To identify the targeting domains of syntaxin 6 responsible for its localization to the trans-Golgi network (TGN), we examined the subcellular distribution of enhanced green fluorescent protein (EGFP) epitope-tagged syntaxin 6/syntaxin 4 chimerae and syntaxin 6 truncation/deletion mutants in 3T3L1 adipocytes. Expression of EGFP-syntaxin 6 resulted in a perinuclear distribution identical to endogenous syntaxin 6 as determined both by confocal fluorescence microscopy and subcellular fractionation. Furthermore, both the endogenous and the expressed EGFP-syntaxin 6 fusion protein were localized to a brefeldin A-insensitive but okadaic acid-sensitive compartment characteristic of the TGN. In contrast, EGFP-syntaxin 6 constructs lacking the H2 domain were excluded from the TGN and were instead primarily localized to the plasma membrane. Although syntaxin 4 was localized to the plasma membrane, syntaxin 6/syntaxin 4 chimerae and syntaxin 6 truncations containing the H2 domain of syntaxin 6 were predominantly directed to the TGN. Importantly, the syntaxin 6 H2 domain fused to the transmembrane domain of syntaxin 4 was also localized to the TGN, demonstrating that the H2 domain was sufficient to confer TGN localization. In addition to the H2 domain, a tyrosine-based plasma membrane internalization signal (YGRL) was identified between the H1 and H2 domains of syntaxin 6. Deletion of this sequence resulted in the accumulation of the EGFP-syntaxin 6 reporter construct at the plasma membrane. Together, these data demonstrate that syntaxin 6 utilizes two distinct domains to drive its specific subcellular localization to the TGN.

Eukaryotic cells maintain an array of distinct membrane compartments, each outfitted with a unique collection of integral membrane proteins. These compartments serve multiple functions including the organized delivery of proteins to various intracellular destinations, a process accomplished largely through vesicular trafficking events (1–4). Despite the tremendous membrane lipid and protein flux through these intracellular trafficking nodes, many proteins show a remarkably stable, compartment-specific distribution under steady state conditions. Since membrane proteins are thought to be transported vectorially along the secretory pathway, a given protein may transiently occupy several compartments en route to its final destination. Indeed, delivery of proteins to various intracellular destinations may in some cases require a series of sorting decisions. One critical sorting step occurs at the trans-Golgi network (TGN), where proteins with specific targeting signals are incorporated into vesicles with defined trafficking itineraries (5–7). In addition, retrieval signals are often employed to return wayward proteins back to their resident membrane compartments (8–12). In contrast, membrane proteins without specific sorting signals are transported along the entire secretory pathway and accumulate at the plasma membrane under steady-state conditions (13).

Our understanding of vesicular trafficking is largely based on detailed studies of endoplasmic reticulum to Golgi trafficking and in the docking and fusion of synaptic vesicles with the presynaptic membrane (14–16). From these studies arose the SNARE hypothesis, which proposes that regulated interactions between v-SNAREs of donor membranes and t-SNAREs of acceptor membranes impart specificity to membrane trafficking and fusion events (14–16). However, analysis of specific v- and t-SNARE binding interactions demonstrate a high degree of promiscuity, at least in vitro (17, 18). Although additional accessory proteins may help to ensure binding selectivity, segregating v- and t-SNAREs in specific compartments may contribute significantly to membrane fusion specificity.

To date, 16 mammalian syntaxin family members have been identified, all of which localize to specific membrane compartments along the exocytic and endocytic pathways. The first group of syntaxins identified (syntaxins 1–4) are predominantly restricted to the plasma membrane, where they mediate constitutive and regulated vesicle trafficking events at the cell surface (19–21). In contrast, syntaxins 5, 6, 10, 11, and 16 are localized to different subcompartments within the Golgi apparatus (22–26), whereas syntaxins 7, 12, and 13 are found in the post-Golgi endosomal population (27–29).

Although the mechanisms and protein interaction domains responsible for v-SNARE localization have been investigated, there is less information with regard to the targeting of the syntaxin family of t-SNARE proteins, particularly in mammalian cells. Therefore, to further our understanding of t-SNARE targeting we have examined the functional domains of syntaxin 6, a t-SNARE localized to the TGN (23). We show here that the cytosolic H2 domain of syntaxin 6 plays a major role in TGN localization. Furthermore, the predicted α-helical H2 domain functions in concert with a YGRL plasma membrane retrieval

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** The abbreviations used are: TGN, trans-Golgi network; SNARE, SNAP receptor; v-SNARE, vesicle membrane SNAP receptor; t-SNARE, target membrane SNAP receptor; EGFP, enhanced green fluorescent protein; M6PR, mannose 6-phosphate receptor; TIB, transferrin receptor; Syn4, syntaxin 4; Syn6, syntaxin 6; BFA, brefeldin A; PBS, phosphate-buffered saline; HSP, high speed pellet; LSP, low speed pellet; PM, plasma membrane; CYT, cytoplasm; PCR, polymerase chain reaction; TM, transmembrane.
Fig. 1. The expressed EGFP-syntaxin 6 fusion protein localizes to the trans-Golgi network. Differentiated 3T3L1 adipocytes were either untreated or electroporated (50 μg) with the cDNA encoding for the full-length EGFP-Syn6 fusion protein as described under “Experimental Procedures.” Subsequently, the cells were then either incubated in the absence (Control, panels a, d, and g) or in the presence of 5 μg brefeldin A for 1 h (BFA, panels b, c, and h) or 0.5 μg okadaic acid for 6 h (Okadaic, panels e, f, and i). The cells were fixed and labeled with the giantin polyclonal antibody (panels a, b, and c) or the syntaxin 6 monomeric antibody (panels d, e, and f) and subjected to confocal fluorescence microscopy. In parallel, the fluorescence of the EGFP-Syn6 fusion construct was determined. These are representative fields of cells from three or four independent determinations (magnification, ×60).

EXPERIMENTAL PROCEDURES

Materials—Brefeldin A (Sigma) was prepared as a 5 mg/ml stock in methanol and used at a final concentration of 5 μg/ml. Okadaic acid (Calbiochem) was kept as a 100 μM stock in dimethyl sulfoxide and used at a final concentration of 0.5 μM. Supersignal and Supersignal Ultra enhanced chemiluminescence reagents were purchased from Pierce and used according to the manufacturer’s directions. Syntaxin 4 polyclonal antibody was obtained as described previously (30). The syntaxin 6 monoclonal antibody was purchased from Transduction Laboratories. EGFP polyclonal antibody was purchased from CLONTECH. Transferrin receptor antibody was from Zymed Laboratories Inc. Grb2 antibody was from Santa Cruz. The cation-independent mannose 6-phosphate receptor (M6PR) antibody was a gift from Dr. Richard G. MacDonald, University of Nebraska. Antibodies directed against the ferrin receptor antibody was from Zymed Laboratories Inc. Grb2 antibody was from Santa Cruz. The cation-independent mannose 6-phosphate receptor (M6PR) antibody was a gift from Dr. Richard G. MacDonald, University of Nebraska. Antibodies directed against the Golgi specific resident protein giantin were kindly provided by Dr. Isabelle Moosbrugger, Institute of Immunology and Molecular Genetics, Karlsruhe, Germany. Fluorescent secondary antibodies were purchased from Jackson Immunoresearch Laboratories. Horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce. The full-length syntaxin 6 cDNA was obtained by PCR amplification from a mouse fat cDNA library (CLONTECH) and was also provided by Dr. Richard H. Scheller, Stanford University School of Medicine.

Green Fluorescent Protein Fusion Constructs and Syntaxin 6/Syntaxin 4 Chimerae—To generate the amino-terminal EGFP fusion constructs, the polymerase chain reaction was used to introduce appropriate restriction enzyme sites on the 5′ and 3′ termini of cDNAs encoding syntaxins 4 and 6. The PCR products were then cloned in-frame with the EGFP coding sequence of the pEGFP-C series vectors (CLONTECH). Syntaxin chimeras and internal deletion constructs were generated using the PCR-based overlap extension method as described (31). Truncations of the syntaxin 6 cDNAs were generated by PCR using internal primers that hybridized to the specific region of interest. The PCR products were then cloned in frame with EGFP. To generate the Syn6(YGRL/234) and Syn6(AGRL/234) constructs, synthetic oligonucleotides encoding the sequences TDYGRDLDR or TDGRDLDR were cloned in-frame with the syntaxin 6 transmembrane domain.

Intracellular Membrane Localization of EGFP-Syntaxin 6—Previous studies in several fibroblast cell lines have indicated that syntaxin 6 is predominantly localized to the TGN (23, 34, 35). To examine the distribution of syntaxin 6 in 3T3L1 adipocytes, we compared the localization of endogenous syntaxin 6 with the Golgi marker giantin by immunofluorescence confocal microscopy (Fig. 1, panels a and d). Giantin was pri-
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Fig. 2. Schematic representation of syntaxin 6 truncation/deletion and syntaxin 6/syntaxin 4 chimeric proteins used in this study. The syntaxin 6 sequence is shown in white, whereas the syntaxin 4 sequence is depicted in gray shading. EGFP was fused to the amino terminus of all constructs. The numbers in parentheses refer to the amino acid residue at the splice junctions, or the amino acid position at which the proteins were truncated. The 7-amino acid sequence TDRYGRL was deleted in the construct Syn6ΔYGRL. The 63-amino acid H2 domain was deleted in the construct Syn6ΔH2. The H1, H2 and HA, HB, HC, and Hcore domains are predicted α-helical coiled-coil secondary structural domains in syntaxin 6 and syntaxin 4, respectively.

The H2 Domain of Syntaxin 6 Confers TGN Localization—As previously observed, the endogenous syntaxin 6 protein as well as expression of EGFP-Syn6 displayed the typical perinuclear distribution characteristic of the TGN as well as a relatively low level of plasma membrane localization (Fig. 3, panels a and b). In contrast to the TGN localization of syntaxin 6, both endogenous syntaxin 4 (data not shown) and EGFP-syntaxin 4 was localized predominantly to the plasma membrane (Fig. 3, panel c). Since integral membrane proteins lacking specific targeting signals localize to the plasma membrane by a default mechanism (13), we reasoned that chimeric syntaxin 6/syntaxin 4 proteins would allow us to identify sequence motifs within syntaxin 6 necessary for TGN localization. Using this approach, expression of the EGFP-Syn6(231)/Syn4(261) chimera resulted in a predominant perinuclear localization, whereas expression of the Syn6(160)/Syn4(185) chimera was mainly localized to the plasma membrane (Fig. 3, panels c and d). The only distinct domain between these two chimeras that could account for perinuclear localization was the syntaxin 6 H2 domain. Therefore, we next examined two syntaxin 6 truncations, EGFP-Syn6Δ160, which contains the H2 and transmembrane domains, and EGFP-Syn6Δ234, which contains only the transmembrane domain. EGFP-Syn6Δ160 showed a pronounced perinuclear distribution in addition to a plasma membrane localization, whereas EGFP-Syn6Δ234 resulted in a diffuse intracellular signal along with a plasma membrane...
Fig. 3. Localization of expressed EGFP-syntaxin 6, EGFP-syntaxin 4, and several EGFP-syntaxin 6/syntaxin 4 chimeras and truncations in 3T3L1 adipocytes. Differentiated 3T3L1 adipocytes were electroporated (50 μg) with the indicated EGFP-fusion constructs as described under “Experimental Procedures.” The cells were then fixed and subjected to confocal fluorescence microscopy. These are representative field of cells from three or four independent determinations (magnification, ×60).

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Localization (Fig. 3, panels f and g). Furthermore, to demonstrate that the syntaxin 6 H2 domain was sufficient to confer a perinuclear distribution, we also examined the localization of a chimeric protein containing only the syntaxin 6 H2 domain fused to the syntaxin 4 transmembrane domain (Fig. 3, panel h). Consistent with the other chimeric and deletion constructs, EGFP-Syn6(161–234)/Syn4(TM) displayed a strong perinuclear localization.

To ensure that the perinuclear distribution of the EGFP-Syn6(231)/Syn4(261), EGFP-Syn6(161–234)/Syn4(TM), and EGFP-Syn6(Δ160) constructs were indicative of the Golgi, we next examined the co-localization of these expressed fusion proteins with giantin or endogenous syntaxin 6 (Fig. 4). Since the resolution afforded by confocal fluorescence microscopy is not sufficient to distinguish between the Golgi stacks and TGN, the distribution of giantin should overlap that of syntaxin 6. As expected, co-localization of giantin with EGFP-Syn6 demonstrated an identical perinuclear distribution (Fig. 4A, panels a–c). Similarly, there was a strong correspondence between the intracellular perinuclear distribution of giantin with the EGFP-Syn6(231)/Syn4(261) chimera (Fig. 4A, panels d–f). In addition, since the EGFP-Syn6(Δ160) fusion only contains the transmembrane and H2 domains of syntaxin 6, we were able to co-localize this fusion protein with endogenous syntaxin 6 (Fig. 4B). As is apparent, the expressed EGFP-Syn6(Δ160) construct displayed a distribution similar to endogenous syntaxin 6 (Fig. 4B, panels a–c). Similarly, the EGFP-Syn6(161–234)/Syn4(TM) fusion protein was also predominantly co-localized with endogenous syntaxin 6. However, it is also important to note that both EGFP-Syn6(Δ160) and Syn6(161–234)/Syn4(TM) displayed a greater degree of plasma membrane localization than endogenous syntaxin 6, full-length EGFP-Syn6, or the Syn6(Δ160) fusion proteins (see below).

In any case, to confirm that these constructs were localized to the TGN rather than throughout the Golgi complex, cells were treated with BFA or okadaic acid. Treatment with BFA resulted in a more spherical and concentrated perinuclear distribution of EGFP-Syn6, EGFP-Syn6(231)/Syn4(261), EGFP-Syn6(Δ160), and EGFP-Syn6(161–234)/Syn4(TM), consistent with TGN localization (Fig. 5, panels a, b, d, e, g, h, j, and k). In contrast, disruption of both the Golgi stack and TGN with okadaic acid resulted in the complete loss of perinuclear localized EGFP-Syn6, EGFP-Syn6(231)/Syn4(261), EGFP-Syn6(Δ160), and EGFP-Syn6(161–234)/Syn4(TM) (Fig. 5, panels c, f, i, and h).

To further verify the localization of the key syntaxin constructs by an independent and more quantitative method, we used differential centrifugation to isolate subcellular membrane fractions from 3T3L1 adipocytes (Fig. 6). Cells were first electroporated with the EGFP fusion constructs, then collected, pooled, and centrifuged as described under “Experimental Procedures.” This protocol yielded HSP, LSP, PM, and cytosolic (CYT) fractions. To assess the efficacy of the fractionation procedure, we used several endogenous marker proteins known to localize to specific intracellular membrane compartments. The cation-independent M6PR is a marker for late endosomes and lysosomes and was partitioned in the HSP fraction. In contrast, TRβ is primarily a marker for early endosomes but is also found in the TGN and plasma membrane and was partitioned predominantly into the LSP fraction, with lower levels in the PM fraction. As expected, syntaxin 4 partitioned mostly with the PM fraction, whereas syntaxin 6 partitioned mostly with the LSP fraction, with low but detectable levels in the PM fraction. The small cytosolic protein Grb2 was detected almost exclusively in the CYT fraction.

Consistent with confocal fluorescence microscopy, EGFP-Syn6 was distributed in a similar pattern as that of the endogenous syntaxin 6 protein. The majority of the endogenous syn-
The EGFP-Syn6/Syn4(261) construct was electroporated into 3T3L1 adipocytes, yielding HSP, LSP, PM, and CYT fractions. The fractions were then subjected to confocal fluorescence microscopy. These are representative field of cells from three or four independent determinations (magnification, ×60).

**FIG. 6.** Determination of syntaxin 6 localization by subcellular fractionation. Differentiated 3T3L1 adipocytes were independently electroporated (50 μg) with the cDNA encoding the full-length EGFP-Syn6 (panels a, b, and c), the EGFP-Syn6/Syn4(261) (panels d, e, and f), the EGFP-Syn6(231)/Syn4(261) (panels g, h, and i) or the EGFP-Syn6(161–234)/Syn4(TM) (panels j, k, and l) fusion proteins as described under "Experimental Procedures." Subsequently, the cells were incubated in the absence (Control, panels a, d, g, and j) or in the presence of 5 μM brefeldin A for 1 h (BFA, panels b, e, h, and k) or 0.5 μM okadaic acid for 6 h (Okadaic, panels c, f, i, and l). The cells were fixed and subjected to confocal fluorescence microscopy. The marker proteins used were as follows: M6PR, TfR, Syn4, Syn6, EGFP-Syn6, Grb2, HSP, LSP, PM, and CYT. The Syntaxin 6 H2 Domain Cooperates with a Plasma Membrane TDRYGRLDRE Retrieval Sequence—Although the H2 domain can impart TGN localization, Western blot analysis revealed that the H2 domain alone may not direct TGN targeting as efficiently as the full-length syntaxin 6 or EGFP-Syn6(231)/Syn4(261) fusion protein. The relatively high levels of the EGFP-Syn6(Δ160) and Syn6(Δ161–234)/Syn4(TM) constructs detected at the cell surface (Fig. 6) may reflect the absence of a potential plasma membrane internalization or retrieval sequence, which could lead to accumulation of these truncated proteins at the plasma membrane. In this regard, it has been reported that a YQRL sequence can function as a tyrosine-based plasma membrane internalization signal (37, 38, 45, 50). Syntaxin 6 contains a highly related TDRYGRL-DRE sequence or mutant (TDRAGRLDRE) with the TDRYGRLDRE sequence or mutant (TDRAGRLDRE) with the just the transmembrane domain of syntaxin 6 to generate the chimeric constructs Syn6(YGRL/Δ234) and Syn6(AGRL/Δ234), respectively (Fig. 2).

We initially compared the localization of EGFP-Syn6 and EGFP-Syn6(Δ160) with EGFP-Syn6(ΔYGRL), EGFP-Syn6(ΔH2), and EGFP-Syn6(ΔYGRL/ΔH2) deletion constructs (Fig. 7). As previously observed, expression of EGFP-Syn6 resulted in a predominant TGN localization with little plasma membrane labeling, comparable to the distribution of endogenous syntaxin 6 (Fig. 7, panel a). Although EGFP-Syn6(Δ160) also resulted in a strong TGN localization, there was also a significant increase in the amount localized to the plasma membrane (Fig. 7, panel b). Similarly, the Syn6(ΔYGRL) construct, which carries an internal deletion of the TDRYGRL sequence also showed strong TGN and plasma membrane localization (Fig. 7,
DISCUSSION

Subcellular compartmentalization is a defining feature of eukaryotic cells and is necessary to organize the intracellular environment into functionally distinct sets of membrane-bound organelles. In order to manage the large amount of protein and lipid flux through this vast array of cellular compartments, membrane trafficking and fusion events must be tightly regulated and highly specific. As originally conceived, the SNARE hypothesis proposed that fusion between distinct membrane structures involves specific pairing of v- and t-SNARE partners in the vesicle and target membranes, respectively (14–16, 51, 52). However, recent evidence suggests that v- and t-SNAREs can interact promiscuously in vitro to form stable, SDS-resistant complexes (17, 18). In addition, in vivo studies have indicated that overexpression of certain t-SNARE isoforms can compensate for the genetic loss of other t-SNARE isoforms, apparently through interactions with noncognate v-SNARE molecules (53, 54). These results have led to the suggestion that additional proteins, such as members of the Rab family of small GTPases, may be important determinants of membrane fusion specificity (55).

Given the tremendous need for maintaining fusion specificity, there are likely to be multiple mechanisms for ensuring that the appropriate membrane compartments participate in the fusion process. In addition to control mechanisms involving protein-protein based interactions, localization of distinct
SNARE partners to define intracellular compartments could contribute substantially to membrane fusion specificity by spatially segregating the fusogenic SNARE molecules. Indeed, sequestering SNARE molecules such as the syntaxin family of t-SNARE proteins in specific compartments may have the effect of demarcating the precise sets of membranes that will have the potential to participate in a particular fusion process. Consistent with this notion, all the known v- and t-SNARE proteins are localized to specific membrane compartments and are not randomly distributed (56, 57). Thus, by identifying the localization signals in these molecules, it may be possible to directly test the hypothesis that restricting SNAREs to specific compartments contributes to and/or defines membrane fusion specificity.

To address this issue, we have begun to dissect the molecular basis for the subcellular compartmentalization of syntaxin 6. Taking advantage of several deletion mutations and syntaxin 6/syntaxin 4 chimeric proteins, in conjunction with established Golgi markers and selective disruptors of the Golgi stacks versus the TGN, we have identified the H2 domain (amino acids 166–228) of syntaxin 6 as a specific TGN localization domain. This domain is adjacent to the transmembrane segment and is predicted to adopt a 63-amino acid amphipathic α-helical structure (23). Although the binding partner(s) for the syntaxin 6 H2 domain have not been identified, the corresponding domain of syntaxin 1 can bind α-SNAP, VAMP2, and SNAP25 through coiled-coil domain interactions (58). In this regard, amphipathic α-helical domains have also been reported to function as sorting signals for VAMP2 and the interleukin 2 receptor β chain (59, 60), presumably by interacting with specific protein partners. Thus, it seems likely that the syntaxin 6 H2 domain mediates TGN localization through interactions with a retention receptor. At present, syntaxin 6 has been shown to interact with several other TGN proteins (GS32, VAMP4, and VPS45) and has been reported to colocalize with clathrin and the AP1 complex by immunogold electron microscopy (61–63). However, it is not known whether these interactions involve the syntaxin 6 H2 domain or if they are sufficient, or even necessary for TGN targeting. Future studies will be required to clarify these issues.

Based upon the data presented in this report, the H2 domain is clearly an important signal for localizing syntaxin 6 to the TGN. However, the Syn6(Δ160) and Syn6(161–234)/Syn4(TM) constructs, which contain just the H2 and TM domains, accumulated at the plasma membrane to a significantly greater extent compared with full-length EGFP-syntaxin 6. Inspection of the syntaxin 6 amino acid sequence revealed the presence of a potential tyrosine-based sorting signal YGRLocmidway between the H1 and H2 predicted α-helical domains. Similar tyrosine-based motifs have been implicated in the localization of various proteins to different membrane compartments including endosomes, lysosomes, basolateral cell surface membranes, and the TGN (50, 64–68). Although deletion of this tyrosine-based sequence did not prevent TGN localization, there was a detectable increase of the reporter construct at the plasma membrane. In fact, subcellular fractionation analysis indicated that similar proportions of plasma membrane/TGN localization occurred for the YGRL deletion (Syn6(ΔYGR)), the syntaxin 6 deletion (Syn6(Δ160)), and the syntaxin 6 H2 domain/syntaxin 4 transmembrane domain chimera (Syn6(161–234)/Syn4(TM)), all of which lack the YGRL motif. These data suggest that the H2 domain functions to retain syntaxin 6 in the TGN, whereas the YGRL motif may function to retrieve syntaxin 6 proteins that have escaped to the plasma membrane. Consistent with this interpretation, the Syn6(ΔH2) reporter construct, which lacks just the H2 domain, showed relatively weak plasma membrane localization, apparently because the intact YGRL motif was functioning as an internalization signal. In contrast, the double deletion mutant Syn6(YGRL/AH2) was predominantly plasma membrane localized since both the TGN retention signal and the plasma membrane internalization motif were absent. To further investigate the potential internalization function of the YGRL motif, we fused the YGRL sequence (TDNYRGLDRE) directly to the transmembrane domain of syntaxin 6 (Syn6(ΔYGR/A234)). This reporter construct showed reduced plasma membrane levels and increased perinuclear localization when compared with a control construct wherein the conserved tyrosine was mutated to an alanine (Syn6(ΔGR/A234)). Thus, these data are consistent with the YGRL motif playing a relatively minor role in localizing syntaxin 6 to the TGN by functioning as a plasma membrane internalization signal.

In any case, we can now postulate the following model for the efficient localization of syntaxin 6 to the TGN. Following its initial biosynthesis, syntaxin 6 is transported vectorially along the secretory pathway until it reaches the TGN, where it interacts with a resident retention receptor via the H2 domain. This process results in the trapping of a substantial amount of syntaxin 6 in the TGN. However, a small but significant amount of syntaxin 6 escapes to the cell surface, where the YGRL motif appears to play a role in recycling syntaxin 6 back to the TGN. Although the dynamics of this process remain speculative at present, future studies using time lapse confocal fluorescent microscopy should help to clarify these kinetic events.

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