Central Region of Wheat UCP Acts as a Major Signal for Protein Transport to Mitochondria, but Both Distal Regions can also Drive a Protein Import to Mitochondria

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To identify the region related to the protein transport of mitochondrial uncoupling protein (UCP), we divided wheat UCP (WhUCP1b) protein into three regions (D1, D2 and D3) and fused them with green fluorescent protein (GFP) as an N-terminal signal peptide. Four fusion proteins (WhUCP::GFP, D#::GFP) were expressed in yeast cells to observe the transport of the fusion proteins to mitochondria in vivo. The mitochondrial localization of the four fusion proteins was visualized using GFP fluorescence as a reporter. Western blot analysis also revealed that all four fusion proteins were transported to the mitochondria. The D2 region had the highest protein transport efficiency, and the D1 and D3 regions also had some protein transport activity. Another kind of fusion protein constructed using the D2 region as an internal signal peptide (DSR::D2::GFP) was also successfully targeted to mitochondria. These results demonstrate that the D2 region of WhUCP1b possesses the highest activity directing protein transport to the mitochondria among three regions, and that the protein transport activity of the D2 region is independent on its position within the fusion protein.

Key Words: mitochondria, transport, uncoupling protein, signal peptide, wheat.

Introduction

Most mitochondrial proteins are encoded in the nuclear genome, and translated on cytoplasmic ribosomes as preproteins whose N-terminus usually contains a signal peptide that plays an important role in the transport of preproteins to the mitochondria (Schatz and Dobberstein 1996, Voos et al. 1999). The N-terminus signal peptide (NSP) binds mainly to the Tom20 receptor protein on the outer membrane of the mitochondrion (Söllner et al. 1989, Moczko et al. 1992, Kanaji et al. 2000) and is then transferred to the TOM channel, which is a protein complex consisting of Tom22 (Kiebler et al. 1993, Lithgow et al. 1994), Tom5 and Tom40 (Dietmeier et al. 1997). Then the preprotein crosses the inner membrane via the TIM23 complex and is finally imported into the matrix by the Tim44-Hsp70 motor system (Ungermann et al. 1996, Cyr 1997, Moro et al. 1999). After translocation of the preprotein into the matrix, NSP is usually removed by a processing peptidase (Schatz and Dobberstein 1996). On the other hand, there are several nuclear-encoded mitochondrial proteins lacking NSP. However, these proteins without NSP are also specifically targeted to the mitochondria, which indicates that there is a transport pathway different from that for preproteins with an NSP.

The mitochondrial uncoupling protein (UCP) is one of the mitochondrial carrier proteins located at the mitochondrial inner membrane and its genetic information is encoded in the nuclear genome. So far, four isoforms of the UCP gene have been identified in mammals (Bouillaud et al. 2001), and plant UCP genes have also been isolated from potato (Laloi et al. 1997), Arabidopsis thaliana (Maia et al. 1998, Watanabe et al. 1999), skunk cabbage (Ito 1999), wheat (Murayama and Handa 2000) and rice (Watanabe and Hirai 2002). UCP mediates thermogenesis in mammalian cells by dissipating protons instead of synthesizing ATP (Klingenberg 1999). The amino acid sequence of UCP contains three repeat units of about 100 amino acids each that are similar to each other. Each unit consists of two transmembrane domains and a typical mitochondrial transporter signature between the transmembrane domains (Palmieri 1994). UCP is translated in the cytoplasm and then transported to the mitochondria although it lacks an NSP, indicating that the information for the transport of this protein to the mitochondria is contained within the internal mature sequence. However, an internal signal peptide (ISP) that plays a significant role in transport of UCP to mitochondria.
has not been clearly identified yet.

To determine the region within wheat UCP acting as the protein import signal to mitochondria, we analyzed mitochondrial localization of proteins constructed by fusing three regions of wheat UCP to green fluorescence protein (GFP) and expressing them in yeast cells.

**Materials and Methods**

**Preparation of plasmid constructs**

The pYES2.1 expression vector containing the V5 epitope and the histidine tag at the C-terminus (Invitrogen, USA) was used to express the fusion protein in yeast cells. Five kinds of plasmid constructs were used in this study (Fig. 1). The whole wheat UCP gene, WhUCP1b (DBJ Acc. No. AB042429) was amplified using the p1 and p6 primer pair (Table 1). The amplified fragments were digested with SalI and NcoI, and then ligated in-frame with the corresponding site of the pTH-2/GFP expression vector CaMV35SPro::sGFP(S65T)::NOSter (a kind gift from Drs. Yasuo Niwa and Hirokazu Kobayashi, University of Shizuoka) (Chiu et al. 1996). The WhUCP::GFP fragment was amplified from pTH-2/WhUCP::GFP plasmid DNA using the p1 and p7 primer pair. The amplified fragments were ligated in-frame with the pYES2.1 yeast expression vector (pYES2.1/WhUCP::GFP, Fig. 1). Four kinds of plasmid constructs used to identify the region responsible for protein transport to mitochondria were prepared as follows.

The WhUCP1b protein was divided into three regions (D1, D2 and D3) corresponding to amino acids positions 1-104aa, 105-191aa and 192-280aa, respectively. Each region contained a conserved sequence of mitochondrial carrier proteins between two transmembrane domains. The DNA fragments corresponding to the D1, D2 and D3 regions were amplified from the WhUCP1b cDNA clone using primer pairs p1-p2, p3-p4 and p5-p6, respectively. The amplified fragments were digested with SalI and NcoI, and then ligated in-frame to the corresponding site of the pTH-2/GFP expression vector. The pTH-2/GFP vectors harboring the amplified fragments were digested with SalI and NotI, and then the excised fragments were ligated into the corresponding site of the pBluescriptII SK+ plasmid vector (Stratagene, USA) (pSK/D1::GFP, pSK/D2::GFP and pSK/D3::GFP plasmids using primer pairs p1-p7, p3-p7 and p5-p7, respectively. The amplified fragments were finally ligated in-frame to the pYES2.1 yeast expression vector (pYES2.1/D1::GFP, pYES2.1/D2::GFP, pYES2.1/D3::GFP, Fig. 1).

The red fluorescent protein (DsRed) gene was amplified from the pDsRed expression vector (Clontech, USA) using primer pair p8-p9. The amplified fragment was digested with Apal and SalI, and then ligated into the corresponding site of pSK/D2::GFP plasmid. The fragments corresponding to the DSR::D2::GFP were amplified from pSK/DSR::D2::GFP plasmid DNA using primer pair p7-p8, and then ligated in-frame to pYES2.1 expression vector (pYES2.1/DSR::D2::GFP, Fig. 1).

**Yeast transformation and induction of fusion protein**

The yeast strain INVSc1 (Invitrogen, USA) was used in this study. This strain is a diploid strain that is auxotrophic for histidine, leucine, tryptophan and uracil. The pYES2.1 expression vector contains the GAL1 promoter, whose transcription is repressed in the presence of glucose (West et al. 1984). Transcription is induced by replacing the glucose with galactose as a carbon source (Giniger et al. 1985).

Yeast transformation was carried out using an S. c. Easy Comp Transformation Kit (Invitrogen, USA) and the recombinants were selected on the SDA-URA medium plates (BIO101, USA). The induction was carried out in 50 ml of induction medium (0.67% yeast nitrogen base without amino acids with ammonium sulfate; 1% raffinose; 2% galactose; 0.01% each adenine, arginine, cysteine, leucine, lysine, threonine and tryptophan; 0.005% each aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine and valine) for 24 h.

**Observation of the yeast cells expressing the fusion proteins under a fluorescence microscope**

Mito Tracker Red CMXRos (Molecular Probes, USA), which specifically stains mitochondria, was added to the induction medium in which the recombinants expressing

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**Table 1. Primer sequences used to prepare plasmid constructs**

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>p1</td>
<td>D1-5F</td>
<td>TCCCCGTGACAAATGGCGACGGC</td>
</tr>
<tr>
<td>p2</td>
<td>D1-3R</td>
<td>CTTGCCATGGAAGTGGCATACTAC</td>
</tr>
<tr>
<td>p3</td>
<td>D2-5F</td>
<td>GATTGGTCGACCTTAATGAACAG</td>
</tr>
<tr>
<td>p4</td>
<td>D2-3R</td>
<td>CTAGAACCATGGGTTAAATGTCG</td>
</tr>
<tr>
<td>p5</td>
<td>D3-5F</td>
<td>CCACGGTCGACAAATTAACAGT</td>
</tr>
<tr>
<td>p6</td>
<td>D3-3R</td>
<td>CTGGTCCATGTTAAAGACATTAC</td>
</tr>
<tr>
<td>p7</td>
<td>GFP-3R</td>
<td>CTGTACAGCTCGTCACTG</td>
</tr>
<tr>
<td>p8</td>
<td>DSR-5F</td>
<td>GCCGCAAGTCGACTCTAGAG</td>
</tr>
<tr>
<td>p9</td>
<td>DSR-3R</td>
<td>CGGCCGCTAAAGGAACAGATGG</td>
</tr>
</tbody>
</table>

Underlines indicate restriction enzyme sites.
WhUCP::GFP or D#::GFP fusion proteins were cultured, and the culturing was continued for 1 h. Then the cells were harvested, washed once with distilled water (dH2O) and resuspended in an adequate volume of dH2O. Recombinants expressing the DSR::D2::GFP fusion protein were harvested and washed once with dH2O. The pellet was resuspended in an adequate volume of 40 mM phosphate buffer (pH 7.2). The GFP images were observed with a Nikon E600 fluorescence microscope through GFP(R)-BP filter (Nikon, Japan). DsRed and Mito Tracker Red were detected with a G-2A filter (Nikon, Japan).

Isolation of mitochondrial and cytoplasmic fractions from yeast cells

Yeast mitochondria were isolated according to a previously reported method (Daum et al. 1982) with a slight modification. When the crude mitochondrial fraction was collected from the yeast homogenate by centrifugation, the supernatant was stored as the crude cytoplasmic fraction.

Western blot analysis

Proteins from the crude cytoplasmic and mitochondrial fractions were separated by electrophoresis on a 15% SDS polyacrylamide gel and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, USA). Anti-V5-HRP antibody conjugated to horseradish peroxidase (Invitrogen, USA) was incubated with the membrane for hybridization with the fusion protein at a final dilution of 1:5000. The reactive protein was detected using an ECL reagent (Amersham Pharmacia Biotech, UK) according to the manufacturer’s instruction. The membrane was exposed to an X-ray film.

Results

Visual analysis by fluorescence microscopy of yeast cells expressing fusion proteins

The WhUCP::GFP fusion protein was expressed in yeast cells to confirm that the WhUCP protein was transported to mitochondria in yeast cells. Many green spots were detected in yeast cells expressing the WhUCP::GFP fusion protein (Fig. 2A), and mitochondria were observed clearly as red fluorescent spots in yeast cells stained with Mito Tracker Red (Fig. 2B). The green spots of GFP in yeast cells expressing the WhUCP::GFP fusion protein overlapped well with the mitochondrial spots indicated by Mito Tracker Red staining of the same field (Fig. 2A and 2B), which showed that the WhUCP protein could be transported to mitochondria. To identify the region within WhUCP required for the protein transport to the mitochondria, we expressed three different fusion proteins in yeast cells and the localization of each fusion protein was examined in vivo. In yeast cells expressing the D1::GFP and the D3::GFP fusion proteins, green fluorescence of GFP could be detected (Fig. 2C and 2G) although the GFP spots of these fusion proteins were dim and a long exposure time was needed for clear detection in both cases. However, the mitochondrial spots stained with Mito Tracker Red (Fig. 2D and 2H) overlapped with the green spots in the GFP image (Fig. 2C and 2G) in the same field. This indicated that the D1::GFP and D3::GFP fusion proteins were transported to the mitochondria. On the other hand, the yeast cells expressing D2::GFP fusion protein showed light green fluorescent spots of GFP (Fig. 2E). These green fluorescent spots in the GFP image of D2::GFP-expressing cells overlapped well with the mitochondrial particles stained with Mito Tracker Red in the same field (Fig. 2E and 2F). This result demonstrated that the D2::GFP fusion protein was also transported to mitochondria.

The fusion protein (DSR::D2::GFP), in which the D2 region was introduced between DsRed and GFP, was expressed in yeast cells. Green and red fluorescent spots were detected clearly in yeast cells expressing the DSR::D2::GFP fusion protein (Fig. 3A and 3B). The fluorescent spots

Fig. 2. Visual analysis of yeast cells expressing the fusion protein WhUCP::GFP (A, B), D1::GFP (C, D), D2::GFP (E, F) or D3::GFP (G, H). GFP images (A, C, E and G) and Mito Tracker Red images (B, D, F and H) were obtained using a GFP(R)-BP filter and a G2-A filter, respectively. The GFP and Mito Tracker Red images shown were obtained from the same field. The white bar in panel H indicates 2 µm.

Fig. 3. Fluorescent images obtained with GFP (A) and DsRed (B) in yeast cells expressing DSR::D2::GFP. The images show the same field. The white bar in panel B indicates 1 µm.
observed in the GFP image overlapped precisely with the red spots in the DsRed image. This indicated that the DSR::D2::GFP fusion protein was transported to mitochondria, even though the D2 region was located in the central part of the fusion protein.

Immunological detection of fusion proteins in yeast mitochondria

To analyze further the details of the protein transport activity to mitochondria in the three repeat regions of WhUCP1b, we prepared cytoplasmic and mitochondrial fractions from the yeast cells, and detected the fusion protein by Western blot analysis using anti-V5 epitope antibody. In Figure 4A, the lane containing the mitochondrial fraction (M) prepared from yeast cells expressing WhUCP::GFP fusion protein showed a clear band about 65 kDa in size, which corresponds to the predicted molecular weight of the WhUCP::GFP fusion protein, although a faint band of the same size was also detected in the cytoplasmic fraction lane (C). The Western blot of the cytoplasmic and mitochondrial fractions from yeast cells expressing D#::GFP showed the bands of around 45 kDa in all lanes (Fig. 4B). The molecular weights of these proteins detected by Western blot analysis corresponded well to the estimated molecular weights of D#::GFP. The fusion protein containing the D2 region, D2::GFP, showed the highest transport efficiency to mitochondria. The other two fusion proteins (containing either the D1 or D3 region) also showed some transport activity (Fig. 4B). These results of Western blot analysis demonstrated that all three regions of WhUCP1b that contained the conserved sequence of mitochondrial carrier proteins between the two transmembrane regions possessed the ability to promote protein transport to mitochondria.

Discussion

The mitochondrial uncoupling protein (UCP) is a nuclear-encoded mitochondrial protein without an NSP. It is still unknown how this nuclear-encoded mitochondrial protein without an NSP is transported specifically to the mitochondria. In this study, we divided the wheat UCP protein into three regions, each of which contained two transmembrane domains and the consensus sequence commonly found in mitochondrial carrier proteins, in order to investigate which region of the WhUCP1b protein directs the protein transport to the mitochondria. In vitro protein transport experiments using the nuclear-encoded mitochondrial carrier proteins, rat UCP1 and yeast ADP/ATP carrier protein (AAC1) (Liu et al. 1999) have previously been reported. However, there have been no reports of in vivo experiments examining the protein transport activity of mitochondrial carrier proteins such as UCP. Recently, reporter gene systems using fluorescent proteins such as GFP or DsRed have been developed, and these fluorescent proteins allowed us to observe the localization of the fusion protein in vivo directly. We employed a reporter system using fluorescent proteins to clarify the localization in the yeast cells of expressed fusion proteins consisting of one of the UCP regions plus the fluorescent protein. The WhUCP::GFP, D#::GFP and DSR::D2::GFP fusion proteins showed mitochondrial localization in yeast cells clearly under a fluorescence microscope (Fig. 2 and Fig. 3) although the fluorescence of GFP in yeast cells expressing D1::GFP and D3::GFP was dim. The results of Western blot analysis demonstrated the protein transport of all fusion proteins to mitochondria, but the levels of protein transport of fusion proteins containing D1 or D3 region were clearly lower than those of WhUCP::GFP and D2::GFP. In this study we expressed plant UCP in yeast cells to check the protein transport activity to mitochondria, assuming that the basic transport system is the same in yeast and plant cells, because mitochondrial carrier proteins including UCP are ubiquitous in eukaryotic cells (plants, fungi and animals). However, experiments using plant cells will be needed to exclude the possibility of a plant-specific modifying factor of protein transport.

Each member of the mitochondrial carrier protein family, including UCP and AAC, contains three repeat units. Several in vitro studies on the protein transports of mitochondrial carrier proteins, UCP and AAC, have been reported. Two groups concluded that the first repeat unit was important for the protein transport to mitochondria (Liu et al. 1988, Smagula and Douglas 1988), but others reported that the second or third repeat unit was required for the protein transport (Schleiff and McBride 2000, Endres et al. 1999). Our results of fluorescent microscopic observation and Western blot analysis revealed that all of the fusion proteins tested in this study were transported to the mitochondria (Fig. 4). D2 region showed the highest protein transport efficiency to mitochondria (Fig. 4B), but other repeat regions of

\[ \text{WhUCP::GFP} \quad \text{(kDa)} \quad \text{D1::GFP} \quad \text{(kDa)} \quad \text{D3::GFP} \quad \text{(kDa)} \]

\[ \begin{array}{cccc}
\text{Fig. 4. Detection of fusion proteins in mitochondrial and cytoplasmic fractions prepared from yeast cells expressing WhUCP::GFP (A) and D#:GFP (B) by Western blot analysis. The proteins of the mitochondrial fraction (lanes M) and cytoplasmic fraction (lanes C) were blotted (3.15 µg/lane), and then the fusion proteins were detected using anti-V5 antibody. The molecular weights of standard markers are indicated on the left.} \\
\end{array} \]
WhUCP1b could also direct foreign protein transport to mitochondria. Thus all three regions of WhUCP may contribute to the transport of the intact WhUCP1b to mitochondria. Multiple signals were found to cooperate for the transport of intact AAC protein to mitochondria (Wiedemann et al. 2001) and this finding for AAC protein transport supports our hypothesis for the transport of UCP. Although all three repeat regions of WhUCP1b have a common structure specific to mitochondrial carrier proteins, two transmembrane domains and a mitochondrial transporter signature between them, the amino acid sequences have only about 30% of similarity to each other. Therefore it is difficult to estimate the critical structures or amino acid residues for the protein transport to mitochondria based on the sequence comparison of the three repeat regions.

Transport of a foreign protein to mitochondria using NSPs has been reported (Chaumont et al. 1994). In contrast to NSPs which must be located at the N-terminus to function, ISPs can be located anywhere within a fusion protein. In this study, we used the D2 region of WhUCP1b as a potential ISP by locating it in the central region of the fusion protein (DSR::D2::GFP), to transport two different proteins to the mitochondria. Moreover, it may be possible to transport a foreign protein containing the ISP of WhUCP1b at the C-terminus to mitochondria, although we did not carry out such experiments here. These results suggest that the biological and physiological properties of mitochondria might be altered by transporting two different proteins into the mitochondria simultaneously. For example, mitochondrial function might be improved by transporting a particular enzyme that functions as a heterodimer, such as maleic dehydrogenase (Doebley et al. 1986). Improvement of the protein transport efficiency by using ISPs and production of recombinant plants by introducing two different proteins into mitochondria based on the sequence comparison of the three repeat regions.

Acknowledgments

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Literature Cited


