Sorting Motifs in the Intracellular Domain of the Low Density Lipoprotein Receptor Interact with a Novel Domain of Sorting Nexin-17*

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The low density lipoprotein (LDL) receptor plays a major role in maintaining human plasma cholesterol levels and mutations in the gene cause familial hypercholesterolemia. The LDL receptor (LDLR) pathway has been well characterized, but little is known of proteins involved in its complex intracellular sorting and trafficking. Sorting nexin 17 (SNX17) has recently been implicated in LDLR intracellular trafficking. We show here that endogenous SNX17 is highly expressed in several cell types and is localized partially in early endosomes. We found that the PX domain of SNX17 is required for its endosomal localization but does not interact directly with the LDL receptor. A novel domain containing a FERM-like domain of SNX17 is needed for its interaction with the LDL receptor. Mutations in the NPXY motif of the LDL-receptor cytoplasmic tail that disrupt internalization also disrupt its interaction with SNX17, whereas mutations elsewhere had little effect. When transiently overexpressed in Chinese hamster ovary cells, SNX17 localized to large vesicular structures and disrupted normal trafficking of the LDL receptor in a PX domain-dependent manner. These results suggest that SNX17 plays a role in the cellular trafficking of the LDL receptor through interaction with the NPXY motif in its cytoplasmic domain and interaction of the PX domain with subcellular membrane compartments.

The low density lipoprotein (LDL) receptor mediates cellular uptake and degradation of extracellular LDL by high affinity binding of LDL to receptors on the cell surface followed by internalization of the receptor-ligand complex in clathrin-coated vesicles. These vesicles then release their protein coat and fuse with other vesicles to form early endosomes, where acidification causes dissociation of the ligand-receptor complex. The ligand is transported to the lysosomes and degraded, while the LDL receptor recycles back to the plasma membrane, specifically to the basolateral surface in polarized cells, either directly from the early endosomes or via recycling endosomes (1). Much of what is known about the LDL receptor pathway has come from studies of its malfunction in cells from patients with familial hypercholesterolemia, who usually have mutations in the LDL receptor gene that affect many different aspects of the pathway. The LDL receptor pathway also malfunctions in individuals with null mutations in the gene for a novel adaptor protein called ARH (from autosomal recessive hypercholesterolemia) that is essential for internalization of the receptor from the cell surface (2, 3).

Apart from ARH, little is known about other proteins involved in the complex pathways of intracellular sorting and trafficking of the LDL receptor. Based on the effects of single amino acid substitutions, the carboxyl-terminal cytoplasmic domain of the LDL receptor appears to contain the necessary information for many aspects of LDL-receptor trafficking, including clustering in clathrin-coated pits, internalization, recycling, and sorting of the receptor to the basolateral membrane in polarized cells. For example, an FDNPVY motif has been shown to be essential for internalization of the receptor, because mutations in this motif result in LDL receptors that fail to cluster in clathrin-coated pits. The FDNPVY motif of the LDL receptor binds to the PTB domain of ARH in vitro, providing further evidence that ARH functions as an accessory protein for endocytosis of the LDL receptor. A basolateral sorting signal comprising a proximal motif that overlaps with the NPXY internalization motif and a second distal motif has been shown to be important both for sorting of newly synthesized receptor to the basolateral plasma membrane and for sorting of the receptor after internalization (4). Mutations in this region of the LDL receptor that interfere with its basolateral sorting have also been identified in patients with familial hypercholesterolemia (5), but the one or more proteins that contribute to the sorting machinery have yet to be identified conclusively.

The existence of novel genetic defects in internalization prompted us to investigate additional proteins involved in the intracellular trafficking and sorting of the LDL receptor. Using the yeast two-hybrid system to screen a human liver cDNA library with the cytoplasmic domain of the LDL receptor, we identified a novel protein that interacted strongly with the cytoplasmic domain of the LDL receptor (6). Florian and colleagues (7) identified this same protein in a yeast two-hybrid screen with the cytoplasmic domain of P-selectin. These authors assigned the protein to the sorting nexin family as sorting nexin-17 (SNX17), based on the presence of a characteristic PX domain (8). Stockinger and co-workers (9) identified the murine

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† The abbreviations used are: LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; SNX, sorting nexin; PX, phox; FERM, protein 4.1, Ezrin, radixin, moesin; GST, glutathione S-transferase; GFP, green fluorescent protein; RFP, red fluorescent protein; CHO, Chinese hamster ovary; Ab, antibody; AP-2, adaptor-protein complex 2; EGF, epidermal growth factor.
homologue of SNX17 as a protein that bound to the cytoplasmic tail of murine LR-8, another member of the LDL-receptor gene family. These authors reported that stable expression of SNX17 in mouse embryonic fibroblasts increased the internalization index of LDL receptor-mediated uptake of LDL and concluded that SNX17 must be involved in trafficking of the LDL receptor. To investigate how SNX17 functions in intracellular trafficking, we have mapped the domains or residues in the amino terminus that are important for their interaction and have investigated which domains of SNX17 determine its subcellular localization.

MATERIALS AND METHODS

Plasmid Constructs—pLexA (LexA-(1-202), HIS3, Amp") was used for the expression of all bait proteins in yeast. The cDNA for the whole cytoplasmic tail (nucleotides 2441–2817) of the LDLR was amplified and cloned as an EcoRI/Smal fragment into the pLexA EcoRI site downstream of the LexA sequence to produce pLexA-LDLR/CT. pLexA-LDLR/CT contains the LDLR cytoplasmic tail cDNA (nucleotides 2441–2509) with a STOP codon introduced at codon 812. Each of the NPVY (amino acids 804–807) residues in pLexA-LDLR/CT were individually replaced with alanine by site-directed mutagenesis (Unique Site Elimination mutagenesis kit, Amersham Biosciences, UK), in some cases with plasmids kindly provided by H. Hobbs (10). SNX17 mutant constructs were amplified by PCR and cloned as EcoRI or EcoRI/XhoI fragments into pEGFP (Invitrogen) to produce a fusion protein with GFP at the amino terminus of SNX17 or into pcDNA3.1 (Invitrogen). They were also cloned into pEGFP (Invitrogen) to produce a fusion protein (RFP) at the amino-terminal end of SNX17. The RFP mamma-protein with GFP at the amino terminus of SNX17 or into pcDNA3.1 modified to produce a fusion protein with monomeric red fluorescent protein (RFP) at the amino-terminal end of SNX17. The RFP mamma-lion expression vector, a kind gift of Drs. P. Cullen and D. Stephens (Bristol), was constructed by inserting the coding sequence of mono-meric RFP (11) into pEGFP (Invitrogen) to produce a fusion protein with GST at the amino terminus of SNX17 or into pcDNA3.1 modified to produce a fusion protein with GST-luciferase.

Analysis of Fusion Proteins—Saccharomyces cerevisiae EGY48[pop-lacZ] was grown and used for all transformations as described by the supplier (Yeast Protocols Handbook, Clontech, Basingstoke, UK). For analysis of fusion proteins, yeast extracts were prepared from mid log phase cultures of transformants in rich medium with galactose and raffinose at 30 °C and assayed for total protein content (D280), protein assay kit, Bio-Rad, Hemel Hempstead, UK). Extracts were fractionated by SDS-PAGE, followed by transfer using a wet blotting technique and blocked in 1% (w/v) bovine serum albumin and 0.05% (w/v) saponin. When antibodies were used, cells were incubated with primary antibodies for 1 h in Citriabbit anti-EL (CLD Biociences, UK), diluted 1/40) and then with Alexa 488/588-conjugated goat anti-mouse/ rabbit IgG (highly cross-absorbed; Molecular Probes Europe BV, Leiden, Netherlands, diluted 1/100–1/200) for 1 h, both at ambient temperature. Coverslips were mounted on slides with Vectashield anti-fade mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories Inc., Peterborough, UK). Cells were viewed by using a Leica confocal microscope using the 100× oil objective, and the images were analyzed using Leica confocal software (Leica Microsystems (UK) Ltd., Milton Keynes, UK).

CHO cells were seeded into wells containing sterilized coverslips at ~50% confluence, then transiently transfected with the FuGENE-plasmid

RESULTS

Screen of a Liver Yeast Two-hybrid Library for Proteins Interacting with the Cytoplasmic Tail of the LDL Receptor—When ~1.8 × 10^6 clones of a human liver cDNA library were screened with amino acid residues 789–839 of the LDLR cytoplasmic tail, 31 clones reproducibly showed strong to moderate interaction. Of these, thirteen (42%) encoded a known cDNA (14), now named sorting nexin-17 (SNX17) (7), whereas the rest were classed as probable false positives (15). No growth occurred when yeast were transfected with pB42AD-SNX17 alone or together with empty pLexA vector, and no blue color was seen in yeast transformed with pLexA-CT and pB42AD-SNX17 but grown in the absence of the inducer. Direct physical interaction between SNX17 and the LDLR cytoplasmic domain was confirmed in a pull-down assay with the LDLR-cyttoplasmic tail fused to immobilized GST and SNX17 translated in vitro. As shown in Fig. 1, SNX17 bound to the GST-LDLR cytoplasmic tail, but not to GST alone or to an unrelated GST fusion protein (GST-luciferase).

The NPVY Motif in the LDL Receptor Is Required for Its Interaction with SNX17—Because it has been suggested that SNX17 is involved in LDL receptor trafficking, we investigated the effect of mutations in the LDLR that have been shown to influence different aspects of its trafficking, for example internalization (10, 16) or basolateral sorting (4). To analyze their effect on the ability to interact with SNX17,
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stable transformants of yeast expressing the SNX17/activation domain construct were co-transformed with wild type or mutant pLexA-LDLR/CT mutants and assayed for β-galactosidase activity. All the variants were expressed at approximately the same level (Fig. 2A).

Truncation of the cytoplasmic tail at residue 812 (LDLR E812X) reduced the strength of interaction with SNX17 to −30% of that with the complete tail (Fig. 2B). This reduction in binding was considerably greater than the reported effect of this same mutation on internalization of the LDL receptor (74% of normal (10)). Nonetheless, with the value for the truncated tail set at 100%, the effect of replacing each of the NPVY residues in the cytoplasmic tail with alanine on the interaction between the truncated tail and SNX17 was very similar to that observed previously on internalization (Fig. 2C), in that substitution of Asn-804, Pro-805, or Tyr-807 with alanine reduced the interaction, to 20% of the wild-type protein. Expression of a series of truncations of this protein revealed that regions at both ends are critical for interaction with the LDL receptor (Fig. 2B). At the amino-terminal end of the fragment, removal of residues 113–137 reduced the interaction to 7.8% of control. At the carboxyl-terminal end, removal of 45 residues increased its ability to interact with the LDL-receptor tail 2-fold, whereas loss of another 36 residues, leaving residues 113–389, resulted in interaction of 113% of control, which was abolished by removal of 56 additional residues. Thus the smallest fragment that retained the ability to interact strongly with the LDL-receptor cytoplasmic tail was the region comprising residues 113–389. Pull-down assays of the SNX17 fragments translated in vitro by immobilized GST-LDLR cytoplasmic tail fusion protein provided independent qualitative confirmation of these findings (Fig. 5).

Endogenous Expression of SNX17 Protein—Immunoblotting of extracts of human skin fibroblasts, Epstein-Barr virus immortalized lymphocytes, HepG2, or HeLa cells with a rabbit anti-serum raised against an amino-terminal peptide of SNX17 revealed a single major band of 47 kDa and similar to that of the protein expressed from the cDNA in heterologous cells. When fibroblast extracts were subjected to centrifugation at 150,000 × g, some SNX17 was present in the membrane pellet, although the majority of the protein was cytosolic (Fig. 6). In contrast, virtually all the LDL receptor protein and the majority of adaptor-protein complex 2 (AP-2) were found in the membrane pellet. Confocal immunofluorescence microscopy of human skin fibroblasts stained with specific antibodies revealed that a proportion of endogenous SNX17 was vesicular, where it co-localized with EEA1, suggesting that it was associated with early endosomes (Fig. 7A). Not all EEA1 structures contained SNX17, but all vesicular SNX17 appeared to be in EEA-containing structures. It was not possible to show specific interaction between endogenous SNX17 and endogenous LDL receptors; in our hands, protein interactions with intact endogenous LDL receptor protein are difficult to demonstrate, because stringent conditions are required to solubilize this integral membrane protein.
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The PX Domain of SNX17 Determines Its Localization in Early Endosomes—To investigate which domains of SNX17 are required for normal endosomal localization but implied that the PX domain may be critical, as has been seen for the endosomal localization of other sorting nexins. For example, substitution of a critical lysine residue in the PX domain of SNX1 has been shown to disrupt its ability to bind to phosphatidylinositol 3-phosphate and thereby localize in early endosomes (20). Comparison of the sequences of SNX1, SNX4, and SNX17 with the p40Pro sequence indicated that this lysine residue was conserved in all four sequences (Fig. 8). To explore this, we also expressed SNX17 fused to the cytoplasmic terminus of monomeric red fluorescent protein (11). The subcellular distribution of RFP-SNX17 was similar to that of endogenous SNX17. When we expressed full-length RFP-SNX17 with the conserved lysine residue (RFP-SNX17K62A) substituted with alanine, the mutant RFP-SNX17K62A protein was similarly expressed at a similar level to RFP-SNX17, but was located entirely in the cytoplasm (Fig. 9A). However, an intact PX domain alone was insufficient to allow endosomal localization, because RFP fused to the PX and FERM-like domains (residues 1–274; RFP-SNX17K62A) was also cytosolic, showing the same pattern in the cells as RFP alone. Clearly the full-length intact protein is required for normal subcellular localization via interactions through the PX domain. We also found that the LDL receptor partially co-localized with RFP-SNX17 when the two proteins were transiently co-expressed in the cells (Fig. 9B). However, neither RFP-SNX17K62A nor RFP-SNX17K62A co-localized with the LDL receptor, showing that the presence of SNX17 in endosomes was a requirement for co-localization with the LDL receptor (Fig. 9B). Expression of the normal or mutant RFP fusion proteins at a moderate level did not appear to disrupt LDL receptor localization in the cells.

We hypothesized that the truncated form of SNX17 that binds strongly to the LDL receptor in vitro, but does not localize to endosomes, might interfere with LDL receptor trafficking as a dominant negative inhibitor of the LDL receptor pathway. We tested this hypothesis in various ways. First, we examined the localization of endogenous LDL receptor protein in fibroblasts transfected with normal and mutant SNX17 constructs, but immunofluorescence detection of endogenous LDL receptor protein was insufficiently sensitive to determine whether trafficking was disrupted. We also compared the uptake of BODIPY®-LDL by untransfected cells and cells transfected with different SNX17 constructs but were unable to detect any quantitatively significant difference in the amount of BODIPY®-LDL in the cells (data not shown). Prolonged overexpression of SNX17-FLAG in CHO cells resulted in the formation of large abnormal vesicular structures (Fig. 9C, panels a and d) reminiscent of the disrupted endosomes seen in cells seen in cells overexpressing Rab-5 (21) or sorting nexin-1 (20). Overexpression of SNX17113–425-FLAG (Fig. 9C, panel b) or SNX17275–470-FLAG (Fig. 9C, panel c) did not disrupt the endosomal pattern in the cells. These abnormal structures were never seen in skin fibroblasts, probably because these cells did not tolerate prolonged overexpression of SNX17. When full-length SNX17-FLAG was co-expressed with the LDL receptor in CHO cells, some LDL receptor was also trapped in the large abnormal endosomal structures, although the two proteins were not completely co-localized (Fig. 9C, panels e, h, and k).
When residues SNX17113–425-FLAG were overexpressed with the LDL receptor, LDL receptor protein accumulated at the plasma membrane, but it was not co-localized with the SNX17 mutant (Fig. 9C, panels f, i, and l), despite the fact that this fragment binds most strongly in vitro with the LDL-receptor cytoplasmic domain. Although these data suggest that overexpression of SNX17113–425-FLAG disrupts LDL receptor trafficking, these experiments must be interpreted with care, because disruption of endosomes will inevitably affect LDL receptor distribution in the cells.

**DISCUSSION**

In this report we have confirmed that the cytoplasmic tail of the LDL receptor interacts strongly with a novel protein previously described as the predicted protein KIAA0064 (14). Our data support the view that SNX17 is a member of the sorting nexin family and may be involved in the intracellular sorting of the LDL receptor. Despite sharing strong PX domain sequence identity with SNX1, the remaining part of the protein, including the domain that interacts with the LDL-receptor cytoplasm-
indicated in proteins were eluted from the beads and subjected to SDS-PAGE, as
post-nuclear extracts (extracts of human skin fibroblasts.), and the middle panel
SNX17, and a soluble fraction; samples equivalent to one volume of original extract were separated by SDS-PAGE and im-
sor cytoplasmic tail. GST fusion proteins comprising GST alone (GST) or GST fused to the cytoplasmic tail of the LDL receptor (GST-LDLR-CT/807Y) were adsorbed to glutathione-Sepharose beads and incubated with 35S-labeled SNX17 proteins translated in vitro. Bound proteins were eluted from the beads and subjected to SDS-PAGE, as indicated in lanes 1–7. Upper panel, autoradiograph of gel showing input of [35S]methionine-labeled SNX17; middle panel, autoradiograph of gel showing labeled SNX17 eluted from GST (left) or GST-LDLR-CT (right); lower panel, Coomassie Blue stain of gel showing GST (left) or GST-LDLR-CT eluted.

immunoblotting of endogenous SNX17 in fractionated extracts of human skin fibroblasts. Post-nuclear extracts (T) of human skin fibroblasts (cells from 7–9-cm diameter dishes in 0.5 ml of buffer) were further fractionated by centrifugation to produce a membrane pellet (M) and a soluble fraction (S); samples equivalent to one volume of original extract were separated by SDS-PAGE and immunoblotted with specific antisera to the LDL receptor (top panel), SNX17 (middle panel), and the β-subunit of adaptor protein-2 (bottom panel). Bound antibody was detected by incubation with horseradish peroxidase-conjugated secondary antibody followed by a chemiluminescence assay.

5'-end of the PD041839 that is weakly similar to the FERM domain (p = 0.056), this is mainly due to the presence in SNX17 of short motifs identical to motifs found in FERM proteins.

Human SNX1 was first identified by its ability to interact with the lysosomal targeting signal of the epidermal growth factor (EGF) receptor (23). It was suggested to be involved in protein sorting because of its sequence similarity to yeast Vps5, a vacuolar protein known to be essential for correct sorting of carboxypeptidase-Y (24). The SNX 2–6 proteins show strong similarity across their entire length, with the exception that SNX3 is shorter than the others, but more recent assignments to this rapidly growing family only show strong sequence similarity in the region of the PX domain. Sorting nexins characteristically localize to endosomes, predominantly early endosomes, in some cases via interaction between their PX domain and phosphoinositides in the endosomal membrane (reviewed in Ref. 25). We have found that endogenous SNX17 localizes to endosomes and that this is disrupted by substitution of a critical lysine residue, as has been found for SNX1 (20). We found that, when overexpressed, SNX17 is found in large endosomal structures similar to those seen in cells overexpressing rab-5a, which the authors concluded were abnormally enlarged endosomes formed because further transport was blocked (21).

Overexpression of SNX15 has also been found to disrupt endosomal morphology and to interfere with normal trafficking of proteins between plasma membrane and recycling endosomes (26, 27). Stockinger et al. (9) measured uptake and degradation of 125I-labeled LDL by stably transfected cells expressing myc-tagged SNX17, and concluded that LDL internalization and degradation, but not binding, was increased, the difference being expressed as a 2-fold increase in the internalization index. We have failed to observe consistent changes in either cellular levels of the LDL receptor or in LDL receptor-mediated catabolism of LDL by cells transiently overexpressing SNX17; however, such experiments are difficult to interpret for several reasons. First, not all the cells express the construct, and second, as we have shown, overexpression of SNX17 appears to disrupt cell structure. In our hands, cells stably expressing SNX17 at moderate levels appear to take up and degrade LDL normally (data not shown). This is not surprising in view of our finding that endogenous SNX17 is a highly expressed protein and presumably moderate levels of heterologous normal or mutant protein cannot compete with the normal pathway. We have attempted to down-regulate endogenous SNX17 expression with small interference RNA but have not yet been successful in reducing expression significantly. Experiments to produce a conditional knock-out are underway.

The role of sorting nexins in endosomal trafficking of proteins appears to vary. It can involve targetting a protein for lysosomal degradation, for example the EGF receptor, or in recycling a receptor back to the plasma membrane, for example the transferrin receptor. Several members of the sorting nexin family have been shown to interact with transmembrane receptors involved in cell signaling (8), in some cases via their carboxyl-terminal domain (23) and in others, via the PX domain (28). Overexpression of SNX1 in monkey CV-1 cells decreased the amount of EGF receptor on the cell surface, probably by enhancing degradation (23), whereas overexpression of SNX5 increased the level of expression of FANCA (29).

The possibility of functionally distinct sub-groups of sorting nexins is further supported by the domain structure of the non-PX part of the protein. Teasdale and co-workers (30) sug-
gested that there are at least three sub-groups of sorting nexins, depending on the presence of multiple, one, or no coiled-coil domains. SNX17 does not fit clearly into any of these subgroups, having an amino-terminal PX domain, but a large novel carboxyl-terminal domain that has no predicted coiled coil domains. It also contains a region with some similarity to a FERM domain that is not seen in other sorting nexins, although this similarity is not strong. This domain is found in the ERM proteins (ezrin, radixin, and moesin), as well as other proteins that are defined as cytoskeletal linking proteins. Although their function is not fully understood, they are located in actin-rich structures associated with the plasma membrane and are believed to attach other proteins to the cell membrane through this domain.

We have shown that the novel PD041839 domain of SNX17, which contains the FERM-like region, specifically interacts with the cytoplasmic tail of the LDL receptor, and that this interaction is disrupted by mutations in the tyrosine-based motif that has been shown to be essential for both internalization and basolateral sorting. Stockinger et al. (9) reported that the NPVY motif in the LDL-receptor was not involved in its interaction with SNX17; this conclusion was based on their observation that Dab-1 bound to the LDL receptor tail, an interaction presumed to be via the NPVY motif, but did not apparently compete for binding to SNX17 in a plate binding assay. We suggest that SNX17 is involved in trafficking the LDL receptor through the early endosomal compartment, although its precise role in the LDL receptor pathway remains to be defined. We hypothesize that it could be involved in sorting the receptor away from the degradation pathway and toward the recycling pathway, perhaps being required for correct basolateral sorting.
Fig. 9. Confocal microscopy showing the effect of substituting a critical lysine residue in the PX domain on the co-localization of RFP-SNX17 with early endosomes or with the LDL receptor. A, human skin fibroblasts were transfected with constructs expressing RFP, RFP-SNX17, RFP-SNX17K62A, or RFP-SNX17,1-274 (PX+FERM-like domains) as indicated, then fixed, permeabilized, and stained 18 h after transfection with mouse anti-EEA1/ALEXA488-conjugated goat antimouse IgG (green, center panels). Left-hand panels show RFP, and the right-hand panels show the RFP/EEA1 overlay. Bar = 10 nm. B, human skin fibroblasts were transfected with constructs expressing RFP, RFP-SNX17, RFP-SNX17K62A, or RFP-SNX17,1-274 (PX+FERM-like domains) as indicated, together with LDLR-GFP. Cells were fixed and permeabilized 18 h after transfection. RFP is shown in the left-hand panels, GFP in the center panels, and the RFP/GFP overlay in the right-hand panels. Bar = 10 nm. C, confocal microscopy showing effects of overexpression of SNX17 on intracellular architecture. Constructs of SNX17 with a FLAG tag at the carboxyl terminus were transiently expressed alone (a–d) or together with the human LDL receptor (e–m) in CHO IdlA7 cells. Cells were fixed, permeabilized, and incubated with mouse anti-FLAG Ab (a–d), or with mouse anti-FLAG Ab and conjugated rabbit anti-LDL-receptor Ab, as indicated. Bound Ab was detected with Alexa 488-conjugated anti-mouse IgG and Alexa 568-conjugated anti-rabbit IgG. a, full-length SNX17; b, SNX17,13-425; c, SNX17,275-470; d, enlargement of a, showing ring-like structures. e–m, CHO IdlA7 cells were cotransfected with constructs as indicated on the left. Bar = 10 nm.
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