O-glycosylation is essential for intracellular targeting of synaptotagmins I and II in non-neuronal specialized secretory cells

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Summary

We have examined the trafficking of synaptotagmin (Syt) I and II in the mast cell line rat basophilic leukemia (RBL-2H3). We demonstrate that both Syt I and Syt II travel through the plasma membrane and require endocytosis to reach their final intracellular localization. However, N- or C-terminal tagging of Syt II, but not of Syt I, prevents its internalization, trapping the tagged protein at the plasma membrane. Furthermore, a chimeric protein comprising a tagged luminal domain of Syt II fused with the remaining domains of Syt I also localizes to the plasma membrane, whereas a chimera consisting of tagged luminal domain of Syt I fused with Syt II colocalizes with Syt I on secretory granules. We also show that endocytosis of both Syt I and Syt II is strictly dependent on O-glycosylation processing, whereby O-glycosylation mutants of either protein fail to internalize and remain at the plasma membrane. Our results indicate that the luminal domains of Syt I and Syt II govern their internalization capacity from the plasma membrane and identify O-glycosylation as playing a crucial role in Syt trafficking in non-neuronal secretory cells.

Key words: Sorting, Glycosylation, Synaptotagmin, RBL-2H3

Introduction

Synaptotagmins comprise a family of structurally related proteins that are highly conserved through evolution. Synaptotagmins are present in all tissues tested so far, although they may exhibit a tissue specific isoform distribution. Members of this family are type I integral membrane proteins comprising two C2 domains (C2A and C2B) in their cytosolic region (reviewed by Yoshihara et al., 2003; Sudhof et al., 1996). Neuronal synaptotagmins, including Syt I, II, III and VII have been implicated as Ca^{2+} sensors in the control of neurotransmission (Sugita et al., 2002). Specifically, Syt I and Syt II localize to synaptic vesicles, where they mediate Ca^{2+}-triggered exocytosis (Brose et al., 1992; Geppert et al., 1991), whereas Syt III, Syt V and Syt VII localize to the synaptic membrane and function as complementary Ca^{2+} sensors (Sugita et al., 2002). In a similar fashion, Syt V and Syt IX localize to secretory granules and function as Ca^{2+} sensors in neuroendocrine PC12 cells (Saegusa et al., 2002; Fukuda et al., 2002; Fukuda, 2004), whereas Syt III and Syt VII function as plasma membrane Ca^{2+} sensors (Shin et al., 2002). In contrast, non-neuronal synaptotagmins acquire diverse intracellular localizations, which are closely linked with their specific functions. For example, unlike the plasma membrane localization of neuronal Syt III and Syt VII, their non-neural counterparts respectively localize to early endosomes and lysosomes where they function to regulate endocytic trafficking (Grimberg et al., 2003; Martinez et al., 2000). Syt II, which localizes to synaptic vesicles in neurons, resides at late endosomes/lysosomes in cells of the immune system including mast cells (Baram et al., 1999) and neutrophils (Lindmark et al., 2002) whereas Syt IX localizes to the endocytic recycling compartment (Haberman et al., 2003). To add even more complexity, Syt I, which localizes to synaptic vesicles or synaptic-like microvesicles (SLMV) in neuronal cells is targeted exclusively to the plasma membrane when transfected into CHO, HEK293 or BHK21 cells (Feany et al., 1993; Han et al., 2004). However, in non-neuronal secretory cells, such as the mast cells (Baram et al., 1998), β cells (Lang et al., 1997) or the parotid (Levius et al., 1997), Syt I localizes to secretory granules. These data therefore indicate that synaptotagmins comprise sorting signals, which are recognized in a cell type-specific fashion, presumably by cell-specific machineries, to direct them to their final intracellular destinations. Therefore, internalization from the plasma membrane might serve a crucial step in defining the final localization of these homologues. Indeed, both internalization signals and inhibitory signals co-embedded within synaptotagmin C2 domains as well as N-glycosylation of the luminal domain have been proposed to govern synaptotagmin internalization (Han et al., 2004; Dasgupta and Kelly, 2001; Jarousse and Kelly, 2001). Alternatively, in non-neuronal secretory cells, synaptotagmins might be delivered to their secretory granule destination directly from the trans-Golgi network (TGN) bypassing the plasma membrane and the need for internalization. In the present study, we have addressed these possibilities by monitoring the biosynthetic pathways of

Syt I and Syt II in cultured mast cells (rat basophilic leukemia, RBL-2H3). We demonstrate that both Syt I and Syt II travel through the plasma membrane during their biosynthetic pathways and therefore require internalization to reach their final intracellular localizations. We show that the luminal domain dictates whether Syt will internalize or remain at the plasma membrane and identify O-glycosylation as the posttranslational modification, which is ultimately required to allow internalization from the plasma membrane.

Materials and Methods

Antibodies

Antibodies used include monoclonal anti-HA (clone 16B12, Babco, Berkeley antibodies company); polyclonal anti-HA (Y-11, Santa Cruz Biotechnology); monoclonal anti-T7 (Novagen); monoclonal antibodies directed against the N-terminal region of Syt II (a generous gift from M. Takahashi, Mitsubishi-Kasei Institute of Life Sciences, Tokyo, Japan); polyclonal anti-C2A-Syt IX antibodies (Fukuda et al., 2002); monoclonal anti-serotonin antibodies (Dako, Denmark); polyclonal antibodies directed against Syt II (Santa Cruz Biotechnology); polyclonal antibodies directed against $G\alpha i_2$ (AS10, a generous gift from A. Spiegel, NIH, Bethesda, MD); monoclonal anti-GFP antibodies (Roche Diagnostics); horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG and Rhodamine, FITC, Cy2- or Cy3-conjugated donkey anti-rabbit, anti-mouse or anti-goat IgG (Jackson Research Laboratories, West Grove, PA).

Reagents

Tunicamycin, chloroquine and DNase I were from Sigma-Aldrich; T4 DNA Ligase and *N*-glycosidase F were purchased from Roche Diagnostics. Texas Red-conjugated human Tfn was obtained from Molecular Probes (Eugene, OR).

Cell culture

RBL-2H3 (hereafter referred to as RBL) cells were maintained in adherent cultures in DMEM supplemented with 10% FCS in a humidified atmosphere of 5% CO_2 at 37°C.

DNA constructs

Plasmids used in this study include pEF-T7-Syt I, pEF-BOS-T7-Syt I (T26/A) and pEF-BOS-T7-Syt I (T15/16/A) (Fukuda and Mikoshiba, 2000; Fukuda, 2002) and pcDNA3-Syt II (Baram et al., 1999). HA-Syt II was produced by PCR amplification of Syt II cDNA using the following primers: 5′-CTAGCTAGCATGAGAAACATCTTCAAGAGGA-3′ (sense) and 5′-CCGCTCGAGCTACTTGTTCTTGCCCAGA-3′ (antisense). The PCR product was cut with *Nhe*I and *Xho*I and ligated in-frame into a pcDNA3-HA vector, kindly provided by Y. Zick (Weizmann Institute of Science, Israel). To generate HA-tagged Syt I/Syt II, Syt I N-terminal domain corresponding to amino acids 1-180 was amplified using primers: 5′-CTAGCTAGCATGGTGAGTGCCAGTCATCCT-3′ (sense) and 5′-AGACTTTGACGTATGGATCCGATGTA-3′ (antisense). A *Bam*HI restriction site was introduced by inserting a silent mutation. These primers were used to amplify by PCR the desired domain, using Syt I cDNA as a template. Syt II C-terminal domain corresponding to amino acids 183-422 was generated using primers: 5′-TGGCACGTCGGATCCTTACGTCAAA-3′ (sense) which again includes a *Bam*HI restriction site, introduced by inserting a silent mutation and 5′-CCGCTCGAGCTACTTGTTCTTGCCCAGA-3′ (antisense), using Syt II full-length cDNA as template. PCR products were digested with *Nhe*I/*Bam*HI and *Bam*HI/*Xho*I, respectively, ligated to produce the chimeric *Nhe*I-Syt I/Syt II-*Xho*I cDNA, which was then subcloned into the pcDNA3-HA vector, digested with *Nhe*I/*Xho*I.

HA-Syt II/Syt I chimeras (A-C) were produced by amplifying the region corresponding to Syt II amino acids 1-60 (A); 1-86 (B) and 1-183 (C) using the sense primer, 5′-CTAGCTAGCATGAGAAACATCTTCAAGAGGA-3′ and the antisense primers, 5′-CCACGGTGGCAATGGGATCTTGTTGATCTCATT-3′ (A); 5′-GAACAAACATTTCTTACAGATGCAGAAGCA 3′ (B); 5′-TTTGACGTAAGGATCCGACGTGCCA-3′ (C). A *Bam*HI restriction site was introduced to the latter antisense primer by inserting a silent mutation. These primers were used to amplify the desired domains by PCR, using Syt II cDNA as a template. The region corresponding to Syt I amino acids 56-403 was amplified using sense primer 5′-CCATTGCCACCGTGGGCCTTAATAG-3′. The region corresponding to Syt I amino acids 80 to 403 was amplified using sense primer 5′-GTCTGTAAGAAATGTTTGTTCAAAAAGAAAAAC-3′. The region corresponding to Syt I amino acids 180-403 was amplified using primers 5′-TACATCGGATCCATACGTCAAAGTCT-3′ (sense) that includes a *Bam*HI restriction site, again introduced by inserting a silent mutation and the antisense primer 5′-CCCTCGAGTTACTTCTTGACAGCCAGCAT-3′. PCR products were digested with *Nhe*I-*Xho*I, *Nhe*I/*Bam*HI or *Bam*HI/*Xho*I and ligated to produce the chimeric *Nhe*I-SytII/SytI-*Xho*I cDNAs, which were then subcloned into the pcDNA3-HA vector.

To generate Flag-Syt II-GFP the sequences encoding the Flag tag were inserted into the 5′-end of Syt II cDNA, whereas the 3′-end was ligated to the 5′-end of GFP cDNA to encode a fusion protein. A glycine linker was inserted between Syt II and GFP as described previously (Saegusa et al., 2002). The tagged cDNAs were subcloned into pShooter vector (Invitrogen, San Diego, CA) and verified by DNA sequencing. Syt II-GFP was produced by digesting the Flag-Syt II-GFP construct with *Bam*HI/*Not*I. Syt II (T17/18/19/A) was created using QuikChange™ site-directed mutagenesis kit (Stratagene) with the sense primer, 5′-ATTGTGGCTCCGGCCGCCGCCGCAGCCACAATGCCTCTG-3′ and antisense primer, 5′-CAGAGGCATTGTGGCTGCGGCGGCGGCCGGAGCCACAAT-3′. cDNAs were subcloned into pcDNA3 and verified by DNA sequencing.

Cell transfection

Stable and transient transfections of RBL cells were performed as previously described (Grimberg et al., 2003; Baram et al., 1999; Haberman et al., 2003).

Subcellular fractionation of RBL cells

Cell fractionation on linear sucrose gradients was performed as previously described (Grimberg et al., 2003; Baram et al., 1999; Haberman et al., 2003).

Cell lysates

RBL cells (1×10^7) were lysed in lysis buffer comprising 50 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM $NaVO_4$, 1 mM PMSF and a cocktail of protease inhibitors (Boehringer Mannheim, Germany). Following a 10-minute incubation on ice, lysates were cleared by centrifugation at 9000 ***g*** for 15 minutes at 4°C. The cleared supernatants were mixed with 5× Laemmli sample buffer, boiled for 5 minutes, and subjected to SDS-PAGE and immunoblotting.

N-glycosidase F treatment

Cells were lysed in lysis buffer comprising 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM PMSF and a cocktail of protease inhibitors and boiled for 5 minutes with 1% SDS.

Following centrifugation at 9000 ***g*** for 15 minutes at 4°C, aliquots of cleared supernatants were divided into two tubes and incubated with or without 0.6 U *N*-glycosidase F for 18 hours at 37°C, prior to SDS-PAGE and immunoblotting (Barzilay et al., 2004).

Laser confocal microscopy

RBL cells (2×10^5 cells/ml) were grown on 12-mm diameter glass coverslips. For immunofluorescence processing cells were washed twice with PBS and fixed for 15 minutes at room temperature in 3% paraformaldehyde/PBS. Cells were subsequently washed three times with PBS and permeabilized for 30 minutes with PBS, 5% FCS, 2% BSA, 0.1% Triton X-100. Cells were subsequently incubated for 1 hour at room temperature with the primary antibodies diluted in PBS, 5% FCS, 2% BSA, 0.1% Triton X-100, washed three times in PBS and incubated for 30 minutes in the dark with the appropriate secondary antibody (Cy2-, Cy3-, Rhodamine- or FITC-conjugated donkey anti-rabbit, anti-mouse or anti-goat IgG, at 1:200 dilution in PBS, 5% FCS, 2% BSA, 0.1% Triton X-100). Coverslips were subsequently washed in PBS and mounted with Gel Mount mounting medium (Biomedica, Foster city, CA). Samples were analyzed using a Zeiss laser confocal microscope (Oberkochen, Germany).

Results

Differential targeting of Syt I and Syt II in RBL cells

Previously, we demonstrated that fractionation on linear sucrose gradients of Syt I-transfected RBL cell homogenates results in the segregation of Syt I and Syt II, whereby Syt I cofractionates with histamine-containing secretory granules and Syt II with amine-free secretory lysosomes (Baram et al., 1999; Baram et al., 1998). In an effort to identify the domain that contains the differential sorting determinants, we generated chimeric proteins containing the N-terminal, transmembrane and juxtamembrane domains of Syt I (amino acids 1-180) or Syt II (amino acids 1-183) fused with the remaining cytosolic domain of the alternative Syt (see Fig. 1). To be able to distinguish these proteins from endogenous Syt II, we tagged the chimeras with an HA epitope at their N-termini. This choice was based on our previous findings that epitope tagging of Syt homologues at their N-terminus does not interfere with their glycosylation or any other characteristics (Fukuda and Mikoshiba, 2000). As controls and because the antibodies directed against Syt II fail to detect Syt II by immunofluorescence, we also prepared N-terminally tagged versions of the wild-type (WT) proteins and examined their cellular distributions at steady-state.

Staining of T7-Syt I-transfected cells with an antibody directed against the T7 tag demonstrated that the majority of T7-Syt I acquired a vesicular intracellular localization (Fig. 2A, a), although some membrane staining could also be detected. However, to our surprise, HA-Syt II localized exclusively to the plasma membrane (Fig. 2A, b). Membrane localization of HA-Syt II does not seem to result from gross overexpression of this protein as HA-Syt II localized exclusively to the plasma membrane also when transfected into Syt II-knocked down cells (data not shown). Finally, as already mentioned, the antibodies directed against Syt II fail to detect Syt II by immunofluorescence, however, we have noticed that an antibody, which was raised against the C2A domain of Syt IX and fails to recognize any endogenous proteins in RBL cells, does label over-expressed Syt IX or Syt II. Thus, these antibodies specifically recognize Syt II in cells that overexpress only Syt II and we used them to examine the intracellular localization of overexpressed, non-tagged Syt II by immunofluorescence. This analysis revealed that in sharp contrast to the membrane localization of the N-terminally tagged Syt II, non-tagged Syt II displayed an intracellular vesicular localization (Fig. 2A, c). To substantiate these results, we also fractionated T7-Syt I or HA-Syt II-expressing RBL cells on continuous sucrose gradients and analyzed the fractions for the presence of Syt I or Syt II. Such analyses revealed that the majority of T7-Syt I migrates with high-density fractions (fractions 16-23) and co-fractionates with the second peak of β-hexosaminidase activity, which we have previously identified (Baram et al., 1999) as secretory granules (Fig. 2B). In contrast, HA-Syt II migrated with lighter fractions (fractions 12-19), which also contained the G-protein Gαi2 (Fig. 2B), which we have previously shown to localize at the plasma membrane (Baram et al., 1999; Grimberg et al., 2003). This distribution was strikingly different from that of non-tagged which cofractionates with the first peak of β-hexosaminidase activity (Baram et al., 1999). Taken together these results indicate that although N-terminal tagging of Syt I does not affect its cellular localization, N-terminally tagged Syt II fails to reach its correct intracellular localization.

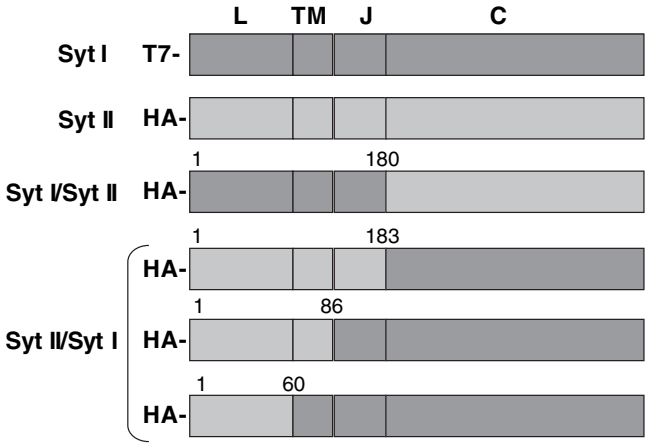

Fig. 1. Schematic representation of constructs used in this study. The diagrams show the positions of the chimeric proteins comprising Syt I and Syt II. C, cytosolic domain; HA, hemagglutinin; J, juxtamembrane domain; L, luminal domain; T7, 11 residue peptide (MASMTGGQQMG) derived from the major capsid protein of the T7 phage; TM, transmembrane domain.

Intracellular distribution of chimeric Syt I and Syt II

Examining the cellular distribution of the N-terminally tagged Syt I and Syt II chimeric proteins revealed that HA-Syt I/Syt II acquired a vesicular intracellular localization (Fig. 3A, a). Moreover, staining of double transfected cells has demonstrated that HA-Syt I/Syt II colocalizes with T7-Syt I in intracellular vesicles (Fig. 3A, a-c). Double staining of HA-Syt I/Syt II and the secretory granule marker serotonin established a clear colocalization, therefore confirming that both T7-Syt I and the chimeric protein HA-Syt I/Syt II are directed to secretory granules (Fig. 3B, a-c). In stark contrast, the chimeric protein HA-Syt II/Syt I, which comprises the first 183 amino

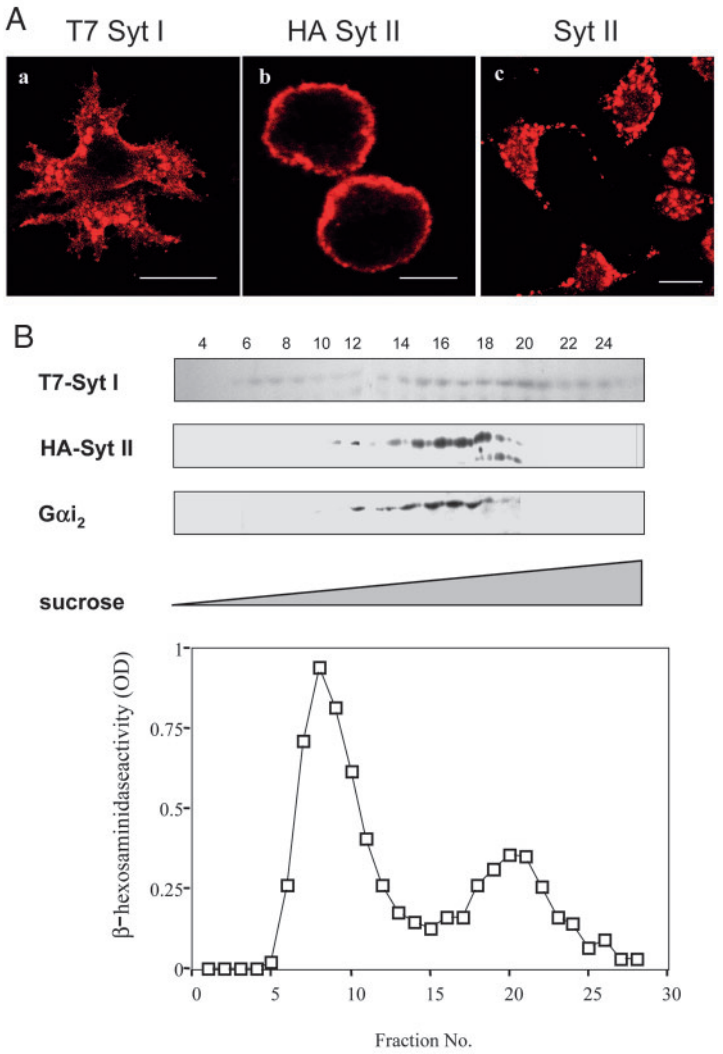

Fig. 2. Cellular localization of Syt I and Syt II. (A) RBL cells transiently transfected with T7-Syt I, HA-Syt II or stably transfected with Syt II were grown on glass coverslips for 24 hours. Cells were subsequently labeled with anti-T7 monoclonal antibodies (a), anti-HA monoclonal antibodies (b) or anti-C2A-Syt IX polyclonal antibodies (c) followed by Cy3-conjugated donkey anti-mouse or Rhodamine-conjugated donkey anti-rabbit IgG. Cells were processed for immunofluorescent staining and visualized by confocal microscopy. (B) Subcellular fractionation of T7-Syt I or HA-Syt II-transfected RBL cells. Cell homogenates derived from RBL cells transfected with either T7-Syt I or HA-Syt II cDNA were fractionated on continuous sucrose gradients as described previously (Baram et al., 1999). Fractions were collected from the top, subjected to SDS-PAGE and immunoblotted with anti-T7, anti-HA or anti-$Gi_2\alpha$ antibodies. Fractions were also examined for β-hexosaminidase activity (presented as OD at 405 nm). Bar, 10 μm.

acids of Syt II, localized exclusively to the plasma membrane (Fig. 3C, a).

To better define the domain that includes the dominant sorting signals, we also generated chimeric proteins comprising the luminal and transmembrane domain (amino acids 1-86) or only the luminal domain (amino acids 1-60) of Syt II fused to the juxtamembrane and remaining cytosolic domain of Syt I (Fig. 1). These chimeras also localized exclusively to the plasma membrane (Fig. 3C, b and c), indicating that information embedded within the luminal domain dictates the plasma membrane localization of Syt II.

To corroborate these results further, we have also studied the cellular distribution of these chimeric proteins by fractionation on sucrose gradients. Consistent with the data obtained by immunofluorescence, HA-Syt I/Syt II migrated at fractions 16-25, where it cofractionated with the second peak of β-hexosaminidase activity that correspond to secretory granules (Fig. 4). HA-Syt II/Syt I migrated at fractions 14-19, which include the plasma membrane (Fig. 4). Notably, all these constructs were analyzed both in transiently and in stably transfected RBL cells and no differences in localization were detected. These results therefore indicate that sorting signals present in the luminal/transmembrane/juxtamembrane domains of Syt I and Syt II dictate whether or not these proteins acquire an intracellular localization or remain at the cell surface.

The biosynthetic pathways of Syt I and Syt II

Trafficking of Syt I and Syt II to secretory granules or lysosomes might follow a direct route from the TGN, or an indirect route including TGN transport to the plasma membrane and internalization into endosomes prior to delivery to their final destinations. Therefore, N-terminal tagging might either deviate Syt II from its direct route of trafficking to an indirect one, or it might block internalization of Syt II, which otherwise occurs. To distinguish between these possibilities, we analyzed the biosynthetic pathways of T7-Syt I and HA-Syt II. To this end, 1 hour after transfection with the desired cDNA, cells were incubated for 2 hours at 20°C, in the presence of CHX (100 μg/ml), in order to block protein synthesis but allow accumulation of the synthesized proteins in the Golgi (Matlin and Simons, 1983; Griffiths et al., 1985). Because prolonged incubation with CHX displayed cell toxicity, at the end of incubation at 20°C, the medium was replaced and the cells delivered to 37°C for monitoring their localization by immunofluorescence. As expected at time zero, both proteins are detected at a perinuclear localization, reminiscent of the Golgi (Fig. 5a,d). However, 1 hour after transfer to 37°C, plasma membrane localization is clearly detected whereas at steady state, 24 hours post transfection, both T7-Syt I and non-tagged Syt II acquired an intracellular vesicular distribution (Fig. 5c,f).

Previous studies have demonstrated that cycling through the plasma membrane allows proteins to internalize exogenous antibodies directed against luminal domains. Therefore, we investigated whether T7-tagged Syt I could internalize exogenous anti-T7 antibodies and direct them to the secretory granules. Indeed, following binding and 24 hours' incubation at 37°C, the exogenously added antibodies were clearly localized to intracellular vesicles (Fig. 5g).

We also examined the biosynthetic pathway of HA-Syt I/Syt II in the absence or the presence of inhibitors of endocytosis. At time zero this chimera was found distributed between the Golgi and the plasma membrane (Fig. 6a). However, following a 3-hour incubation at 37°C, in the absence of inhibitors, this chimera acquired a vesicular structure (Fig. 6b). In contrast,

when endocytosis was blocked by either chloroquine (100 μM) or hypertonic sucrose (0.45 M), HA-Syt I/Syt II remained distributed between the plasma membrane and Golgi (Fig. 6c) or solely at the plasma membrane (Fig. 6d), respectively. These results therefore suggest that synaptotagmins are delivered to their intracellular localizations via a pathway that includes the plasma membrane and endosomes as intermediates. Therefore N-terminal tagging of Syt II prevents its internalization from the plasma membrane during its biosynthetic route of trafficking.

Intracellular distribution of Syt II-GFP

Because N-terminal tagging of Syt II was found to affect its cellular localization, we created a chimera of Syt II fused at its C-terminal end with GFP and compared its localization with that of Flag-Syt II-GFP. We expected to find Flag-Syt II-GFP at the plasma membrane and Syt II-GFP at an intracellular localization. However, surprisingly, both fusion proteins, irrespective of the N-terminal tag, localized exclusively to the plasma membrane (Fig. 7A). To make sure that the plasma membrane location of tagged Syt II was not due to general inhibition of endocytosis, we explored the ability of tagged Syt II-transfected cells to endocytose transferrin (Tfn). These experiments have demonstrated that overexpression of tagged Syt II does not impair endocytosis as the cells are capable of internalizing exogenously added Tfn (Fig. 7B).

Both N- and C-terminally tagged Syt II are N-glycosylated

The majority of endogenous and overexpressed non-tagged Syt II migrates as 90 kDa immunoreactive proteins on SDS-PAGE, although lower molecular mass proteins of 75 and 65 kDa could also be detected (Fig. 8A). In contrast, the major band present in cells transfected with HA-Syt II cDNA co-migrated with the smaller 75 kDa immunoreactive Syt II protein (Fig. 8A). Syt II-GFP as well as Flag-Syt II-GFP displayed similar sizes, where the largest form acquired an apparent molecular mass of 105 kDa (Fig. 8B). Considering the size of GFP, the molecular mass of Syt II in these tagged forms then also corresponds to 75 kDa. In mast cells (Baram et al., 1999), as well as in neutrophils (Lindmark et al., 2002), Syt II acquires a molecular mass (80-90 kDa) that is higher than that of its neuronal counterpart (~65 kDa). We have postulated that this difference in molecular size might reflect distinct cell-specific posttranslational modifications, which in turn might be involved in cellular targeting (Baram et al., 1999). In this context, protein glycosylation is an attractive candidate. In particular, N-glycosylation was implicated as an essential requirement for internalization of Syt I in neuronal tissue (Han et al., 2004). Consistent with this view, Western blot analyses of cell lysates derived from T7-Syt I-transfected RBL cells has revealed the presence of two proteins: a 53 kDa protein, which probably represents an immature form of Syt I, and a 67 kDa form, which corresponds to its mature, fully glycosylated form (Fig. 8C). In contrast, the Syt I mutants, T7-Syt I(T26/A) and T7-Syt I(T15/16/A), which we have perviously shown to lack N- or O-glycosylation, respectively (Fukuda, 2002), included the 53 kDa immature protein as well as proteins of 61 and 58 kDa, respectively (Fig. 8C). These results therefore confirm that both N- and O-glycosylation affect the molecular size of Syt I. Because Syt II tagging is associated with both a reduced molecular size and failure to

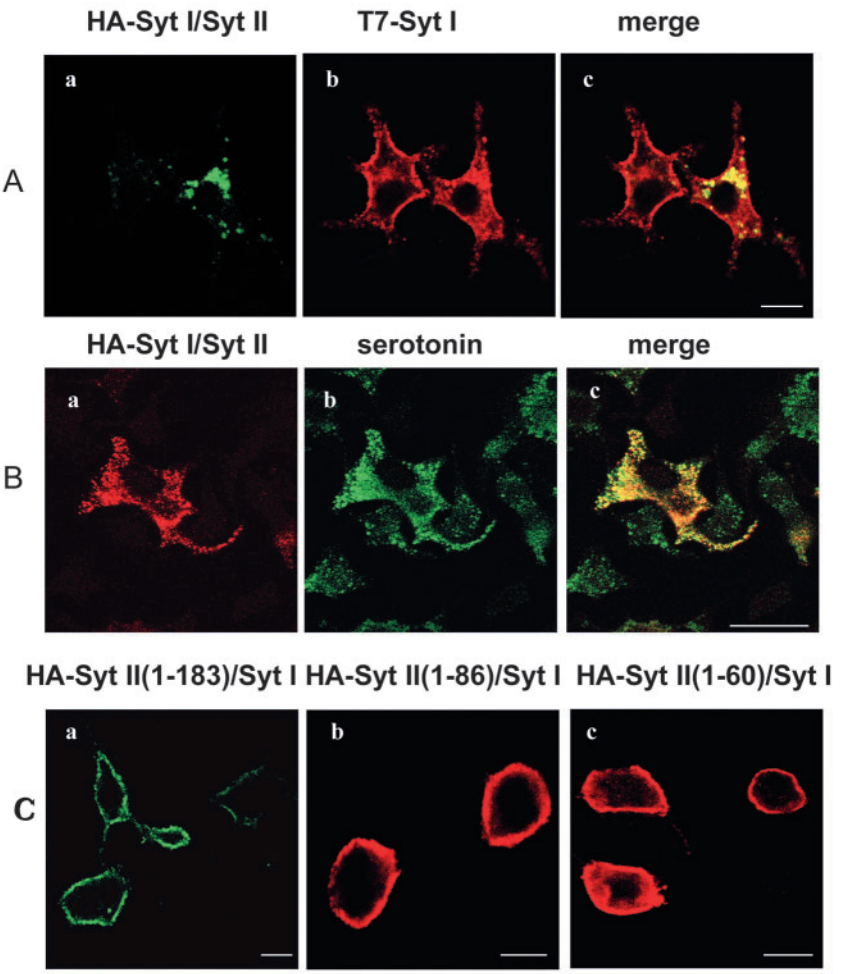

Fig. 3. Visualization of the cellular localization of Syt I and Syt II chimeric proteins. (A) RBL cells transiently double transfected with HA-Syt I/Syt II and T7-Syt I cDNA were grown on glass coverslips and labeled with polyclonal anti-HA and monoclonal anti-T7 antibodies followed by Rhodamine-conjugated donkey anti-mouse and FITC-conjugated donkey anti-rabbit IgG. (B) RBL cells transiently transfected with HA-Syt I/Syt II cDNA were grown on glass coverslips and double stained with polyclonal anti-HA and monoclonal anti-serotonin antibodies followed by Rhodamine-conjugated donkey anti-rabbit and FITC-conjugated donkey anti-mouse IgG. (C) RBL cells, transiently transfected with HA-Syt II (1-183aa)/Syt I (a), HA-Syt II (1-86aa)/Syt I (b) or HA-Syt II (1-60aa)/Syt I (c) cDNA, were grown on glass coverslips and stained with monoclonal anti-HA antibodies. Cells were processed for immunofluorescent staining and visualized by confocal microscopy. Bar, 10 μm.

internalize, we explored the possibility that tagging of Syt II prevents its N-glycosylation and therefore its internalization from the plasma membrane. For this purpose, we investigated how tunicamycin, which is known to block N-glycosylation, and *N*-glycosidase F, which releases all *N*-glycans from protein backbones, affect the molecular mass of tagged Syt II.

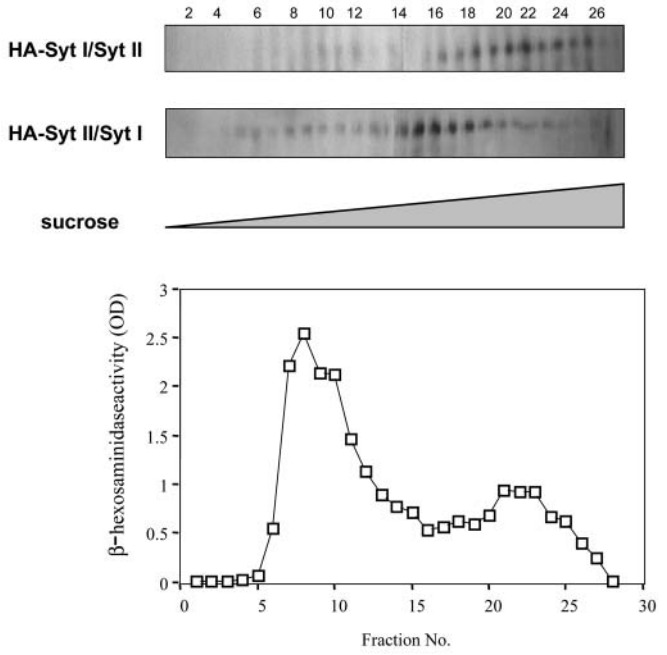

Fig. 4. Subcellular fractionation of HA-Syt I/Syt II- or HA-Syt II/Syt I-expressing RBL cells. Cell homogenates derived from RBL cells transiently transfected with HA-Syt I/Syt II or HA-Syt II(1-183aa)/Syt I cDNA were fractionated on continuous sucrose gradients as described previously (Baram et al., 1999). Fractions were collected from the top, subjected to SDS-PAGE and immunoblotted with anti-HA antibodies. Fractions were also examined for β-hexosaminidase activity (presented as OD at 405 nm).

In the absence of tunicamycin, T7-Syt I and HA-Syt II appeared as two proteins, probably represent fully glycosylated and partially or non-modified proteins (Fig. 9A). Notably, the amount of partially or non-modified proteins appeared to vary between different experiments for reasons that we presently do not understand. The chimeric proteins emerged as single bands (Fig. 9A). Tunicamycin treatment caused a modest but reproducible reduction in the apparent molecular sizes of all mature proteins (Fig. 9A). These results therefore suggested that consistent with our previous results (Fukuda and Mikoshiba, 2000), all the tested proteins are N-glycosylated irrespective of whether they are N-terminally tagged. Furthermore, *N*-glycosidase F treatment reduced the apparent molecular sizes of all proteins tested, consistent with the notion that all are capable of acquisition of N-linked glycosylation (Fig. 9B).

To substantiate these results further we also subjected the GFP-tagged versions of Syt II to *N*-glycosidase F treatment. Such treatment resulted in a reduction in the apparent molecular sizes of both Syt II-GFP and Flag-Syt II-GFP (Fig. 9C). In sharp contrast, the molecular mass of neither Syt II(T34/A)-GFP nor Flag-Syt II(T34/A)-GFP was affected by tunicamycin or *N*-glycosidase F treatment (Fig. 9D,E), consistent with their inability to acquire N-glycosylation. Finally, both wild-type proteins display molecular sizes greater than those of their N-glycosylation mutants (Fig. 9F). Therefore, plasma membrane localization of N- or C-terminally tagged Syt II or Syt II/Syt I chimeras is not due to lack of N-glycosylation.

O-, but not N-glycosylation, is essential for Syt internalization from the plasma membrane

Consistent with the fact that Syt I and Syt II are N-glycosylated only at a single site (Fukuda and Mikoshiba, 2000), tunicamycin as well as *N*-glycosidase F treatment caused only a modest reduction in their apparent molecular sizes. However, the molecular size of HA-Syt II is ~10 kDa lower than that of non-tagged Syt II (Fig. 8). Both Syt I and Syt II include several putative O-glycosylation sites and Syt I was shown to undergo O-glycosylation in a VAMP-2-dependent fashion (Fukuda, 2002). As O-glycosylation plays an important role in the sorting of type I membrane proteins (Scheiffele and Fullekrug, 2000; Huet et al., 2003; Alfalah et al., 1999), we sought to investigate whether O-glycosylation is required for the intracellular targeting of Syt I and Syt II in RBL cells.

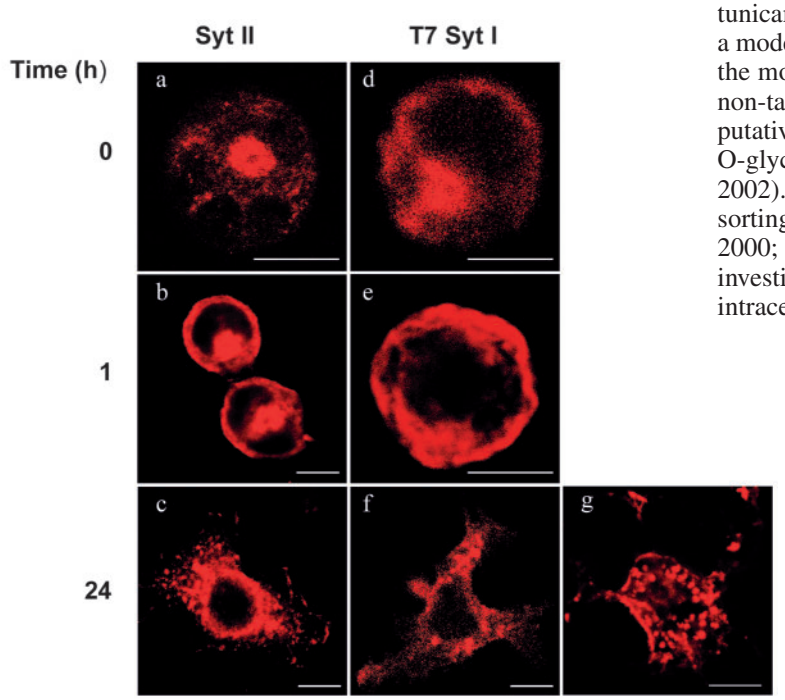

Fig. 5. Biosynthetic routes of Syt I and Syt II. RBL cells, transiently transfected with Syt II (a-c) or T7-Syt I (d-g) cDNA, were grown on glass coverslips for 1 hour (a-f) or 5 hours (g) and then incubated for 2 hours at 20°C (a-f) or for 1 hour at 4°C in the presence of monoclonal antibodies directed against T7 (1 μg/ml) (g). Cells were subsequently transferred to 37°C (time zero) and incubated for the indicated time periods. Cells were stained with polyclonal antibodies directed against C2A-Syt IX (a-c) or with monoclonal antibodies directed against T7 (d-f) followed by Cy3-conjugated secondary antibodies. Cells were visualized by laser confocal microscopy. Bar, 5 μm.

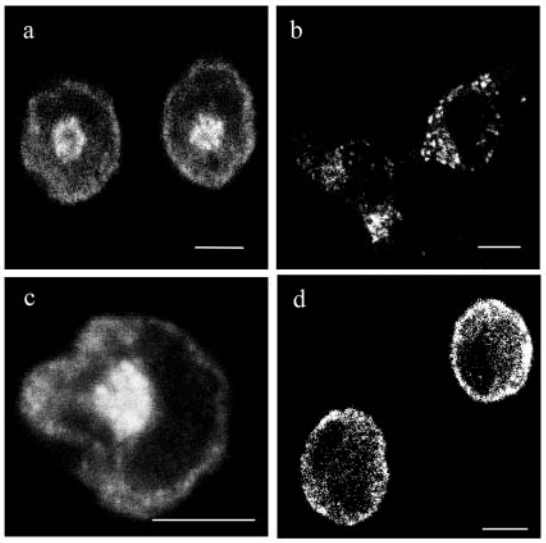

Fig. 6. Effect of inhibitors of internalization on HA-Syt I/Syt II trafficking. RBL cells transiently transfected with HA-Syt I/Syt II cDNA were grown on glass coverslips for 1 hour and subsequently incubated for 2 hours at 20°C (a). Cells were then warmed to 37°C for 3 hours in the absence (b) or presence of chloroquine (100 μM) (c) or sucrose (0.45 M) (d). Cells were subsequently stained with monoclonal anti-HA antibodies followed by Cy3-conjugated secondary antibody. Cells were processed for immunofluorescence and visualized by laser confocal microscopy. Bar, 5 μm.

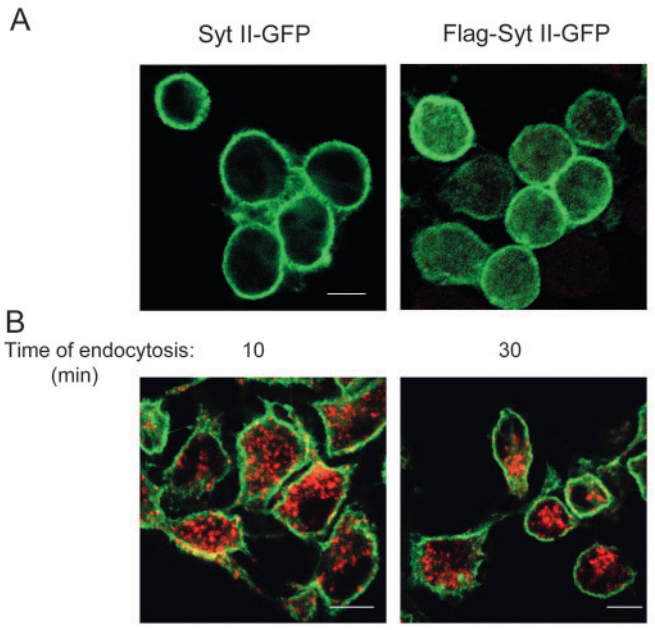

Fig. 7. Cellular localization of GFP-tagged Syt II. RBL cells stably transfected with Syt II-GFP (A) or Flag-Syt II-GFP (A,B) were grown on glass coverslips and either left untreated (A) or were serum starved for 1 hour and subsequently incubated for the indicated time periods with Texas Red-conjugated Tfn (50 μg/ml) (B). Cells were fixed and visualized by laser confocal microscopy. Bar, 10 μm.

Like its wild-type counterpart, T7-Syt I(T26/A), the N-glycosylation mutant of Syt I, acquired an intracellular vesicular localization (Fig. 10a). Moreover, fractionation of

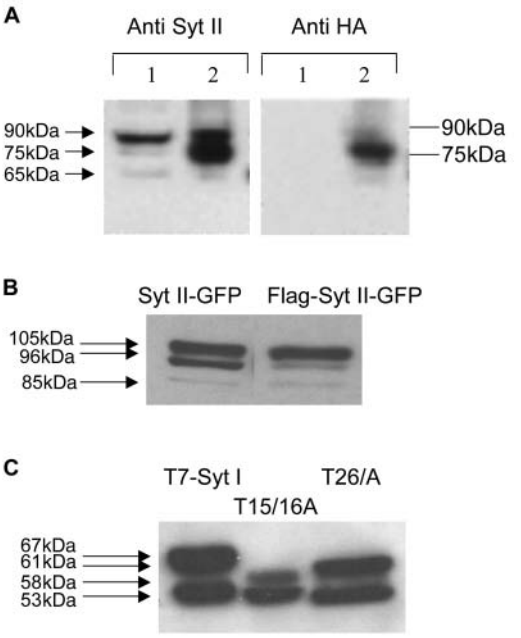

Fig. 8. Western blot analysis of HA-Syt II expressed in RBL cells. Cell extracts (80 μg) derived from RBL cells stably transfected with either (A) non-tagged Syt II (1) or HA-Syt II (2) cDNA, or (B) Syt II-GFP or Flag-Syt II-GFP cDNA or (C) transiently transfected with T7-Syt I, T7-Syt I(T15/16/A) or T7-Syt I(T26/A) cDNA, were resolved by SDS-PAGE and subjected to immunoblotting with either monoclonal anti-Syt II or anti-HA antibodies as indicated (A), or anti-GFP antibodies (B), or anti-T7 antibodies (C). The positions of the molecular mass markers are shown on the right in A. Arrows point to fully, partially or non-modified immunoreactive proteins.

transfected cells on linaer sucrose gradients confirmed that T7-Syt I(T26/A) cofractionates with secretory granules (not shown). In contrast, the O-glycosylation mutant T7-Syt I(T15/16/A) failed to reach its intracellular localization and remained localized at the plasma membrane (Fig. 10b). To assess the importance of O-glycosylation to internalization of Syt II, we generated mutants in which we replaced all the putative O-glycosylation sites of Syt II [both Flag-Syt II(T17/18/19/A)-GFP and non tagged Syt II(17/18/19/A)]. Consistent with the notion that tagged Syt II is lacking O- but not N-glycosylation, SDS-PAGE analysis of the molecular size of the O-glycosylation mutant Flag-Syt II(T17/18/19/A)-GFP has indicated that its molecular mass was similar to that of the wild-type protein but larger than that of the N-glycosylation mutant (Fig. 9F). Moreover, immunofluorescence analysis of cells transfected with the corresponding non-tagged mutant revealed that although untagged, this mutant also localized exclusively to the plasma membrane (Fig. 10c). These results therefore implicate O-glycosylation as an essential post-translational modification for internalization from the plasma membrane of both Syt I and Syt II.

Discussion

Previous studies have demonstrated that unlike its synaptic vesicle or SLMV localization in neurons or neuroendocrine cells, Syt I localizes to the plasma membrane in non-neuronal

cells such as fibroblasts or epithelial cells (Feany et al., 1993; Han et al., 2004). The ability of Syt I to internalize in neuronal tissue is tightly linked to its ability to undergo N-glycosylation (Han et al., 2004). We have previously demonstrated that when transfected into RBL mast cells, Syt I is directed to secretory granules analogous to the situation in neuronal cells (Baram et al., 1998). Moreover, Syt II, which is endogenously expressed in mast cells, also localizes to intracellular secretory vesicles (Baram et al., 1999). These results therefore indicate that at least certain non-neuronal cells might use mechanisms similar to that of neurons to direct Syt I and Syt II to intracellular secretory organelles. Consistent with this notion, Syt I was also reported to reside on secretory granules in insulin secreting β cells (Lang et al., 1997) and in the parotid gland (Levius et al., 1997). Therefore, in specialized secretory cells, including the mast cells, Syt I does localize to secretory vesicles. However, unlike synaptic vesicles and SLMV, whose biogenesis involves protein transport from the TGN to the plasma membrane and internalization either directly as synaptic vesicles/SLMV or through an endosomal intermediate (Shi et al., 1998), biogenesis of secretory granules involves direct delivery from the TGN to secretory granules bypassing the plasma membrane (Tooze, 1998), Therefore, the intracellular localization of Syt I and Syt II in specialized secretory cells, such as the RBL cells, might either indicate that like neurons, secretory cells can internalize Syt I and Syt II from the plasma membrane, or alternatively, that their trafficking does not involve endocytosis. To distinguish between these possibilities we investigated whether the plasma membrane is an intermediate step in the biosynthetic pathways of Syt I and Syt II in RBL cells. Indeed, based upon our results, both Syt I and Syt II travel through the plasma membrane during their biosynthetic pathways and are therefore likely to require endocytosis in order to reach their final destinations. Consistent with this notion, we show that inhibitors of endocytosis, prevent HA-Syt I/Syt II, a chimera, which takes a similar route to Syt I, from reaching the secretory granules. Therefore, in common with neurons and neuroendocrine cells, RBL cells are able to internalize both Syt I and Syt II from the plasma membrane. Notably, both Syt I and Syt II were shown to bind the clathrin adaptor complex AP-2 (Zhang et al., 1994; Haucke et al., 2000). Whether AP-2 is indeed required for internalization of these synaptotagmins during their biosynthetic trafficking is currently under investigation. Although we do not know whether Syt I also travels through the plasma membrane in

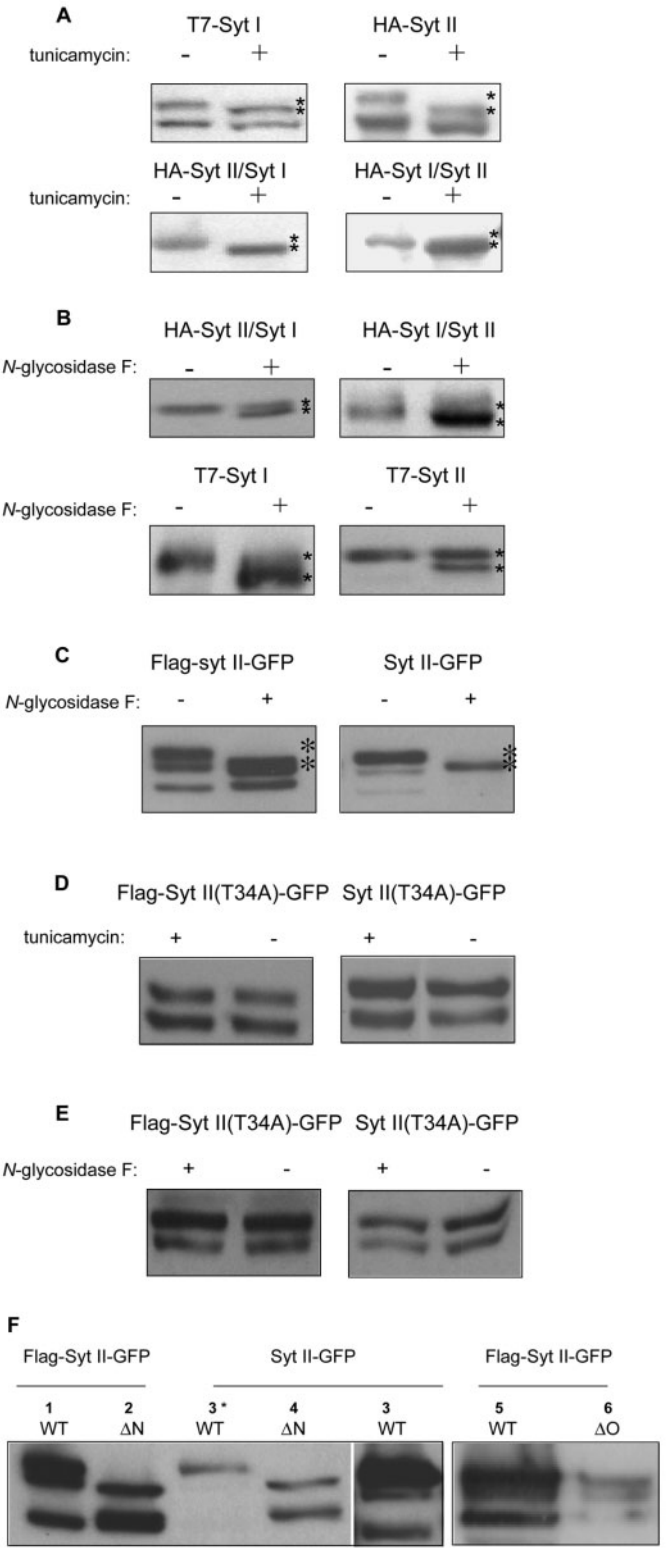

Fig. 9. Analysis of glycosylation of tagged Syt I and II. (A) RBL cells transiently transfected with T7-Syt I, HA-Syt II or the chimeras HA-Syt I/Syt II and HA-Syt II(1-183aa)/Syt I cDNA were grown for 2 hours at 37°C and subsequently left untreated or incubated for 24 hours in the presence of tunicamycin (10 μg/ml). Cell lysates were resolved by SDS-PAGE and probed with either anti-T7 or anti-HA antibodies, as indicated. (B) Cell lysates derived from RBL cells transfected with T7-Syt I, HA-Syt II, HA-Syt I/Syt II and HA-Syt II(1-183aa)/Syt I cDNA were treated with *N*-glycosidase F or buffer as described in Materials and Methods. Lysates were subjected to SDS-PAGE and probed with anti-T7 or anti-HA antibodies. (C) Cell lysates (80 μg) derived from RBL cells stably transfected with Syt II-GFP or Flag-Syt II-GFP were treated with *N*-glycosidase F, resolved on SDS-PAGE and probed with anti-GFP antibodies. The asterisks in panels A-C indicate proteins that were reduced by tunicamycin or *N*-glycosidase F treatment. (D) RBL cells transfected with Syt II(T34/A)-GFP or Flag-Syt II(T34/A)-GFP were subjected to tunicamycin or (E) *N*-glycosidase F treatment as described above. (F) Lysates (80 μg) derived from RBL cells transiently transfected with Flag-Syt II-GFP (WT)(1, 5) or Flag-Syt II(T34/A)-GFP (ΔN)(2), or Syt II-GFP (WT)(3) or Syt II(T34/A)-GFP (ΔN)(4) or Flag-Syt II(T17/18/19/A)-GFP (ΔO) (6) were subjected to SDS-PAGE and probed with anti-GFP antibodies. Lane 3 is a longer ECL exposure time of the lane marked 3*.

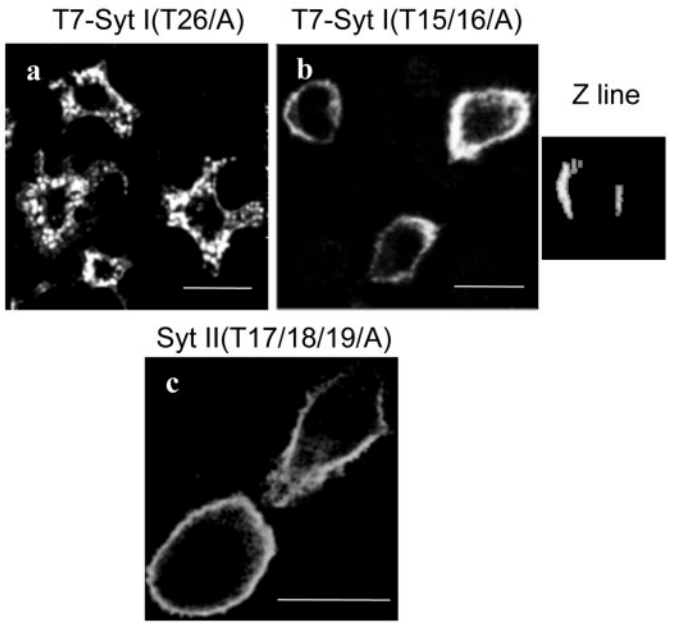

Fig. 10. Visualization of T7-Syt I and Syt II glycosylation mutants. RBL cells transiently transfected with T7-Syt I(T26/A) (a) or T7-Syt I(T15/16/A) (b) or Syt II(T17/18/19/A) (c) were grown on glass coverslips for 24 hours and subsequently labeled with anti-T7 monoclonal antibodies (a,b) or polyclonal antibodies directed against C2A-Syt IX (c) followed by the relevant Cy3-conjugated (a,c) or Cy2-conjugated secondary antibodies. Cells were processed for immunofluorescent staining and visualized by confocal microscopy. The *z*-line image is of the same field as in b and confirms the plasma membrane localization of the ΔO mutant. Bar, 10 μm.

endocrine cells, our results are consistent with the intimate connection that exists between the exocytic and the endocytic pathways in mast cells. These cells contain several types of secretory granule (Sannes and Spicer, 1979; Jamur et al., 1986; Raposo et al., 1997; Baram et al., 1999), all of which are lysosome related (Griffiths, 1996; Dell'Angelica et al., 2000). Indeed, previous studies have already demonstrated that cargo internalized by the RBL cells can reach secretory granules and exocytose in a regulated fashion (Xu et al., 1998). An 80 kDa integral membrane protein of the secretory granules appears at the plasma membrane after exocytosis, from where it is internalized and recycled back to the secretory granules (Bonifacino et al., 1989). Finally, TPA-induced down-regulation of protein kinase Cα (PKCα) results in trafficking of the enzyme to endosomes followed by its appearance on the secretory granules (Peng et al., 2002). Collectively these results indicate that one route of reaching secretory granules in RBL cells involves protein transport from the TGN to the plasma membrane and internalization into endosomes. However, unlike internalization of Syt I in neuronal tissue, where N-glycosylation is an essential factor, this posttranslational modification is unnecessary for internalization of Syt I in RBL cells. This conclusion is based upon our findings that an N-glycosylation mutant of Syt I maintains its intracellular localization. In contrast, our results provide unequivocal evidence for a crucial role of O-glycosylation in mediating Syt I internalization. We show that the O-glycosylation mutant Syt I(T15/16/A) remains at the plasma membrane and fails to internalize in RBL cells. In a similar fashion, non-tagged Syt II, which is mutated at its putative O-glycosylation sites [Syt II(T17/18/19/A)] fails to internalize and remains at the plasma membrane (Fig. 10c). We do not presently know how O-glycosylation promotes internalization. Studies are currently underway to resolve this issue. In this context it is interesting to note that Syt I undergoes O-glycosylation in a VAMP-2-dependent fashion (Fukuda, 2002), which in turn might occur only in specialized secretory cells, in which VAMP-2 is expressed.

Another rather unexpected result, which emerged from this study, is the finding that N as well as C-terminal tagging of Syt II, but not of Syt I, prevents its internalization. Although not proven here, the significantly lower molecular mass of HA-Syt II and Syt II-GFP is consistent with the idea that tagging of Syt II interferes with its O-glycosylation and thereby prevents its internalization. However, we cannot completely exclude alternative possibilities. Whatever the reason, this result calls for caution in using tagged synaptotagmins to study their trafficking and consequent function.

Our present results clearly designate the luminal domain as playing a crucial role in Syt trafficking. However, it is important to note that despite their high degree of homology (~80%), Syt I and Syt II are targeted to distinct secretory vesicles in RBL cells. Specifically, Syt I is targeted to amine-containing secretory granules and Syt II to amine-free secretory lysosomes (Baram et al., 1999; Baram et al., 1998). This differential localization suggests the existence of additional and unique sorting determinants within the cytosolic domains of Syt I and Syt II. However, we show that a chimeric protein comprising the luminal domain of Syt I fused with the cytosolic domain of Syt II colocalizes with Syt I at the secretory granules. Therefore, our results demonstrate that the luminal domain not only governs the internalization of Syt I and presumably Syt II, but it also indirectly overrides and dominates signals localized within the cytosolic domain thus demonstrating a hierarchy of sorting determinants.

In conclusion, although sorting motifs present in the cytosolic domains of synaptotagmins have been implicated in binding of endocytosis adaptor proteins including the clathrin adaptor complex AP-2 (Zhang et al., 1994; Haucke et al., 2000) and stonins (Walther et al., 2001), it is the luminal domain that dictates whether Syt will internalize and where it will be delivered to in both neuronal and non-neuronal specialized secretory cells. Specifically, glycosylation appears to mediate sorting by the luminal domain: N-glycosylation in neuronal tissue and O-glycosylation in non-neuronal tissues.

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References

Alfalah, M., Jacob, R., Preuss, U., Zimmer, K. P., Naim, H. and Naim, H. Y. (1999). O-linked glycans mediate apical sorting of human intestinal sucrose-isomaltase through association with lipid rafts. *Curr. Biol.* **9**, 593-596.

Baram, D., Linial, M., Mekori, Y. A. and Sagi-Eisenberg, R. (1998). Ca^{2+}-

dependent exocytosis in mast cells is stimulated by the Ca^{2+} sensor, synaptotagmin I. *J. Immunol.* **161**, 5120-5123.

Baram, D., Adachi, R., Medalia, O., Tuvim, M., Dickey, B. F., Mekori, Y. A. and Sagi-Eisenberg, R. (1999). Synaptotagmin II negatively regulates Ca^{2+}-triggered exocytosis of lysosomes in mast cells. *J. Exp. Med.* **189**, 1649-1658.

Barzilay, E., Ben-Califa, N., Supino-Rosin, L., Kashman, Y., Hirschberg, K., Elazar, Z. and Neumann, D. (2004). Geldanamycin-associated inhibition of intracellular trafficking is attributed to a co-purified activity. *J. Biol. Chem.* **279**, 6847-6852.

Bonifacino, J. S., Yuan, L. and Sandoval, I. V. (1989). Internalization and recycling to serotonin-containing granules of the 80K integral membrane protein exposed on the surface of secreting rat basophilic leukaemia cells. *J. Cell Sci.* **92**, 701-712.

Brose, N., Petrenko, A. G., Sudhof, T. C. and Jahn, R. (1992). Synaptotagmin: a calcium sensor on the synaptic vesicle surface. *Science* **256**, 1021-1025.

Dasgupta, S. and Kelly, R. B. (2003). Internalization signals in Synaptotagmin VII utilizing two independent pathways are masked by intramolecular inhibitors. *J. Cell Sci.* **116**, 1327-1337.

Dell'Angelica, E. C., Mullins, C., Caplan, S. and Bonifacino, J. S. (2000). Lysosome-related organelles. *FASEB J.* **4**, 1265-1278.

Feany, M. B., Yee, A. G., Delvy, M. L. and Buckley, K. M. (1993). The synaptic vesicle proteins SV2, synaptotagmin and synaptophysin are sorted to separate cellular compartments in CHO fibroblasts. *J. Cell Biol*. **123**, 575-584.

Fukuda, M. (2002). Vesicle-associated membrane protein-2/synaptobrevin binding to synaptotagmin I promotes O-glycosylation of synaptotagmin I. *J. Biol. Chem*. **277**, 30351-30358.

Fukuda, M. (2004). RNA interference-mediated silencing of synaptotagmin IX, but not synaptotagmin I, inhibits dense-core vesicle exocytosis in PC12 cells. *Biochem. J.* **380**, 875-879.

Fukuda, M. and Mikoshiba, K. (2000). Calcium-dependent and -independent hetero-oligomerization in the synaptotagmin family. *J. Biochem.* **128**, 637-645.

Fukuda, M., Kowalchyk, J. A., Zhang, X., Martin, T. F. and Mikoshiba, K. (2002). Synaptotagmin IX regulates Ca^{2+}-dependent secretion in PC12 cells. *J. Biol. Chem.* **277**, 4601-4604.

Geppert, M., Archer, B. and Sudhof, T. C. (1991). Synaptotagmin II. A novel differentially distributed form of synaptotagmin. *J. Biol. Chem.* **266**, 13548-13552.

Griffiths, G. M. (1996). Secretory lysosomes-a special mechanism of regulated secretion in haemopoietic cells. *Trends Cell. Biol*. **6**, 329-332.

Griffiths, G., Pfeiffer, S., Simons, K. and Matlin, K. (1985). Exit of newly synthesized membrane proteins from the trans cisterna of the Golgi complex to the plasma membrane. *J. Cell Biol*. **101**, 949-964.

Grimberg, E., Peng, Z., Hammel, I. and Sagi-Eisenberg, R. (2003). Synaptotagmin III is a critical factor for the formation of the perinuclear endocytic recycling compartment and determination of secretory granules size. *J. Cell Sci.* **116**, 145-154.

Haberman, Y., Grimberg, E., Fukuda, M. and Sagi-Eisenberg, R. (2003). Synaptotagmin IX, a Possible Linker Between the Perinuclear Endocytic Recycling Compartment and the Microtubules. *J. Cell Sci*. **116**, 4307-4318.

Han, W., Rhee, J. S., Maximov, A., Lao, Y., Mashimo, T., Rosenmund, C. and Sudhof, T. C. (2004). N-glycosylation is essential for vesicular targeting of Synaptotagmin 1. *Neuron* **41**, 85-99.

Haucke, V., Wenk, M. R., Chapman, E. R., Farsad, K. and de Camilli, P. (2000). Dual interaction of synaptotagmin with mu2- and alpha-adaptin facilitates clathrin-coated pit nucleation. *EMBO J.* **19**, 6011-6019.

Huet, G., Gouyer, V., Delacour, D., Richet, C., Zanetta, J. P., Delannoy, P. and Degand P. (2003). Involvement of glycosylation in the intracellular trafficking of glycoproteins in polarized epithelial cells. *Biochimie* **85**, 323-330.

Jamur, M. C., Vugman, I. and Hand, A. R. (1986). Ultrastructural and cytochemical studies of acid phosphatase and trimetaphosphatase in rat peritoneal mast cells developing in vivo. *Cell Tissue Res.* **244**, 557-563.

Jarousse, N. and Kelly, R. B. (2001). The AP-2 binding site of Synaptotagmin 1 is not an internalization signal but a regulator of endocytosis. *J. Cell Biol.* **154**, 857-866.

Lang, J., Fukuda, M., Zhang, H., Mikoshiba, K. and Wollheim, C. B. (1997). The first C2 domain of synaptotagmin is required for exocytosis of insulin from pancreatic beta-cells: action of synaptotagmin at low micromolar calcium. *EMBO J.* **16**, 5837-5846.

Levius, O., Feinstein, N. and Linial, M. (1997). Expression of synaptotagmin I in rat parotid gland. *Eur. J. Cell Biol*. **73**, 81-92.

Lindmark, I. M., Karlsson, A., Serrander, L., Francois, P., Lew, D., Rasmusson, B., Stendahl, O. and Nusse, O. (2002). Synaptotagmin II could confer Ca(2+) sensitivity to phagocytosis in human neutrophils. *Biochim. Biophys. Acta*. **1590**, 159-166.

Martinez, I., Chakrabarti, S., Hellevik, T., Morehead, J., Fowler, K. and Andrews, N. W. (2000). Synaptotagmin VII regulates Ca(2+)-dependent exocytosis of lysosomes in fibroblasts. *J. Cell Biol.* **148**, 1141-1149.

Matlin, K. S. and Simons, K. (1983). Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell* **34**, 233-243.

Peng, Z., Grimberg, E. and Sagi-Eisenberg, R. (2002). Suppression of Synaptotagmin II restrains phorbol ester-induced down-regulation of protein kinase Cα by diverting the kinase from a degradative pathway to the recycling endocytic compartment. *J. Cell Sci.* **115**, 3083-3092.

Raposo, G., Tenza, D., Mecheri, S., Peronet, R., Bonnerot, C. and Desaymard, C. (1997). Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol. Biol. Cell* **8**, 2631-2645.

Saegusa, C., Fukuda, M., and Mikoshiba, K. (2002). Synaptotagmin V is targeted to dense-core vesicles that undergo calcium-dependent exocytosis in PC12 cells. *J. Biol. Chem.* **277**, 24499-24505.

Sannes, P. L. and Spicer, S. S. (1979). The heterophagic granules of mast cells: dipeptidyl aminopeptidase II activity and resistance to exocytosis. *Am. J. Pathol.* **94**, 447-457.

Scheiffele, P. and Fullekrug, J. (2000). Glycosylation and protein transport. *Essays Biochem.* **36**, 27-35.

Shi, G., Faundez, V., Roos, J., Dell'Angelica, E. C. and Kelly, R. B. (1998). Neuroendocrine synaptic vesicles are formed in vitro by both clathrin-dependent and clathrin-independent pathways. *J. Cell Biol.* **143**, 947-955.

Shin, O. H., Rizo, J. and Sudhof, T. C. (2002). Synaptotagmin function in dense core vesicle exocytosis studied in cracked PC12 cells. *Nat. Neurosci.* **5**, 649-656.

Sudhof, T. C. and Rizo, J. (1996). Synaptotagmins: C2-domain proteins that regulate membrane traffic. *Neuron* **17**, 379-388.

Sugita, S., Shin, O. H., Han, W., Lao, Y. and Sudhof, T. C. (2002). Synaptotagmins form a hierarchy of exocytotic Ca(2+) sensors with distinct Ca(2+) affinities. *EMBO J.* **21**, 270-280.

Tooze, S. A. (1998). Biogenesis of secretory granules in the trans-Golgi network of neuroendocrine and endocrine cells. *Biochim. Biophys. Acta* **1404**, 231-244.

Walther, K., Krauss, M., Diril, M. K., Lemke, S., Ricotta, D., Honing, S., Kaiser, S. and Haucke, V. (2001). Human stoned B interacts with AP-2 and synaptotagmin and facilitates clathrin-coated vesicle uncoating. *EMBO Rep.* **2**, 634-640.

Xu, K., Williams, R. M., Holowka, D. and Baird, B. (1998). Stimulated release of fluorescently labeled IgE fragments that efficiently accumulate in secretory granules after endocytosis in RBL-2H3 mast cells. *J. Cell Sci.* **111**, 2385-2396.

Yoshihara, M., Adolfsen, B. and Littleton, T. J. (2003). Is synaptotagmin the calcium sensor? *Curr. Opin. Neurobiol*. **13**, 315-323.

Zhang, J. Z., Davletov, B. A., Sudhof, T. C. and Anderson, R. G. (1994). Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. *Cell* **78**, 751-760.