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Identification and characterization of an endoplasmic reticulum localization motif

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ABSTRACT

Sorting motifs are involved in the transport of diverse proteins. In the present study, we identified a hydrophobic peptide (WRPWRNFWWSIRVPWRRN) that was able to target enhanced green fluorescent protein- or DsRed2-enriched vesicular-like sub-compartments of the endoplasmic reticulum (ER). Analysis of mutation constructs revealed that the sequence WRPWRNFWW was responsible for the ER-targeting activity, and the arginine residue of the peptide is a critical determinant of ER localization. Results from co-immunoprecipitation, glutathione S-transferase pull-down, liquid chromatography-tandem mass spectrometry, and western blotting analyses demonstrated that this motif could bind with the γ2-COP subcomplex of coat protein complex I (COPI), which is involved in the retrieval and transport of ER-resident proteins from the Golgi apparatus to the ER. Overall, we report a new hydrophobic peptide that possesses an arginine-based ER localization motif, which can help elucidate the mechanisms of ER sorting mediated by COPI.

1. Introduction

Protein synthesis is achieved by ribosomes attached to the rough endoplasmic reticulum (ER) membrane [1]. These polypeptides enter the ER lumen, which harbors resident molecular chaperones and folding factors that assist with their maturation [2]. The ER contains a population of resident proteins that perform a variety of organelle-specific functions, including protein and lipid modifications, processing of N-linked glycosylation, vesicle formation, protein sorting, and transport [3].

Coat protein complex I (COPI) vesicles are involved in the retrieval of ER resident proteins from the Golgi apparatus to the ER [4]. COPI consists of a heptameric (α, β, β′, γ, δ, ε, ζ) complex with two main subcomplexes: the γ-COP–δ-COP–ζ-COP–β-COP tetrameric complex, which constitutes the inner layer core, and the α-COP–β′-COP–ε-COP trimeric complex, which forms the outer layer of the COPI coat [5]. Several motifs have been described to be involved in the ER retention of proteins through their interaction with COPI. For instance, the canonical dilysine motifs (KKXX and KXKXX) function through binding with the α-COP subunit of COPI [6–9]. Retrieval of HDEL signals in yeast were shown to require the presence of COPI [10]. The diphenylalanine motif (FF motif) of mammalian p24 and yeast p26 binds with the outer layer of COPI [11], and the aromatic residue was shown to be a critical determinant of the δL interaction with COPI [12]. However, the specific interactions of COPI with different motifs have not yet been clarified. In particular, it is completely unknown how the arginine-based ER localization signals are recognized [13].

The present study was undertaken to assess the possible involvement and significance of arginine motifs in ER targeting, by preparing protein chimeras containing an arginine-encompassing hydrophobic peptide (WRPWRNFWWSIRVPWRRN) and conducting mutational analysis. In addition, the possible role of COPI in the recognition of this hydrophobic peptide was investigated.

2. Materials and methods

2.1. Plasmid construction

The vectors pDsRed2-ER and pEYFP-Golgi were used to co-transfect cells with pEGFP-C1-AP (artificial hydrophobic peptide) or pDsRed2-C1-AP vectors. To generate the hydrophobic peptide–enhanced green fluorescent protein (EGFP) fusion protein, the *Mus musculus* histone acetyltransferase 1 (HAT1; NM_026115.4)

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fragments were amplified by polymerase chain reaction using the primers 5′-G*GAATTC*ATGGCGGCCTTGGAGAAATT and 5′-CG*GGATCC*TCACTCTTGAGCAAGTCGCTCAATGACACG (*Eco*RI and *Bam*HI cutting sites are underlined, respectively), and cloned into the multiple cloning sites of pEGFP-C1 vectors (Clontech).

Complementary oligonucleotides of the hydrophobic peptide (AP) and mutants were synthesized, and the fragments were annealed to form a DNA duplex, which was cloned into the pEGFP-C1 or pDsRed2-C1 vectors (Supplementary Table 1).

Glutathione-S-transferase (GST) fragments were amplified by polymerase chain reaction using the primers 5′-GA*AGATCT*TCCCCTATACTAGGTTATTG and 5′-*GGAATTC*GAATCCGATTTTGGAGGATGGT (*Bg*lII and *Eco*RI sites are underlined, respectively), and cloned into the pEGFP-C1-AP and pEGFP-C1 vectors.

2.2. Cell culture and transfection

HeLa cells (**ATCC Number:** CCL-2) were cultured in Dulbecco's modified Eagle medium (Hyclone), supplemented with 10% fetal calf serum, 100 U of penicillin, and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Plasmid DNA was purified using a plasmid mini kit (A2492, Promega). Transfection was performed using FuGENE HD (E2311, Promega) according to the manufacturer instructions.

2.3. Immunofluorescence staining and microscopy

Indirect immunofluorescence was performed as previously described [14]. Anti-HAT1, anti-LC3, anti-EEA1, anti-LAMP1, anti-LMNB1, and anti-ERp72 antibodies used for immunofluorescence staining were obtained from Proteintech. Images were acquired with a Nikon A1R laser-scanning confocal microscope.

2.4. Binding assay

HeLa cells were transfected with pEGFP-C1-GST or pEGFP-C1-GST-AP vectors. The whole cell lysate was prepared by adding 1 mL of RIPA Lysis Buffer (#20188, Millipore), and then centrifuged at 12,000 × *g* for 15 min. Glutathione high-capacity magnetic agarose beads (G0924, Sigma) were incubated with the cell lysate for 3 h at 4 °C. The beads were washed with RIPA buffer four times, and the proteins that bound to the beads were eluted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer.

2.5. Co-immunoprecipitation assays

The co-immunoprecipitation assay was performed as described previously [15] using the anti-GFP antibody (sc-8334, Santa Cruz Biotechnology). In brief, HeLa cells were transfected with the pEGFP-C1-AP, or pEGFP-C1-GST-AP vectors for 48 h, respectively. A total of 500 μg of the cell lysate was subjected to precipitation using a catch-and-release V2.0 reversible immunoprecipitation system kit (#17–500, Millipore) according to the manufacturer's instructions.

2.6. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The proteins bound to the antibody were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gel bands were digested with trypsin. LC-MS/MS analysis was performed at China Beijing Protein Innovation Company. The peptides were subjected to fractionation using an Ultimate 3000 nano-high-performance liquid chromatography system (Dionex), and the gradient-eluted peptides were analyzed using a Q Exactive mass spectrometer (Thermo Scientific). The proteins identified from the pEGFP-C1-GST group were regarded as non-specific proteins. The proteins identified in both the pEGFP-C1-AP group and pEGFP-C1-GST-AP group were selected for further analysis.

2.7. Western blotting analysis

The procedure of western blotting was performed as previously described [16], using anti-γ2-COP antibody (16111-1-AP, Proteintech) and anti-GFP antibody (sc-8334, Santa Cruz Biotechnology). The blotted proteins on the nitrocellulose membranes were visualized with Thermo Scientific Pierce ECL western blotting substrate on a Tanon detection system.

3. Results

3.1. The hydrophobic peptide sequence contains ER localization information

Fluorescence microscopy revealed that EGFP-AP, EGFP-GST-AP, and DsRed2-AP were primarily localized in a vesicular-like compartment. In contrast, the fluorescence of EGFP-GST was evenly distributed throughout the cytoplasm (Fig. 1). The EGFP-AP fusion protein showed a distinct localization from that of the HAT1 protein (Fig. 1). This is because the *HAT1* gene was not in the same reading frame as *EGFP*, and this frameshift mutation resulted in the expression of a hydrophobic peptide sequence (WRPWRNFWWSIRVPWRRN).

To determine the specific vesicular compartment in which the hydrophobic peptide was localized, we further stained for the LC3 (an autophagosome marker), EEA1 (an early endosome marker), LAMP1 (a lysosome marker), LMNB1 (an inner nuclear membrane marker), and ERp72 (an ER lumen marker) vesicle proteins [17] in pEGFP-C1-AP- or pDsRed2-C1-AP-transfected HeLa cells by an indirect immunofluorescence assay. As shown in Fig. 1, DsRed2-AP co-localized with ERp72, but not LC3, EEA1, LAMP1, and LMNB1. These findings indicate that the vesicular compartment of the hydrophobic peptide fusion protein is a sub-compartment of the ER. To validate this observation, pDsRed2-ER (as an ER marker) vectors were co-transfected with pEGFP-C1-AP, and the pEYFP-Golgi (as a Golgi apparatus marker) vectors were co-transfected with pDsRed2-C1-AP vectors in HeLa cells. As shown in Fig. 1, EGFP-AP co-localized with DsRed2-ER, and DsRed2-AP was not localized in the Golgi apparatus. Taken together, these results indicated that the hydrophobic peptide localized in a vesicular-like sub-compartment of the ER, which prompted us to explore the molecular mechanism responsible for its ER localization ability.

3.2. The arginine-based ER localization motif is responsible for ER localization

Based on the current paradigms of ER localization signals, it is possible that the inserted amino acids (AAs) caused the motif WRPWRNFWWSIRVPWRRN to be different from nearly all ER localization signals. Therefore, a deletion mutation study was conducted with the aim of narrowing down the specific motif responsible. As shown in Supplementary Table S1, a series of deletion mutations of the hydrophobic peptide were constructed, termed Δ-1AP, Δ-2AP, Δ-3AP, Δ-4AP, Δ-5AP, and Δ-6AP. These constructs were transfected to HeLa cells, and the subcellular localization of the mutant peptides were analyzed using fluorescence microscopy at 24 h post-transfection. The fluorescence images (Fig. 2A) showed that Δ-1AP, Δ-2AP, Δ-3AP, Δ-4AP, Δ-5AP, and Δ-6AP all had a vesicle-like localization near the nucleus. Therefore,