Intracellular localization of FLAG-peroxisomal protein in Chinese Hamster Ovary (CHO) cells

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Abstract
Epitope tagging is a method of expressing proteins whereby an epitope for a specific monoclonal antibody is fused to a target protein using recombinant DNA techniques. The aim of this study was to sub-clone the peroxisomal protein (PEP) cDNA into a mammalian expression vector leading to the formation of a chimeric *PEP*-cDNA containing the FLAG epitope. The *FLAG-PEP* recombinant cDNA was constructed using the method of splicing by overlap extension polymerase chain reaction (SOE PCR) and inserted into the pUcD2SRaMCSHyg eukaryotic expression vector. To investigate the intracellular localization of the PEP protein that was linked to the *FLAG* tandem, the constructed plasmid was used for transient transfection of the Chinese hamster Ovary (CHO) cells. The CHO cells that were transfected with the recombinant plasmid showed peroxisomal localization of FLAG-PEP as was previously shown for catalase.
Key words: PEP cDNA; Peroxisomes; PTS1 signal; Transfection.

INTRODUCTION

Peroxisomes are ubiquitous organelles found in almost all eukaryotes and function to rid the cell of toxic substances such as peroxides. Peroxisomes were discovered in 1966 by the pioneering activities of the Belgian biologist Christian De Duve (De Duve, 1969). These organelles perform many biochemical functions of lipid metabolism, including β-oxidation of very long chain fatty acids, biosynthesis of structural ether lipids such as plasmalogens that are abundant in the central nervous system, interconversion of cholesterol to bile acids, and glyoxylate transamination (Furuki *et al*., 2006; Shimizu *et al*., 1999). Mature peroxisomes are spherical, with diameters of between 0.5 and 1.0 μm. Peroxisomes are single membrane organelles with defined granular matrices (Latruffe and Vamecq, 2000). The size and number of peroxisomes depend on the type of cell, organism and environment. The physiological importance of peroxisomes arises as a result of lethal peroxisome biogenesis disorders (Wanders, 2004; Vizeacoumar *et al*., 2004). A group of autosomal recessive diseases including the Zellweger syndrome, rhizomelic chondrodysplasia punctata, and neonatal adrenoleukodystrophy, are known in which multiple peroxisomal metabolic pathways are dysfunctional because peroxisome biogenesis is compromised (Purdue and Lazarow, 2001; Subramani, 1998; van den Bosch *et al*., 1992; Lazarow and Fujiki, 1985). With respect to the biogenesis of peroxisomes and considering that peroxisomes lack DNA, all peroxisomal proteins are synthesized on cytoplasmic free polysomes and are post-translationally transported to pre-existing

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peroxisomes (Lazarow and Fujiki, 1985). Two types of peroxisome-targeting signals (PTS) for directing the matrix proteins into peroxisomes have been identified: the C-terminal Serine-Lysine-Leucine (SKL) and its conserved variants representing the peroxisome-targeting signal type I (PTS1) for most proteins (Baker and Sparkes, 2005; Baker *et al*., 2000; Miura *et al*., 1992; Gould *et al*., 1989), and the N-terminal cleavable nonapeptide, -(R/K)(L/V/I)X_5(H/Q)(L/A)-, present in several proteins such as 3-ketoacyl-CoA thiolase (thiolase III) of the fatty acid -oxidation pathway representing peroxisome-targeting signal type II (PTS2), (Singha *et al*., 2004; Osumi *et al*., 1991; Swinkels *et al*., 1991). Membrane proteins are sorted to peroxisomes by targeting signals distinct from PTS1 or PTS2.

One of the peroxisomal matrix proteins, termed peroxisomal protein (PEP), has already been cloned in mouse by Ferrer-Martinez *et al*. (2002). The PEP structure is formed by 209 amino acids, consisting of a C-terminal tail of tripeptides: Serine, Lysine, Isoleucine (SKI) closely resembling SKL, the consensus sequence for PTS1 (Ferrer-Martinez *et al*., 2002).

Studies have shown that PEP expression in the mouse embryo is different in various tissues, its reason being unclear (Ferrer-Martinez *et al*., 2002). This study was aimed at defining the function of PEP and identifying its interacting partners. Hence, the *PEP*-cDNA was inserted downstream of the *FLAG* gene in the pUcD2SRaMCSHyg eukaryotic expression vector to express the tagged-PEP protein. The resulting product was used for the purpose of transient transfection analysis and subsequent identification of its localization in the Chinese hamster ovary CHO-K1 cells.

MATERIALS AND METHODS

Construction of pUcD2SRaMCSHyg-*FLAG-PEP*

The coding region of *PEP (PEP*-cDNA) (Tanhaei *et al*., 2008), was tagged with the *FLAG* tandem at its upstream region, by a two step reaction of splicing overlapping extension polymerase chain reaction (SOE-PCR). The amplified *FLAG*-PEP fragment was inserted into the pUcD2SRaMCSHyg vector (Ghaedi *et al*., 1999) in order to construct the pUcD2SRaMCSHyg-*FlAG-PEP* vector under the regulation of SRα promoter. This vector contains a hygromycin resistant gene, which is suitable for the stable expression of cloned genes in mammalian cells.

SOE-PCR was performed in an Eppendorf Mastercycler gradient thermal cycler, which is described below. Primers, used during this study, were ordered from the Bioneer company (Korea) and are presented in Tables 1 and 2.

PCR conditions

Step 1: The aim of the first step of the PCR is production of *PEP* and *FLAG* fragments. *PEP*-cDNA was amplified using pEGFP/*PEP* (Tanhaei *et al*., 2008) as template, with primers introducing

Table 1. Primer sequences for PEP amplification in order to construct *FLAG-PEP*. Forward primer introduces the *Bam*HI restriction site at the 5' end of PEP and reverse primer introduces the *Apa*I restriction site at the 3' end of PEP. Restriction sites are bolded.

F	5’ AT**GGATCC**TGCCCCCAGGGCCGTCCGCCT 3’ *Bam*HI	
R	5’ AAAA**GGGCCC**TCATATCTTGCTGCGGAGGAGA 3’ *Apa*I	

Table 2. Primer sequences for *FLAG* amplification in order to construct *FLAG-PEP*. Reverse primer, at 5’ end, encompasses nine nucleotides complementary to the head of PEP cDNA and *Bam*HI restriction site respectively. Restriction sites are bolded.

F	5’ ATAAGAAT**GCGGCC**GCCACCATGGATTACAAGGAC 3’ *Not*I	
R	5’ CTGGGGGCA**GGATCC**CAAGCTTATCGTCGTCGTC 3’ *Bam*HI	

*Bam*HI and *Apa*I restriction sites at the 5' and 3' ends, respectively (Table1). The *FLAG* coding sequence was also amplified using pUcD2SRaMCSHyg/*FLAG-PEX3* (Ghaedi *et al*., 2000) as template, with a forward primer introducing the *Not*I restriction site at its 5' end and a reverse primer containing the *Bam*HI restriction site (Table 2). The amplified products of this step, being 647bp (*PEP*) and 77 bp (*FLAG*) in length were purified by the QIAprep Spin Miniprep kit (Qiagen, Germany) and used as templates in step 2 of the PCR procedure.

Step 2: In the second step of PCR, both of the amplified *FLAG* and *PEP* fragments were used as templates and *FLAG-PEP* was amplified using the *FLAG* forward primer and *PEP* reverse primer. The resulting product of this step was the *FLAG-PEP* chimeric cDNA, being 700 bp in length.

The pUcD2SRaMCSHyg vector (Ghaedi *et al*., 2000) and the amplified *FLAG-PEP* fragment were cut with *Not*I/*Apa*I restriction enzymes (Fermentas, Germany) and were then ligated. Transformation was carried out immediately using competent JM105 *Escherichia coli* cells (Fermentas, Germany). Insert check analysis was carried out on grown colonies the next day in order to select those that contain the constructed vector. Plasmid extraction from bacterial colonies was performed by using the Qiagen plasmid miniprep kit (Qiagen, Germany). The recombinant plasmid was subsequently sequenced (Bioneer Company, Korea)

Transient transfection of CHO cells by the pUcD2SRaMCSHyg/*FLAG-PEP* plasmid

Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F-12 Ham (Sigma-Aldrich, USA) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco, USA), 1% (v/v) penicillin/streptomycin and 2 mM L-glutamine (Gibco, USA) under a humidified atmosphere containing 5% CO_2. In this procedure, CHO cells were seeded at 5000 cells/cm^2 onto sterile glass cover slips in 24-well plates. The cells were allowed to attach and grow for one day prior to treatment.

Exponentially growing CHO cells with 50% confluency were transfected with the pUcD2SRaMCSHyg/*FLAG-PEP* vector by using Lipofectamine 2000 (Invitrogen, USA). In each well of the 24-well plates, 0.4 µg of plasmid DNA was diluted in 25 µl of serum-free Opti-MEM I medium (Gibco, USA) containing 1 µl of Lipofectamine 2000. The resulting solutions were mixed and incubated at room temperature for 20 min. Cells in each well were washed with serum-free Opti-MEM I medium immediately before transfection, overlaid with 250 µl of Opti-MEM I medium and 50 µl of DNA-Lipofectamine complex, incubated for 6 h, at 37ºC. The tarnsfection medium was then replaced with DMEM/nutrient mixture F-12 Ham supplemented with 10% (v/v) FCS. For the transient expression experiment, the cells were stained 48 h after transfection with the anti-Flag and anti-catalase antibodies.

Immunofluorescence studies for catalase and FLAG: For immunofluorescence staining, transfected CHO-K1 cells cultured on glass slides were fixed for 30 min in 4% (w/v) paraformaldehyde (Sigma, USA) and permeabilized with 0.2% (v/v) Triton X-100 (Merck, USA) in phosphate buffer saline (PBS). The cells were then incubated for 1 h in a blocking solution made up of PBS containing 20 mg/ml of bovine serum albumin (BSA) (Sigma, USA) and 10% (v/v) goat serum (Chemicon, USA). Subsequently, rabbit polyclonal anti-catalase (1:300, Abcam, UK) and mouse monoclonal anti-Flag (20 µg, Sigma, USA) were diluted in PBS containing 1mg/ml of BSA and then added to the cells which were incubated overnight at room temperature. For the purpose of detection, the cells were incubated the following day for 2 h with the fluorescein Texas red-labeled donkey anti-rabbit IgG (1:400, Amersham Biosciences, USA) and FITC-labeled goat anti-mouse IgG antibodies (1:500, Chemicon, USA), which were diluted as above. Cover slips (Roth, Germany) were mounted on microscope slides using Entellan mounting medium (Merck, USA). The fluorescent pattern was viewed using an Olympus BX5 fluorescence microscope (Olympus, Japan) and the resulting images were then recorded.

RESULTS

Construction of pUcD2SRaMCSHyg-*FLAG-PEP*: The PCRs using pEGFP-*PEP* and

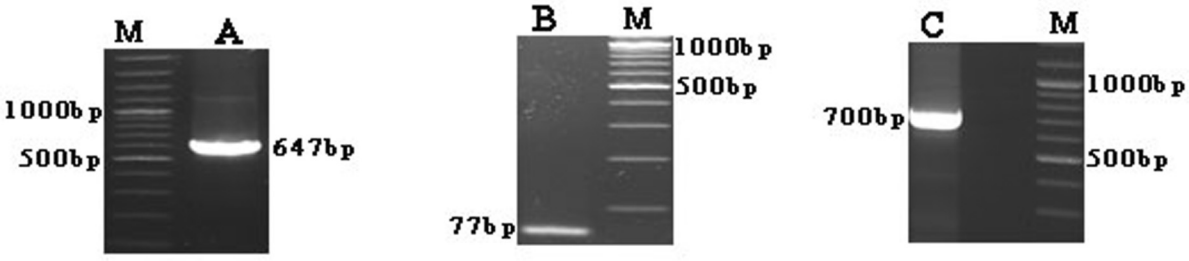

Figure 1. Construction of the eukaryotic expression vector pUcD2SRaMCSHyg-*FLAG-PEP*. A) Amplified *PEP* cDNA fragment. B) Amplification of the *FLAG* coding sequence. C) Amplification of the *FLAG-PEP* fragment. M) DNA size marker (100 bp ladder, Fermentas, Germany).

pUcD2SRaMCSHyg-*FLAG-PEX3* as templates in two separate reactions generated 647 bp (*PEP*) and 77 bp (*FLAG*) fragments, respectively. Restriction sites were added at the ends of *PEP* and *FLAG* fragments (Figs. 1A, 1B). A Second PCR using *PEP*-cDNA and *FLAG* tandem as templates generated a 700 bp fragment that demonstrated amplification of the *FLAG-PEP* fragment (Fig. 1C).

Insert check analysis on colonies which grew one day after transformation with ligated products, demonstrated that the *FLAG-PEP* had been inserted into the pUcD2SRaMCSHyg vector (data not shown). Constructed vectors were extracted from several positive bacterial colonies. Finally, sequencing confirmed that *FLAG-PEP* cDNA was inserted into the vector appropriately and was free from mutation.

Transfection of pUcD2SRaMCSHyg/*FLAG-PEP* into CHO cells and immunostaining: Transient transfection with a plasmid expressing the FLAG-PEP chimeric protein was performed in the CHO-K1 cell line. When the control vector, pEGFP-C1/*PEP*, was expressed in CHO cells, a punctuated pattern was seen (Fig. 2A) as it was previously demonstrated (Tanhaei *et al*., 2008). The recombinant pUcD2SRaMCSHyg/*FLAG-PEP* plasmid encodes a fusion protein in which its amino-terminus corresponds to the FLAG and its carboxy-terminus to the PEP. CHO-K1 cells were transfected with pUcD2SRaMCSHyg/*FLAG-PEP*, fixed, and processed for immunostaining using the anti-FLAG and anti-catalase antibodies. A punctuated pattern, typical of peroxisome staining, was detected after pUcD2SRaMCSHyg/*FLAG-PEP* transfection. The tiny green bright spots were distributed uniformly in the cytoplasm but were completely absent from the nucleus. Red spots for catalase representing a peroxisomal marker were obtained by double staining, in order to verify. The punctuated pattern seen in transfected cells corresponding to the peroxisomal targeting of FLAG-PEP is verified in Figures 2B and 2C, which show the colocalization of FLAG-PEP and catalase. For the sake of clarity, the fluorescent signal that overlaps in Figures 2B and 2C is displayed as a yellow punctuated pattern in Figure 2D. This result confirms the peroxisomal targeting of the chimerical protein FLAG-PEP.

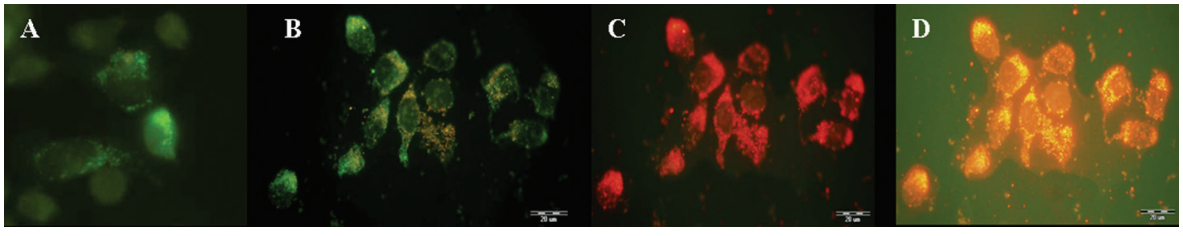

Figure 2. Subcellular localization of the chimeric PEP protein in the CHO-K1 cells. A) Localization of EGFP-PEP. B) Localization of FLAG-PEP. C) Staining of catalase as a peroxisomal marker. D) Merged staining pattern of FLAG and catalase.

DISCUSSION

Unraveling the functional properties of proteins is one of the main interests for biologists. Purification techniques seems to be required as a step for functional characterizations of proteins, which can be achieved by epitope tagging of target proteins. Tagging a protein with an epitope at the N' or C' terminus allows detection of the protein with an antibody that has specific high affinity for this particular sequence (Sung *et al*. 2008; Johnson *et al*. 2002; Zhang *et al*., 2001). Using this approach, researchers can elucidate the size of a tagged protein as well as its intarcellular location, or posttranslational modifications, and its interactions with other proteins. The approach also facilitates protein purification (Zhang *et al*., 2001). Thus it enables them to rapidly and efficiently characterize gene products *in vivo* (Gloeckner *et al*., 2007). Various heterologous expression systems are currently used by researchers for different kinds of cells including yeast, bacteria, insect cells and mammalian hosts. Among them, expression of recombinant eukaryotic proteins in mammalian cell lines has several advantages including rapid characterization of native structure, synthesis, posttranslational modification, and intracellular transportation studies of that protein (Wu and Chiang, 2002; Zhang *et al*., 2001). In addition, mammalian cells are well suited to a variety of recombinant protein studies that analyze the physiological effects of the protein on cellular functions. However, the levels of recombinant proteins which are expressed by mammalian expression systems are lower than those expressed by bacteria, yeast, or insect cells (Zhang *et al*., 2001; Singh *et al*., 2000; Hosfield and Lu, 1998).

There are various tags which are currently being used for production of recombinant proteins in mammalian expression systems. One of the most widely used tags, is the FLAG epitope tag. This tag comprises eight amino acid residues (Asp-Try-Lys-Asp-Asp-Asp-Asp-Lys) which can be detected by the anti-FLAG monoclonal antibody (Huang *et al*., 2001; Zhang *et al*., 2001).

In this investigation, in order to define the function of PEP through its interaction with unknown partners, the *PEP*-cDNA was sub-cloned into a eukaryotic expression vector so as to tag it with the *FLAG* tandem. PEP fused with the tandem of the Flag epitope could then be detected with a monoclonal antibody, specific for this tag. However epitope tagging can interfere with normal protein function or its intracellular sorting, indicating the need for checking of its efficiency (Brizzard and Chubet 2001). Thus transient transfection of CHO cells with the constructed vector was carried out to evaluate intracellular targeting of Flag-PEP. Previous studies have indicated that the PEP protein is a peroxisomal protein containing PTS1 (Tanhaei *et al*., 2008; Ferrer-Martinez *et al*., 2002). PTS1 has been found in approximately half of the peroxisomal proteins, directing PEP's import from the cytosol to the organelle matrix (Sacksteder and Gould, 2000; Subramani, 1998). PTS1 was identified for the first time at the C-terminus of firefly luciferase. The PST1 of most peroxisomal matrix proteins contain three amino acid residues in the form of S/A/C-K/R/H-L/M at their carboxy terminals (Sacksteder and Gould, 2000). In this study, the transient transfection experiment involving the pUcD2SRaMCSHyg/*FLAG-PEP* plasmid was carried out in the CHO cells and subsequent double staining for the FLAG tag and catalase as a peroxisomal marker demonstrated that the PEP is targeted to the peroxisomes, indicating that Flag tagging of PEP does not hamper its peroxisomal localization. This strongly suggests that SKI, the PEP C-terminal tripeptide, behaves as a functional PTS1 in mammalian cells even in the presence of the Flag epitope. Unlike other studies that describes multiple FLAG epitope tagging of expressed proteins for enhanced detection (Hernan *et al*., 2000), only one copy of the *FLAG* cDNA upstream of *PEP* was used successfully in this study, which was detectable under fluorescence microscopy. In order to see the possible role of this domain in the molecular structure of PEP, proteomic analysis of PEP using Flag-PEP in mammalian cell lysates seems to be necessary. Thus this construction (pUcD2SRaMCSHyg/*FLAG-PEP*) can be used for further functional analysis of this protein.

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