at the PM are likely to be required to handle such a heavy vesicle targeting load. This might also explain why two syntaxin isoforms are present at the apical PM. Not only do they together provide sufficient numbers of docking sites, but they may also serve as specific receptors for different vesicle types.

We have also determined that rat spleen and kidney express high levels of endogenous syntaxins 2, 3 and 4. Based on the results presented here and elsewhere [15,47–54], we assume that they are also predominantly expressed at the PM in these tissues. This suggests that cells in both spleen and kidney are actively delivering vesicles to their surfaces. If these molecules are functioning strictly stoichiometrically, one might predict more enhanced rates or numbers of vesicle targeting events relative to liver, given the elevated concentrations of syntaxin 3. Despite the increased concentrations of syntaxins in spleen and kidney, they share a similar molar ratio of Munc-18/syntaxin species with liver. This suggests that the regulation of vesicle fusion provided by these interactions is conserved among tissues and may be functioning stoichiometrically. At present, however, whether Munc-18 isoforms play a positive or negative regulatory role in vesicle targeting is unknown. Mutational analysis of related proteins in yeast, *Drosophila* and *Caenorhabditis elegans* all implicate Munc-18 species as positive regulators of vesicle fusion at the cell surface [55–60], while assays *in vitro* suggest the opposite [61]. This conundrum awaits further investigation.

Syntaxins 2, 3 and 4 are differentially expressed at the hepatocyte PM

Immunological analysis of fractions obtained by biochemical subfractionation has indicated that syntaxins 2, 3 and 4 are all predominantly expressed at the hepatocyte PM. Each syntaxin isoform had a unique distribution in gradients containing separated populations of apically or basolaterally derived PM vesicles. From these patterns we predict that only syntaxin 4 expression is restricted to a single PM domain, the basolateral. Although found in both PM domains, syntaxins 2 and 3 distributed primarily to the apical PM, with a greater enrichment of syntaxin 3 than of syntaxin 2.

These results were confirmed for syntaxins 2 and 4 by immunofluorescent microscopical examination of isolated PM sheets. Syntaxin 4 staining was restricted to the membranes surrounding the bile canaliculi while syntaxin 2 was detected in both domains. Unlike the other resident apical PM proteins, syntaxin 2 staining seemed to include areas that were also

positive for staining with anti-ZO-1 antibodies. Interestingly, rab3B and 13, ras-like GTPases thought to be involved in vesicle targeting, have been detected in regions at or near tight junctions in hepatocytes [62,63] where, it has been proposed, they are involved in regulating the specific delivery of vesicles containing junctional components to the appropriate site at the PM [63]. Whether syntaxin 2 also participates in this process is unknown, but remains an exciting possibility.

In intact hepatocytes, syntaxins 2 and 4 were also observed by indirect immunofluorescence to distribute differentially between the PM domains. In contrast to PM sheets, syntaxin 4 expression was detected at both the apical and basolateral domains. A likely explanation for these disparate results is the differing accessibilities of syntaxin 4 epitopes to the polyclonal antibodies in each of the preparations. The conditions for the isolation of PM sheets are rather gentle and a whole host of peripheral-membrane proteins retain their associations with the PM during purification [34]. In contrast, the rat liver sections must be extracted before fixation in order to detect the syntaxin isoforms. This extraction may be releasing peripherally-associated proteins that are normally associated with syntaxin 4 so that detection of newly exposed epitopes at the apical domain is possible. In fact, detection of syntaxin 4 at the apical PM was observed to increase as the sections were more heavily extracted (C. Finnegan and H. Fujita, unpublished work). Alternatively, the syntaxin 4 molecules may be redistributed upon hepatocyte extraction. A similar explanation reconciles the absence of syntaxin 4 in fractions containing apical PM vesicles when biochemical methods are used. However, because it is difficult to determine the absolute recoveries of proteins in the domain gradient fractions, we cannot exclude or examine that possibility.

The distribution of syntaxin 4 at the PM appears to vary most among the isoforms examined in polarized epithelial cells. In kidney collecting duct cells, syntaxin 4 was found to distribute solely to the apical PM [49]. However, in pancreatic acinar cells and when overexpressed in Madin–Darby canine kidney (MDCK) cells, syntaxin 4 expression was restricted to the basolateral domain [53,54]. Our results strongly argue for the expression of syntaxin 4 in both PM domains in hepatocytes. Syntaxin 2 distributions are similarly inconsistent among tissues. In the polarized hepatic WIF-B cells, syntaxin 2 is expressed solely at the apical PM (H. Fujita, unpublished work), whereas in hepatocytes, pancreatic acinar cells and MDCK overexpressing transfectants syntaxin 2 is found in both domains [53,54]. Only syntaxin 3 distributions appear to be more consistent with apical location, as observed in hepatocytes, pancreatic acinar cells and transfected MDCK cells.

The tremendous variation in the syntaxin PM distributions both among tissue types and among modes of detection (for example, morphological versus biochemical) underscores the importance of using multiple approaches for determining the locality within a given cell type. Our report here serves as the first such example to examine endogenous syntaxin PM distributions quantitatively, biochemically and morphologically in a single cell type. Our observations and those of others clearly indicate that the factors regulating the patterns of syntaxin PM expression are complex. Only upon further examination of the complexities that surround these proteins (e.g. interactions with other proteins, post-translational modifications, biochemical properties) will we understand these seemingly paradoxical results.

Syntaxins are also associated with intracellular membranes

Although predominantly expressed at the hepatocyte PM, significant proportions of syntaxin 3 were found in association with

intracellular membranes. Over 25% of syntaxin 3 was detected in the 'load' fraction (fraction D) of the Golgi flotations. This fraction contains mainly cytosolic proteins, however numerous membrane proteins have also been detected, including those associated with the PM, Golgi apparatus and transport vesicles [37]. These so-called PM syntaxins have been shown to be associated with intracellular compartments in other cell types as well. Syntaxins 2, 3 and 4 have all been shown to co-purify with phagosomes isolated from J774 cells (murine macrophage cell line; [50]). Since phagosomal maturation occurs through fusion with specifically targeted intracellular vesicles, it has been hypothesized that the syntaxins are mediating vesicle recognition at the phagosomal membrane [50]. In MDCK cells overexpressing syntaxin 3 isoforms, a substantial proportion of the syntaxin 3 population was found in association with lysosomes [53]. Endogenous syntaxin 3 was also observed to co-localize with zymogen granules in pancreatic acinar cells [50]. A substantial proportion of syntaxin 4 (33% of total) has also been observed in microsomal preparations from 3T3-L1 adipocytes [52]. Within these fractions, syntaxin 4 appears to be at least partially associated with GLUT4-containing vesicles. Syntaxin 1 isoforms have been found in association with chromaffin granules [64], synaptic vesicles [65–67] and microvesicles from rat gerbil pinealocytes [68]. The functions of these associations are not known but it has been hypothesized that in neurons, syntaxin 1 is not only regulating the docking/fusion of synaptic vesicles at the cell surface but also regulating their recycling [67]. It is compelling to speculate that syntaxin 3 is performing an analogous function in hepatocytes and regulating the itineraries of transport vesicle trafficking.

Syntaxins and the SNARE hypothesis

The data presented here challenge our initial model of the role syntaxins 2, 3 and 4 play in targeting vesicles to the cell surface in polarized epithelial cells. Our simple prediction maintained that if syntaxins are responsible for conferring specificity of vesicle targeting to the correct PM domain, their expression would be limited to a single domain. Although the syntaxins were differentially distributed among the PM domains, their expressions were not restricted and the patterns were overlapping. Similar observations have been made for syntaxins in neurons [65,66,69]. Syntaxin 1 was originally proposed to specify the docking site of synaptic vesicles at the synaptic terminal active zones, but immunolocalization studies revealed that syntaxin 1 expression was not only detected at the synaptic terminal PM but also at the axonal PM. Furthermore, there is no definitive evidence for syntaxin 1 expression at the active zone [65,66,69].

These observations, in combination with ours, are not consistent with syntaxins functioning as the sole determinants of specific targeting to the PM. Other factors, either alone or in concert with the syntaxins, are likely to be involved in regulating targeting specificity. Some candidates include the Munc-18 isoforms, SNAP-25 or SNAP-23 (synaptosomal-associated proteins of 25 or 23 kDa) molecules (other putative t-SNAREs) and rab GTPases. Alternatively, specificity is not provided by a single protein but rather by multimeric complexes, whose specific members are in particular combinations. In this case, the different syntaxin isoforms are necessary but not sufficient for conferring specificity to vesicle delivery in polarized epithelial cell surfaces. The answer awaits further investigation.

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