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An Isoform of the Golgi t-SNARE, Syntaxin 5, with an Endoplasmic Reticulum Retrieval Signal

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The early Golgi t-SNARE (target-membrane–associated soluble-*N*-ethylmaleimide–sensitive factor attachment protein receptor) syntaxin 5 is thought to specify the docking site for both COPI and COPII coated vesicles originating from the endoplasmic reticulum (ER) and COPI vesicles on the retrograde pathway. We now show that there are two forms of syntaxin 5 that appear to be generated from the same mRNA by alternative initiation of translation. The short form (35 kDa) corresponds to the published sequence. The long form (42 kDa) has an N-terminal cytoplasmic extension containing a predicted type II ER retrieval signal. When grafted onto a reporter molecule, this signal localized the construct to the ER. Biochemical fractionation and immunofluorescence microscopy showed that there was less of the long form in the Golgi apparatus and more in peripheral punctate structures, some of which colocalized with markers of the intermediate compartment. The predicted absence of the long form in budding yeast points to a function unique to higher organisms.

INTRODUCTION

The soluble-*N*-ethylmaleimide–sensitive factor (NSF)[1] attachment protein receptor (SNARE) hypothesis has provided a conceptual framework with which to understand the targeting of transport vesicles to specific membranes (for reviews see Bennett *et al.*, 1993; Ferro-Novick and Jahn, 1994; Rothman, 1994; Rothman and Warren, 1994; Scheller, 1995). A vesicle-associated SNARE (v-SNARE) in the transport vesicle interacts specifically with a target-membrane–associated SNARE (t-SNARE) in the target membrane. SNAREs are membrane proteins with most of their mass projecting into the cytoplasm. They are thought to interact via the coiled-coil domains predicted from their sequences (Chapman *et al.*, 1994; Hayashi *et al.*, 1994). Assembly of the SNARE complex is regulated by the Sec1/Sly1 family of proteins (Aalto *et al.*, 1991; Dascher *et al.*, 1991; Ossig *et al.*, 1991; Aalto *et al.*, 1992; Pevsner, 1996) and the Ypt/Rab family of small GTP-binding proteins (Salminen and Novick, 1987; Segev *et al.*, 1988; Novick and Brennwald, 1993; Pfeffer, 1994). The cognate SNARE complex acts as a scaffold for the binding of the ATPase NSF via the soluble NSF attachment proteins (SNAPs) (Sollner *et al.*, 1993b). Hydrolysis of ATP leads to break-up of the SNARE complex and membrane fusion (Sollner *et al.*, 1993a). Variations to this basic model exist that are largely concerned with the point at which NSF and SNAPs act (O'Connor *et al.*, 1994; Mayer *et al.*, 1996).

Though first applied to the targeting of synaptic vesicles to the presynaptic membrane (Pevsner and Scheller, 1994), the SNARE hypothesis was quickly extended to other vesicle targeting steps (Ferro-Novick and Jahn, 1994). The best characterized is that from the endoplasmic reticulum (ER) to the Golgi apparatus. Work in budding yeast has shown that COPI and COPII-coated vesicles, budding from the ER, carry at

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[1] Abbreviations used: COP, coat proteins; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; SNARE, soluble-NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor; t-SNARE, target membrane-associated SNARE; v-SNARE, vesicle-associated SNARE.

least three types of v-SNARE (Bos1p, Bet1p and Sec22p; Bednarek *et al.*, 1995) that interact with the Golgi t-SNARE Sed5p (Sogaard *et al.*, 1994) in a manner dependent upon Ypt1p (Lian *et al.*, 1994; Sogaard *et al.*, 1994) and Sly1p (Sogaard *et al.*, 1994). Retrograde vesicles appear to use a different v-SNARE, Sft1p, to interact with Sed5p (Banfield *et al.*, 1995). Sed5p is required for exocytic transport in both yeast (Hardwick and Pelham, 1992) and mammals (Dascher *et al.*, 1994).

A crucial feature of the SNARE hypothesis is that the t-SNARE must be a resident of the target membrane. This is in marked contrast to the cognate v-SNARE, which must cycle between the two compartments linked by vesicles. The v-SNARE will share the same compartment as the t-SNARE immediately after membrane fusion but is presumably recycled back to the compartment from which it originated so that it can be reincorporated into budding COP vesicles for another round of vesicle transport. The presence of an active t-SNARE in another compartment would compromise compartmental identity. Signals must, therefore, exist to localize t-SNAREs to particular compartments.

Localization signals are best studied for the Golgi t-SNARE Sed5p (Banfield *et al.*, 1994). The mammalian homologue syntaxin 5 (Bennett *et al.*, 1993) is located in a tubulo-reticular network on the *cis*-side of the Golgi stack, as is the transiently expressed *Drosophila* homologue dSed5p. Chimeras between dSed5p and other mammalian syntaxins (from other locations) showed that more than one signal was present (Banfield *et al.*, 1994). The membrane-spanning domain was sufficient to localize the chimeras to the *cis*-Golgi but not necessary. Additional signals were found in the cytoplasmic domain though their precise nature was not determined.

In carrying out further studies on the mammalian homologue syntaxin 5, we noticed, as have others (Hay *et al.*, 1997), that there are two forms of the protein. We found that a range of anti-peptide antibodies recognized an additional longer form in a wide variety of rat tissues. The lack of an in-frame stop codon upstream of the putative start codon in the published sequence (Bennett *et al.*, 1993) prompted us to clone further upstream sequence. We found an N-terminal extension containing a functional ER retrieval signal.

MATERIALS AND METHODS

Antisera

Peptides were coupled to keyhole limpet hemocyanin, emulsified in Freund's complete adjuvant, and used to immunize rabbits. Affinity purification was carried out as described (Harlow and Lane, 1988). The synthetic peptides used were as follows (amino acid residue numbers in the published sequence GenBank accession no. L20822): NHU1 (aa 14–29), NHU2 (aa 104–119), NHU3 (aa 143–156), NHU4 (aa 209–222), NHU5 (TDQGVYLGLSKTQVL), and NHU6 (IPRKRYGSKNTDQG) as indicated in Figure 2.

A recombinant syntaxin 5 was produced by using the QIA-express system (Qiagen, Chatsworth, CA) by following the manufacturer's protocol. The N-terminal His_6 tag was used to purify the recombinant protein, which was then used for immunization. Affinity purification of the rabbit antibodies was performed by using the same recombinant protein bound to ProBond Resin (Invitrogen, San Diego, CA) and then as described (Harlow and Lane, 1988).

cDNA Cloning

Poly(A)$^+$ RNA was isolated from livers of Sprague–Dawley rats (Sambrook *et al.*, 1989) and used for 5′ rapid amplification of cDNA ends (5′-RACE) using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA), the expand long template polymerase chain reaction (PCR) system (Boehringer-Mannheim, Mannheim, Germany), and *Taq* Start antibody (Clontech) by following the manufacturer's protocol. Gene-specific primers used were 5′-CTCGGGACTCTCCTCCCAGAACTA-3′ for the PCR and 5′-CGCGGATCCGAACTGTTCCCGACGGTTCC-3′ for the nested PCR. Adaptor primer 2 (AP2) was modified to contain a *Bam*HI site for subsequent cDNA cloning. A full-length cDNA was constructed by combining the *Bam*HI–*Xba*I fragment from the 5′-RACE product and the *Xba*I–*Bam*HI fragment from the rat syntaxin 5 cDNA (Banfield *et al.*, 1994) and cloning into Bluescript II KS+ (Stratagene, La Jolla, CA).

In Vitro Transcription/Translation

Translation exclusively from the first, newly identified, ATG is ensured by mutating the second ATG to CTG by PCR. Translation from the second ATG is ensured by using the original cDNA (Banfield *et al.*, 1994) and from both ATG sites by using the full-length cDNA. Coupled in vitro transcription/translation was performed by using the TNT T3 coupled reticulocyte lysate system (Promega, Madison, WI) in the absence of microsome membranes. Samples were applied directly onto 10% SDS-PAGE gels or were incubated with 15 μg of affinity-purified anti-NHU4 or -NHU5 antibodies for 1 h at room temperature. The complexes were recovered by using protein A-Sepharose (Pharmacia, Piscataway, NJ) and then applied to 10% SDS-PAGE gels.

DNA Sequence Analysis

The cDNA sequence was determined in both directions by appropriate oligonucleotide primers. Manual sequencing (Sambrook *et al.*, 1989) and automatic sequencing using the Applied Biosystems PRISM dye terminator cycle sequencing system (Perkin Elmer-Cetus, Norwalk, CT) gave identical results.

Cell Culture, Transfection, and Immunofluorescence

HeLa and NRK cells were grown in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum, penicillin, and streptomycin (100 μg/ml), glutamine, and nonessential amino acids (Life Technologies); and Vero cells were grown in minimum essential medium (Life Technologies). Transient transfection and indirect immunofluorescence microscopy were carried out essentially as described (Nilsson *et al.*, 1991). LN2 monoclonal hybridoma culture supernatant antibody against p31 (Clonlab, Biotest AG, Dreieich, Germany) was used to stain transfected cells. Cycloheximide was added to subconfluent monolayers of NRK cells at a concentration of 10 μg/ml for up to 8 h and washed away with phosphate-buffered saline before lysis. Double-label immunofluorescence images were captured on a Princeton charge-coupled devise camera (KMF 1400 chip) through a Zeiss 135TV inverted microscope. The images were processed by using IP Lab Spectrum and Adobe Photoshop software programmes.

SDS-PAGE and Western Blotting

Golgi membranes were isolated from rat liver as described (Slusarewicz *et al.*, 1994a). Various rat tissues were first homogenized by using Polytron PT3000 (Kinematica, Johnson City, TN) before lysis. Cells and tissues were lysed with SDS-PAGE sample buffer without bromophenol blue and dithiothreitol so that protein concentration could be assayed with the BCA protein assay reagent (Pierce, Rockford, IL). Other proteins were methanol-chloroform precipitated (Wessel and Flugge, 1984) before SDS-PAGE (15% gel). After transfer to nitrocellulose membrane (Hybond-C super, Amersham, Arlington Heights, IL), blots were probed with antibodies to syntaxin 5 synthetic peptides, His-tagged recombinant syntaxin 5, calnexin, or p58. Horseradish peroxidase-conjugated secondary antibodies (TAGO Immunologicals, Biosource International, Burlingame, CA) were used and visualized by using the ECL system (Amersham).

Fractionation

Rat liver postnuclear supernatant fractionation was carried out by diluting 1.0 ml of rat liver homogenate (prepared as in Slusarewicz *et al.*, 1994b) with 2.0 ml of 0.25 M sucrose/0.1 M phosphate buffer and centrifuging10 min at 1000 × *g* before loading onto sucrose gradients of 0.5 M-2.0 M in 0.25 M steps. The gradients were centrifuged to equilibrium at 30,000 rpm for 18 h in a Beckman centrifuge using SW40 rotor (100,000 × g_{av}). One-milliliter fractions were taken for further analysis.

RESULTS

The 42-kDa Form of Syntaxin 5

When purified rat liver Golgi membranes were fractionated by SDS-PAGE and Western blotted with anti-peptide antibodies to the C-terminal region of syntaxin 5, near the membrane anchor (NHU4; see Figure 2), one major and two minor proteins were revealed (Figure 1A, lane 2). Prior treatment of the antibodies with the NHU4 peptide abolished staining of all three proteins showing that it was specific (Figure 1A, lane 1). The major protein had an apparent molecular weight consistent with that of the published sequence (Bennett *et al.*, 1993; 35 kDa vs. 34.1 kDa). The minor protein of lower molecular weight (33 kDa, asterisk in Figure 1A) was likely a degradation product, cleaved near the membrane anchor, because it was largely removed by carbonate washing (Figure 1A, lane 3). It was also not found in whole liver extracts (Figure 1B) or NRK cells (Figure 1C). The minor protein of higher molecular weight (42 kDa) was resistant to carbonate washing (Figure 1A, lane 3) but completely sensitive to protease treatment (Figure 1A, lane 4) under conditions where lumenally oriented proteins such as mannosidase II were insensitive (our unpublished results). These results are consistent with the 42-kDa protein being an integral membrane protein, with most of its mass projecting into the cytoplasm, exactly the same topology as syntaxin 5.

The 42-kDa protein was also observed by using three other anti-peptide antibodies raised against different parts of the published sequence (NHU1 to NHU3; Figure 2) and in all cases staining was blocked by pretreatment of the antibody with the peptide to

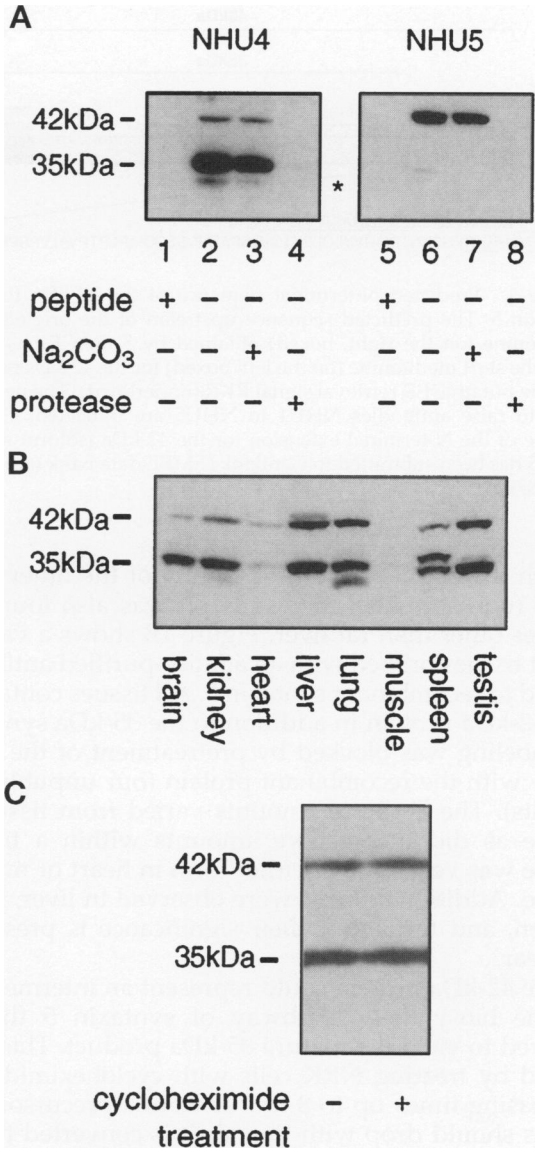


Figure 1. Characterization of the 42-kDa form of syntaxin 5. (A) Golgi membranes from rat liver (25 μg/lane) were fractionated by SDS-PAGE (15% gel) and Western blotted with affinity-purified anti-peptide antibodies that recognized both forms of syntaxin 5 (NHU4) or only the long form (NHU5). Antibody binding was blocked by prior treatment with the specific peptides (lanes 1 and 5). Binding was not affected when Golgi membranes were pretreated with 0.1 M sodium carbonate, pH 11.5 (lanes 3 and 7), but was abolished by pretreatment with 0.05 mg/ml trypsin (lanes 4 and 8). The asterisk indicates a likely proteolytic fragment of the 35-kDa syntaxin 5. (B) Various rat tissues (25 μg/lane) were analyzed for their syntaxin 5 content by SDS-PAGE (15% gel) and Western blotting with affinity-purified antibodies to a recombinant syntaxin 5. In addition to the 35- and 42-kDa forms of syntaxin 5, there were additional forms in liver, lung, spleen, and testis. (C) NRK cells were incubated in the presence or absence of 10 μg/ml cycloheximide for 8 h then fractionated by SDS-PAGE and Western blotted with affinity-purified antibodies to recombinant syntaxin 5.

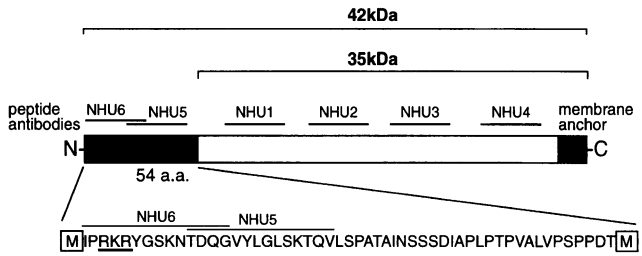


Figure 2. Predicted N-terminal sequence of the 42-kDa form of syntaxin 5. The predicted sequence upstream of the original start methionine (on the right, boxed) obtained by 5′-RACE is shown. Note the start methionine (on the left, boxed) for the 42-kDa isoform and the putative ER retrieval signal RKR (underlined). The peptides used to raise antibodies NHU1 to NHU5 are indicated. The sequence of the N-terminal extension for the 42-kDa isoform of syntaxin 5 has been submitted to GenBank/EMBL data bank (accession no. U87971).

which it was raised but not by any of the other peptides (our unpublished results). It was also found in tissues other than rat liver. Figure 1B shows a variety of rat tissues probed with an affinity-purified antibody raised to recombinant syntaxin 5. All tissues contained the 42-kDa protein in addition to the 35-kDa syntaxin 5. Labeling was blocked by pretreatment of the antibody with the recombinant protein (our unpublished results). The absolute amounts varied from tissue to tissue as did the relative amounts within a tissue. There was very little of either form in heart or muscle tissue. Additional forms were observed in liver, lung, spleen, and testis, but their significance is presently unclear.

The 42-kDa protein could represent an intermediate in the biosynthetic pathway of syntaxin 5 that is cleaved to yield the mature 35-kDa product. This was tested by treating NRK cells with cycloheximide for increasing times up to 8 h. If it were a precursor, the levels should drop with time as it is converted to the mature form. As shown in Figure 1C, this did not happen, because the ratio of the 35-kDa to the 42-kDa forms remained constant over time. Differences in phosphorylation could also not explain the two forms. Our unpublished observations showed no change in the molecular weight of either form of syntaxin 5 when Golgi membranes were incubated with either phosphatases (acid or alkaline) or kinases (in the form of cytosol).

To confirm the identity of the 42-kDa protein as an isoform of syntaxin 5, an anti-peptide antibody was raised to part of the sequence upstream of the predicted start site (NHU5; see Figure 2). We focused on the N terminus because there were no predicted stop codons in the published upstream sequence, which meant that translation from an upstream start codon might be possible, explaining the longer 42-kDa protein. As shown in Figure 1A, the NHU5 anti-peptide antibody only recognized the 42-kDa protein, not the 35-kDa syntaxin 5. Labeling was specifically blocked by the NHU5 peptide (Figure 2, lane 5) and was resistant to carbonate washing (Figure 2, lane 7) but sensitive to protease (Figure 2, lane 8).

The N-Terminal Sequence of the 42-kDa Form

To find the upstream start codon, further sequence was obtained by using 5′-RACE on a poly(A)$^+$ RNA library isolated from rat liver. Six independent clones of different lengths were sequenced. The overlapping sequences were all identical. A predicted in-frame start codon (ATG) was found 162 bases (54 amino acids; Figure 2) upstream of the original start site. An in-frame stop codon (TGA) was located 120 bases further upstream of this new ATG. The predicted N-terminal extension in rat syntaxin 5 showed very high homology (87–90% similarity; 84–89% identity) to human expressed sequence tag database entries AA112327, H21702, H83290, T09399, T30862, T31170, and W96260. Because the predicted molecular weight of the novel syntaxin 5 isoform, starting at the upstream start codon, is 39.8 kDa, similar to the 42 kDa observed by Western blotting (Figure 1), we concluded that the extra sequence obtained by 5′-RACE is the N-terminal sequence of the long form of syntaxin 5.

Alternative Initiation Sites

Northern blot analysis of rat tissues suggested that there was only one mRNA for syntaxin 5 (Bennett *et al.*, 1993) raising the possibility that alternative start sites determine the translation of the two syntaxin isoforms. Evidence in favor of this possibility was obtained by in vitro transcription/translation using cDNAs engineered in such a way that translation could only occur from the first or second ATG (see MATERIALS AND METHODS). Translation from the second ATG was ensured by using the cDNA encoding the original published sequence. Translation from the first ATG involved the use of the full-length cDNA and the changing of the second ATG to CTG (encoding isoleucine). As shown in Figure 3, lane 1, transcription/translation of the full-length cDNA yielded both the 35- and 42-kDa forms of syntaxin 5. Translation from the first ATG yielded the higher 42-kDa (Figure 3, lane 3) form, whereas translation from the second ATG yielded the 35-kDa form (Figure 3, lane 2).

To show that these two proteins are the same as those identified by Western blotting (Figure 1A), they were immunoprecipitated with antibodies to the common part of the syntaxin (NHU4) and to the new N terminus (NHU5). As shown in Figure 3, the NHU4 antibodies precipitated both the 35- and 42-kDa forms (Figure 3, lanes 4–6), whereas the NHU5 antibodies only recognized the 42-kDa form (Figure 3, lanes 7–9).

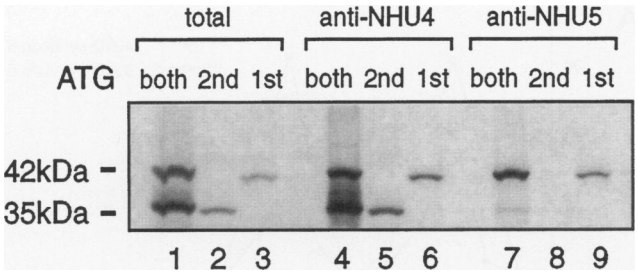


Figure 3. Alternative initiation sites. Syntaxin 5 cDNAs containing both start ATGs (lanes 1, 4, and 7), the second ATG (generating the short syntaxin 5; lanes 2, 5, and 8), or the first ATG (generating the long form of syntaxin 5; lanes 3, 6, and 9) were transcribed and translated in vitro. The products were fractionated directly (lanes 1–3) or after immunoprecipitation with antibodies to both syntaxin forms (NHU4, lanes 4–6) or just to the long form (NHU5, lanes 7–9). Fractionation by 10% SDS-PAGE was followed by fluorography.

Interestingly, a small amount of the 35-kDa form was also precipitated when the full-length cDNA was transcribed and translated, suggesting the formation of heterodimers (Figure 3, lane 7). This presumably reflects the known capacity of syntaxin 5 to form dimers (Banfield *et al.*, 1995).

An ER Retrieval Signal

A possible role for the N-terminal extension in the 42-kDa form of rat syntaxin 5 was revealed by examination of the sequence. As shown in Figure 2, there was a double-arginine motif at positions 4 and 6. Exactly the same motif was found in the human sequence (expressed sequence tag entries T09399, T31170, and T30862).

The double-arginine motif was first identified in human major histocompatibility complex class II invariant chain. Two major forms are synthesized differing by an N-terminal extension of 16 amino acids (Strubin *et al.*, 1984). The form lacking this extension (p31) moves to endosomes, whereas the form containing this extension (p33) is localized to the ER (Lotteau *et al.*, 1990). A double-arginine motif near the N terminus of the extension was shown to be both necessary and sufficient for localizing reporter molecules to the ER. Extensive mutagenesis studies defined a consensus sequence for this motif (Schutze *et al.*, 1994). The double-arginine motif in the N-terminal extension of syntaxin 5 matches this consensus.

To confirm the function of the double-arginine motif in the 42-kDa syntaxin 5, the first 16 amino acids of the N-terminal extension were grafted onto the p31 invariant chain. In another construct, the double-arginine motif (RKR) was replaced by SSS. The original start methionine in p31 was changed to an isoleucine so that only the full-length fusion protein was synthesized. HeLa cells were transiently transfected with these constructs. After 48 h, the cells were fixed, permeabilized, and labeled with antibodies to the lumenal domain of invariant chain followed by secondary antibodies coupled to fluorescein isothiocyanate. As shown in Figure 4, top, p31 was localized to large vacuolar structures previously identified as endosomes (Romagnoli *et al.*, 1993). In marked contrast, the p31 with the N-terminal double-arginine motif was localized to the endoplasmic reticulum, including the nuclear envelope and an extensive reticular network throughout the cytoplasm (Figure 4, middle). This location was determined by the double-arginine motif because the chimeric p31 in which this motif was replaced by SSS was again localized to endosomes (Figure 4, bottom).

Localization of the 42-kDa Isoform

Though the transient transfection studies clearly showed that the double-arginine motif in the 42-kDa syntaxin 5 was both necessary and sufficient to localize a reporter molecule to the ER, this does not mean that the motif is functional in syntaxin 5 in vivo. If it were then the distribution of the two syntaxin 5 forms should differ. This was assessed by fractionation of rat liver and by indirect immunofluorescence microscopy.

Postnuclear supernatants were prepared from rat liver homogenates and sedimented to equilibrium on sucrose gradients. Membranes were recovered from each fraction, and proteins were separated by SDS-PAGE and then Western blotted with antibodies to markers of the intermediate compartment and ER and to the two forms of syntaxin 5. The fractions were also assayed directly for the Golgi marker enzyme β1,4-galactosyltransferase. The results were quantitated using ECL and appropriate internal controls. A typical experiment is shown in Figure 5. The results from three experiments were averaged and are tabulated in Figure 5E.

Both forms of syntaxin 5 were found in two peaks though in different amounts (Figure 5A). More than 60% of the short syntaxin 5 was present in the lighter peak that cofractionated with the Golgi marker β1,4-galactosyltransferase (Roth and Berger, 1982; Figure 5B). The long form of syntaxin 5 was mostly present in the denser peak that overlapped partially with the bulk ER marker calnexin (Wada *et al.*, 1991; Figure 5C) and more closely with the intermediate compartment/*cis*-Golgi network marker, ER-Golgi intermediate compartment (ERGIC) 53/p58 (Saraste *et al.*, 1987; Schweizer *et al.*, 1988; Lippincott-Schwartz *et al.*, 1990; Figure 5D).

These observations were extended by immunofluorescence microscopy. Antibodies to both forms strongly labeled the Golgi apparatus and punctate more peripheral structures (Figure 6A). Because the NHU5 antibody did not label the cells, another an-

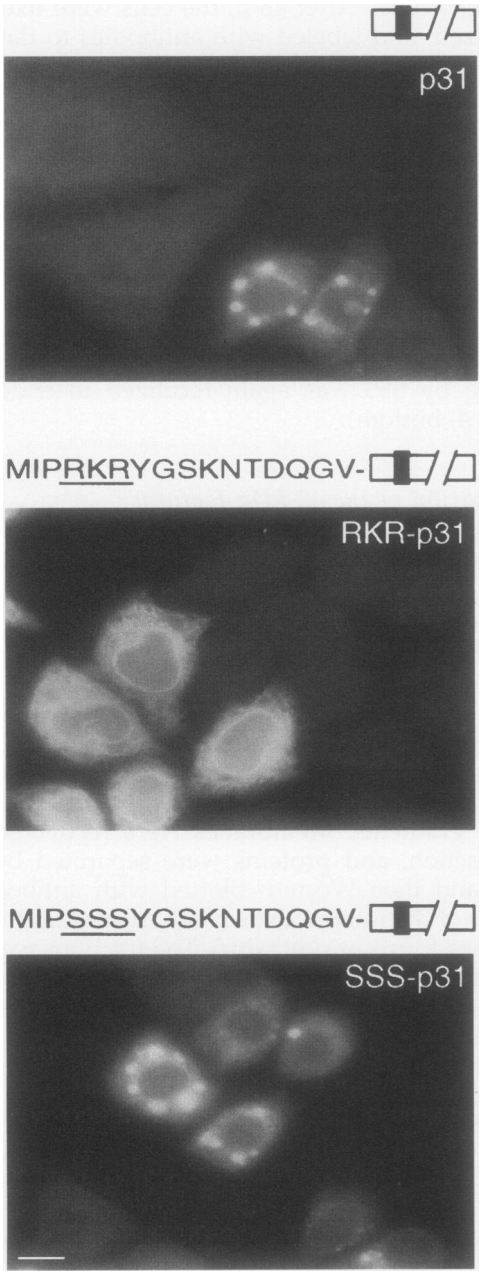
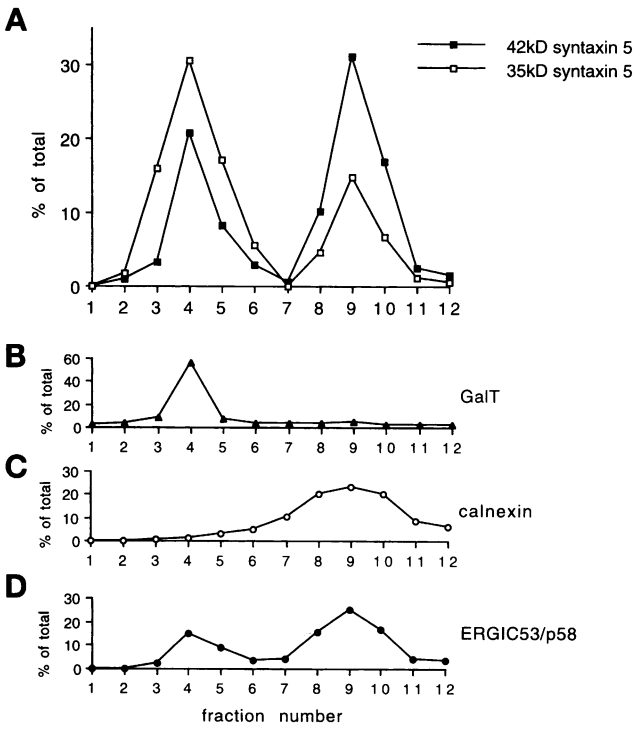


Figure 4. Immunofluorescence microscopy of p31 constructs. HeLa cells were transfected with cDNAs encoding the constructs shown. Top, p31; middle, first 16 N-terminal amino acids of the 42 kDa syntaxin 5 grafted onto the N terminus of p31; bottom, RKR motif changed to SSS. Note that the p31 start methionine was changed to isoleucine in the chimeras to ensure translation only of the full-length constructs. Forty-eight hours after transient transfection, the cells were fixed, permeabilized, and labeled with a monoclonal antibody to p31 followed by secondary antibodies coupled to fluorescein isothiocyanate. Bar, 10 μm.



E

	% of total protein	
	Golgi	ER
35kD syntaxin 5	64.4 ± 0.8	26.4 ± 1.3
42kD syntaxin 5	40.0 ± 5.1	50.4 ± 4.6

Figure 5. Gradient distribution of the syntaxin 5 isoforms. Postnuclear supernatants from rat liver homogenates were sedimented to equilibrium on a 0.5–2.0 M sucrose gradient. The reisolated membranes were fractionated by SDS-PAGE and Western blotted with antibodies to a recombinant syntaxin 5 (A), an ER marker (calnexin; C), or a marker for the intermediate compartment (ERGIC53/p58; D). Results were visualized by using ECL and quantitated. (B) The fractions were assayed directly for the Golgi marker enzyme β1,4-galactosyltransferase (GalT). (E) Average (±SEM) results from three independent experiments.

tibody, NHU6, was raised to a different part of the N-terminal extension (Figure 2) to label uniquely the long form of syntaxin 5. As shown in Figure 6B, the long form was still found in the Golgi apparatus but more appeared to be present in the peripheral structures. These were mostly punctate but occasionally reticular. Double labeling (Figure 7) showed occasional structures in which labeling for the long form of syntaxin 5 was adjacent to that for either the intermediate compartment marker ERGIC53/p58 (Figure 7C, arrows) or β-COP (Figure 7F, arrows). This suggests that there are two closely apposed compartments or different membrane domains within a compartment. Thus, the fractionation and immunofluorescence data strongly suggest that the presence of the N-terminal extension shifts the distribution of syntaxin 5 away from the

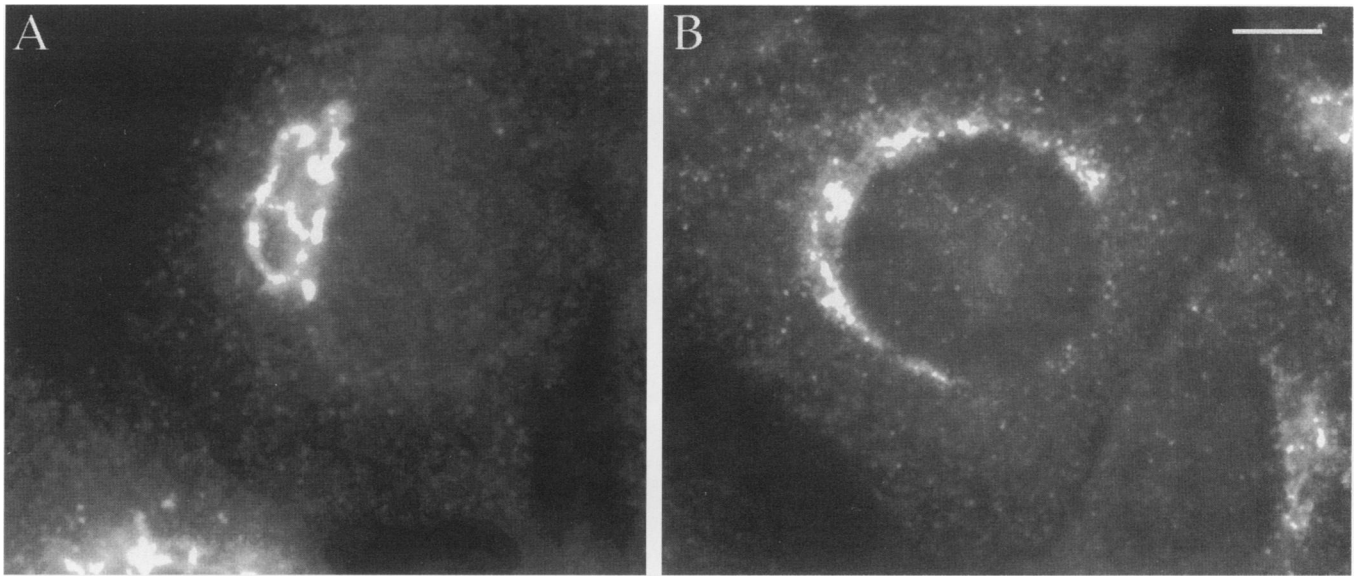


Figure 6. Distribution of syntaxin 5 isoforms by immunofluorescence microscopy. Vero cells were fixed, permeabilized, and labeled with affinity-purified antibodies against a recombinant syntaxin 5 (recognizing both forms; A) or anti-peptide antibodies (NHU6) against the N-terminal extension of syntaxin 5 (only recognizing the long form; B). Bar, 5 μm.

Golgi apparatus and toward more peripheral structures, including the intermediate compartment.

DISCUSSION

By using specific antibodies and sequencing upstream of the published start site, we have identified an isoform of syntaxin 5 with an N-terminal extension. It had the same biochemical properties published for the short form. Its resistance to extraction with sodium carbonate showed that it is a membrane protein. Its complete sensitivity to proteases showed that it had most of its mass on the cytoplasmic side of the membrane. It reacted with all four antibodies raised to the short form and reacted uniquely with two anti-peptide antibodies raised to the predicted N-terminal extension. Treatment for extended times with cycloheximide showed that the long form was not a precursor of the short because no change in the relative amounts was observed.

The two forms appear to be generated from a single mRNA by alternative initiation of translation. Northern blotting suggested a single species of mRNA (Bennett *et al.*, 1993; our unpublished results) and in vitro transcription/translation of the full-length cDNA generated both forms. Translation exclusively from the first ATG yielded the 42-kDa form, whereas translation from the second yielded the 35-kDa form. These were shown by immunoprecipitation to be the same proteins as those observed by Western blotting. Both were precipitated by antibodies to the common part of the sequence but only the 42-kDa form was precipitated by antibodies to the predicted N-terminal extension.

The 42-kDa protein was the minor form. Quantitation of rat tissue blots (Figure 1B) showed that the long form constituted 25–40% of the total syntaxin 5. In NRK cells it was 40–45% (Figure 1C) and, after in vitro transcription/translation in a reticulocyte lysate, 40% (Figure 3, lanes 1 and 4). There was about 40% in rat liver (Figure 1B) but only 10–20% in the Golgi membranes purified from rat liver (Figure 1A). This is because less of the 42-kDa form cofractionated with Golgi membranes when compared with the 35-kDa form (Figure 5, A and B). The minority amounts of the 42-kDa form can best be explained by the alternative initiation sites on the syntaxin 5 mRNA. The bases flanking the initiation site determine the efficiency with which it is recognized by the scanning ribosome. The optimal sequence is gccgcc**A**/Gcc(AUG)**GA**/Cu, the relative importance of each residue being indicated by uppercase boldface type > uppercase type > lowercase type (Grünert and Jackson, 1994). When applied to the syntaxin 5 mRNA, the first start site has only four matches (underlined) to the flanking bases (tgcgactcg[ATG]atc), whereas the second start site has 7 (cccgac**A**ct[ATG]tCc) including an important **A** at position −3. These start sites sequences would predict that less of the long form is translated, in accordance with the experimental results.

Examination of the N-terminal sequence revealed a double-arginine motif that was first identified as an ER retrieval signal in invariant chain and other type II

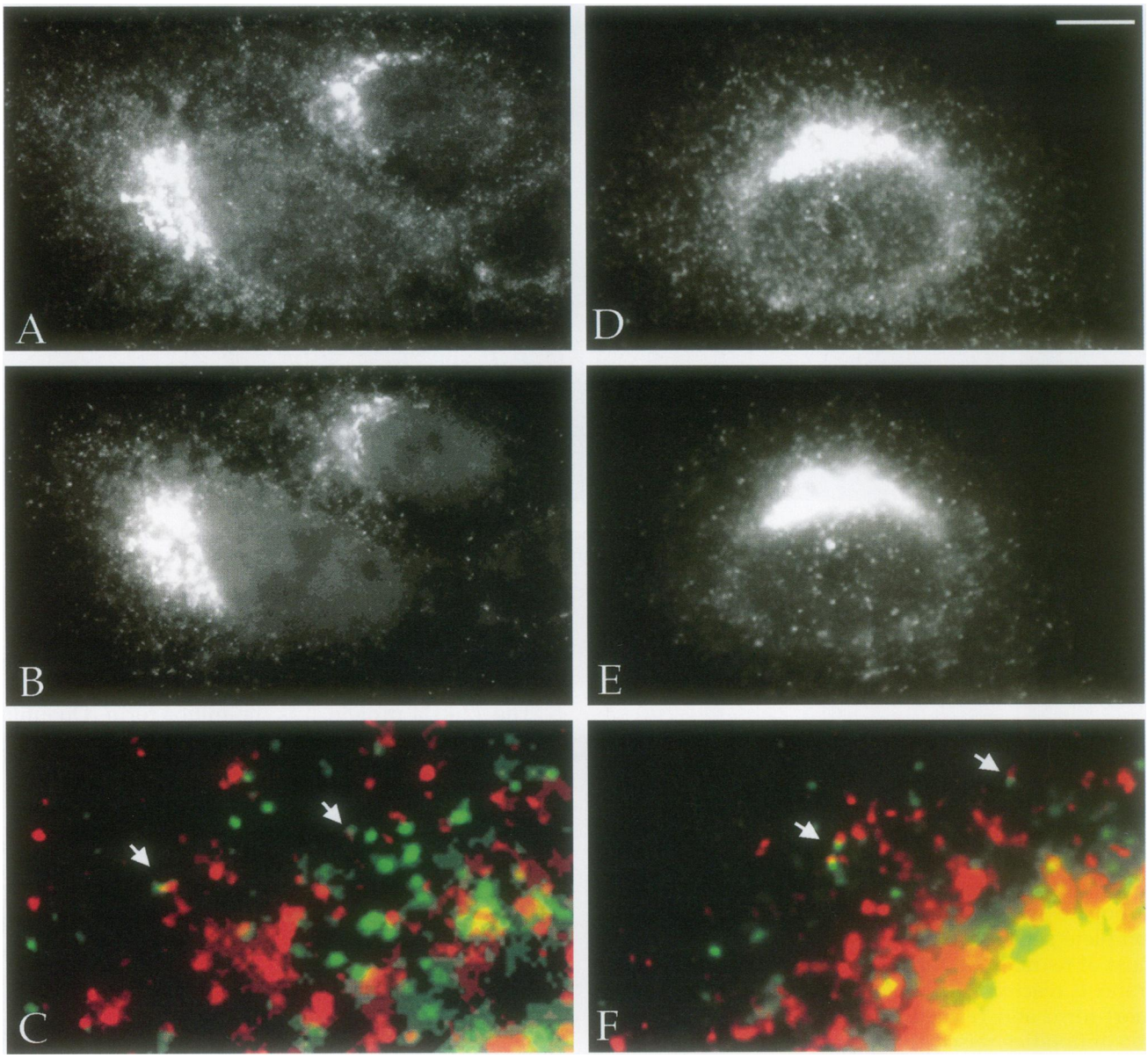


Figure 7. Distribution of the long form of syntaxin 5 relative to ERGIC53/p58 and β-COP. Vero cells were fixed, permeabilized, and double-labeled with affinity-purified anti-peptide antibodies (NHU6) against the N-terminal extension of syntaxin 5 (A and green in C) and a monoclonal antibody against the intermediate compartment marker ERGIC53/p58 (B and red in C) or NHU6 (D and green in F) and monoclonal antibody CM1A10 against β-COP (E and red in F). Merged images (3× magnification) are shown in C and F, and the arrows indicate overlap (yellow) in membrane profiles peripheral to the Golgi. Bar, 5 μm.

membrane proteins (Schutze *et al.*, 1994). Invariant chain exists predominantly in two forms that differ by an N-terminal extension of 16 amino acids containing the motif (Strubin *et al.*, 1986). The motif in the 42-kDa syntaxin 5 was shown to operate as an ER retrieval signal by grafting it onto the end of the p31 invariant chain lacking the motif. Instead of moving to endosomes as p31 normally does, the chimera was localized to the ER. Localization to the ER was dependent on the double-arginine motif because conversion of RKR to SSS restored transport to the endosomes. It is still not clear whether the double-arginine motif acts as a retention or a retrieval signal (Nilsson and Warren, 1994), but the latter seems more likely given the functioning of the closely related double-lysine motif found in type I membrane proteins (Nilsson *et al.*, 1989; Jackson *et al.*, 1990). This motif functions as a retrieval signal (Jackson *et al.*, 1993) by interacting

with the coatomer of retrograde COPI vesicles (Cosson and Letourneur, 1994; Letourneur *et al.*, 1994).

The two isoforms of syntaxin 5 had distinct, though overlapping, distributions in the cell. Biochemical fractionation showed that there was less of the long form in Golgi membranes but more in ER fractions, especially those containing intermediate compartment markers. Immunofluorescence microscopy showed that the long form did colocalize to a small extent with the intermediate compartment markers ERGIC53/p58 or β-COP in the cell periphery, but this was a minority of the total syntaxin 5-positive structures. Most of the peripheral structures did not stain with either of these markers or any others that we have tried. These structures are reminiscent of ER exit sites, but none of the available antibodies (Tang *et al.*, 1997) presently permit double labeling to confirm or deny this suggestion. We also do not yet know whether there is any of the short form of syntaxin 5 in these peripheral structures. This is suggested by the possibility that heterodimers can be formed (Figure 3, lane 7), at least in vitro. We have prepared a tagged version of the short syntaxin 5 stably expressed in HeLa cells but the presence of the myc tag at the N terminus clearly disrupts the cycling behavior of the protein. Definitive studies of the differential location of the two forms must await the generation of tagged C-terminal forms of both proteins.

The ER retrieval signal clearly shifts the distribution of the long form toward more ER-related structures but there appears to be little of the protein in the ER itself. This is not without precedent. Several type I proteins carry a double-lysine motif yet are not located in the ER. The mannose-binding lectin ERGIC53/p58 carries such a motif yet is located in the intermediate compartment and cycles between the ER and the Golgi. The motif appears to be weakened by the presence of an adjacent double-phenylalanine permitting exit from the ER (Itin *et al.*, 1995b). The same may be true for syntaxin 5 so further work will be needed to determine whether other residues in the N-terminal extension are responsible for weakening the ER retrieval signal.

We can only speculate at present on the function of the syntaxin 5 isoforms. It is interesting to note that the long form is not predicted from the budding yeast sequence (Hardwick and Pelham, 1992). There are multiple in-frame stop codons in the cDNA sequence upstream of the putative start codon. The predicted yeast protein sequence, and others (GenBank accession nos. U26648, L20822, and X78219), has what at first sight appears to be an ER retrieval signal but the double-arginine motif is split by an aspartic acid that presumably inactivates its function. If the yeast lacks the long form with an ER retrieval signal, what differences are there between the yeast and mammalian exocytic pathways that might explain the need for the long form in mammals? The most striking difference is the physical separation of the Golgi apparatus and the ER exit sites in mammals. In yeast the distances are very small because of the small size of the cell and the fact that there are multiple dispersed Golgi throughout the cell cytoplasm (Preuss *et al.*, 1992). Vesicles budding from the ER are never far from a Golgi. In mammals, the distances are much longer. Most of the ER exit sites are in the cell periphery (Bannykh *et al.*, 1996) and cargo must be transported 10 μm or more to the Golgi ribbon in the juxtanuclear region (Saraste and Svensson, 1991; Griffiths *et al.*, 1995). Vesicles budding from the ER might need a representative of the Golgi apparatus nearby and such an outpost could be provided by the long form of syntaxin 5. This outpost might be needed to form the tubulo-vesicular intermediates that are transported along microtubules to the juxtanuclear ribbon (Saraste and Svensson, 1991; Balch *et al.*, 1994). The short form of syntaxin 5, which resides mainly in the Golgi ribbon, would then receive cargo from local exit sites. Such a function would certainly explain the enrichment of the long form in peripheral punctate structures that could be part of a recycling pathway analogous to that marked by ERGIC53/p58 (Lippincott-Schwartz *et al.*, 1990; Itin *et al.*, 1995a; Tisdale *et al.*, 1997). A proper test of this function will, however, require antibodies that block syntaxin 5 function after microinjection. This is presently the focus of our attention.

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