A 10 kD BARLEY BASIC PROTEIN TRANSFERS PHOSPHATIDYLCHOLINE FROM LIPOSOMES TO MITOCHONDRIA

by

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A small basic 10 kD abundant protein in barley seeds can convey up to 7% of the phosphatidylcholine in liposomes to potato mitochondria whereas cholesterylolate is not transported. The demonstration of this activity combined with a somewhat more than 50% homology of its primary structure to that of other plant phospholipid transfer proteins are the bases for our naming it a barley lipid transfer protein (LTP).

1. INTRODUCTION

Several years ago an abundant basic protein of circa 10 kD was isolated from barley seeds (caryopses) and the sequence of its 42 amino terminal residues plus the identity of its carboxyl residue determined (7). Simultaneously a full length clone from a barley aleurone cDNA library was characterized (6). A segment of the resulting deduced amino acid sequence matched perfectly with that available for the 10 kD protein suggesting that the latter consists of 91 residues including 8 half-cystines from which

a 26 amino acid signal sequence has been cleaved. The primary structure of this protein was circa 50% homologous with the α -amylase inhibitor I-2 isolated from seeds of Indian finger millet and showed a distant homology with two Bowman Birk protease inhibitors. None of the amylases or proteases tested, however, were inhibited by the 10 kD basic protein (7) which was named probable α -amylase protease inhibitor, PAPI (6).

Tissues from other plants such as maize endosperms plus embryos as well as coleoptiles (4,

8, 9), castor bean endosperms (10), and spinach leaves (5) contain basic proteins with a molecular mass of 9 kD. These proteins mediate in vitro transfer of phospholipids from liposomes to organelles such as mitochondria and chloroplasts (5), and are classified as lipid transfer proteins. Very recently a search in a data bank for additional potential members of this group of proteins disinterred the barley 10 kD basic protein (1). To illustrate, the sequence of the latter was somewhat more than 50% homologous to that of the maize endosperm plus embryo phospholipid transfer protein. This observation suggested a hitherto unexpected function for the 10 kD protein quite different from what its name implied. In this communication we present the results of assays with the barley protein demonstrating its ability to transfer phospholipids between membranes.

2. MATERIALS AND METHODS

2.1. Materials

Barley seeds (*Hordeum vulgare*, cv. Hiproly) were obtained from Carlsberg Plant Breeding and potato tubers (*Solanum tuberosum*, cv. Bintje) from the local market. Methyl-[³H]-choline chloride ($77 \text{ Ci} \times \text{mmol}^{-1}$) and cholesteryl-[1-¹⁴C]-oleate ($50 \text{ Ci} \times \text{mmol}^{-1}$) were from the Radiochemical Centre, Amersham, UK and the egg yolk phosphatidylcholine from Sigma, St. Louis, USA. [³H]-phosphatidylcholine ($0.3 \text{ Ci} \times \text{mol}^{-1}$) was prepared from potato tuber slices incubated with methyl-[³H]-choline chloride as previously described (5). Maize phospholipid transfer protein was purified from three day-old seedlings by CM-Sepharose chromatography as previously described (3).

2.2. Preparation of mitochondria, liposomes and 10 kD barley protein

Mitochondria were isolated from potato tubers by sucrose gradient centrifugation and stored in 10 mM Tris HCl, 250 mM sucrose, 1 mM Na₄EDTA at pH 7.2 (TSE buffer) at a concentration of $10 \text{ mg protein} \times \text{ml}^{-1}$.

A stock of liposomes was prepared: 2.6 μmol egg yolk phosphatidylcholine, 70 nmol

cholesteryl-[1-¹⁴C]-oleate (200,000 dpm) and an aliquot of [³H]-phosphatidylcholine (200,000 dpm) were dissolved in 1 ml of chloroform. After evaporation of the solvent, 5 ml of the TSE buffer was added and the suspension sonicated for 30 min at 20 °C under nitrogen. This suspension can be kept at 0–4 °C for up to 24 h before use.

Protein was purified from barley flour essentially as described previously (7). After the separation on CM-cellulose and Sephadex G75, protein was lyophilized without desalting and stored at -18 °C. Its protein content of 37.3% was determined on the basis of amino acid analysis (7). A sample of desalted 10 kD protein was prepared as described using Sephadex G25 and lyophilization.

2.3. Lipid transfer assay

Liposomes (0.5 ml) were incubated with potato mitochondria (2 mg of protein) in the presence or absence of protein fractions in a total volume of 3 ml as has been described (5). After 30 min incubation at 30 °C, 5 ml of cold TSE buffer was added and the mixture centrifuged at $15,000 \times g$ for 10 min. The mitochondrial pellets were suspended in 0.3 ml of 1% Triton X-100 to which 10 ml of a toluene scintillant was added and the [³H] and [¹⁴C] contents measured using an LKB 1219 Rackbeta liquid scintillation counter (Uppsala, Sweden).

3. RESULTS AND DISCUSSION

When mitochondria were incubated with liposomes without added protein, very little label was recovered in the mitochondrial pellets, that is, only 2 and 1% of the liposomal [³H]-phosphatidylcholine and [¹⁴C]-cholesteryl-oleate radioactivity, respectively (Figure 1). As increasing amounts of the barley 10 kD protein were used in the experimental mixtures, more and more [³H] label was found in the acceptor organelles. This enrichment is due to transfer of phosphatidylcholine molecules from liposomes to mitochondria and not to a coprecipitation of the donor liposomes with the acceptor organelles. The latter possibility is ruled out by the fact that the [¹⁴C] label attributable to

Figure 1. Transfer of phosphatidylcholine from liposomes to mitochondria in the presence of the barley lipid transfer protein. Liposomes prepared from [^3H]-phosphatidylcholine and [^{14}C]-cholesteryl oleate were incubated with mitochondria in the presence of the specified amounts of 10 kD barley protein, whereafter the radioactivity of the mitochondrial pellets was determined as described in Section 2. The results are expressed in A as radioactivity per mitochondrial pellet and in B as nmole lipids transferred per mitochondrial pellet.

cholesteryl oleate recovered in mitochondria did not parallel the increasing amounts of the 10 kD protein. This is in accord with the observation that cholesteryl oleate is not a substrate for plant phospholipid transfer proteins (4). Additional assays carried out with the desalted barley protein gave similar results indicating that the Sephadex G25 desalting step is not necessary for the detection of the activity.

The data of Figure 1A which is typical of five assays reveal that 7% of the liposomal phosphatidylcholine was transferred to mitochondria during the 30 min incubation with the highest amount of 10 kD protein (2 mg). The marked predilection for the phosphatidylcholine moieties of the liposomes is revealed

when the results are replotted on a nmolar basis (Fig. 1B). This value is lower than the 15-20% transfer obtained with the maize phospholipid transfer protein under the same experimental conditions and that reported for the spinach protein (5). The castor bean protein, however, transfers only 8-10%. To what extent the lyophilization process has contributed to the observed activity of the 10 kD barley protein is unknown as none of the other phospholipid transfer proteins have been lyophilized before being assayed. Nevertheless this protein is remarkably stable as activity is measurable after five years storage. Perhaps this attribute is a prerequisite for the function of seed phospholipid transfer proteins. In this connection it can

be noted that the maize protein is thermoresistant (3).

The present experiments establish that the 10 kD basic protein from barley seeds transfers phospholipids *in vitro*. We therefore propose that it is classified as a barley lipid transfer protein (LTP) with the gene symbol *Ltp* replacing the former designations PAPI (6) and *Ipa* (11), respectively. The protein and its messenger are abundant in barley seeds (6, 7). The mRNA is present not only during the latter half of seed development but also in the mature and germinating seed in the aleurone (6, 7), which tissue is characterized by the presence of large numbers of oleosomes (2). Most interestingly the barley lipid transfer protein is not only present in but is also secreted from cultured aleurone layers into the surrounding medium (6) inferring that it may act in the endosperm cells. These observations raise the question of the barley LTP's *in vivo* function. The protein may be required to convey lipids from sites of storage to sites where they are broken down by β -oxidation to provide energy for germination. Alternatively the primary function of this protein may be to transfer specific lipids to developing organelles.

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REFERENCES

1. BERNHARD, W.R. & C.R. SOMERVILLE: Coidentity of putative amylase inhibitors from barley and finger millet with phospholipid transfer proteins inferred from amino acid sequence homology. *Arch. Biochem. Biophys.* 269, 695-697 (1989)
2. CAMERON-MILLS, V. & D. VON WETTSTEIN: Protein body formation in the developing barley endosperm. *Carlsberg Res. Commun.* 45, 577-594 (1980)
3. DOUADY, D., M. GROSBOIS, F. GUERBETTE & J.-C. KADER: Purification of a basic phospholipid transfer protein from maize seedlings. *Biochim. Biophys. Acta* 710, 43-153 (1982)
4. KADER, J.-C.: Lipid-binding proteins in plants. *Chem. Phys. Lipids* 38, 51-62 (1985)
5. KADER, J.-C., M. JULIENNE & C. VERGNOLLE: Purification and characterization of a spinach-leaf protein capable of transferring phospholipids from liposomes to mitochondria or chloroplasts. *Eur. J. Biochem.* 139, 411-416 (1984)
6. MUNDY, J. & J.C. ROGERS: Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: Comparison to the barley amylase/subtilisin inhibitor. *Planta* 169, 51-63 (1986)
7. SVENSSON, B., K. ASANO, I. JONASSEN, F.M. POULSEN, J. MUNDY & I. SVENDSEN: A 10 kD barley seed protein homologous with an α -amylase inhibitor from Indian finger millet. *Carlsberg Res. Commun.* 51, 493-500 (1986)
8. TCHANG, F., P. THIS, V. STIEFEL, V. ARONDEL, M.-D. MORCH, M. PAGES, P. PUIGDOMENECH, F. GRELLET, M. DELSENY, P. BOUILLON, J.-C. HUET, F. GUERBETTE, F. BEAUVAIS-CANTE, H. DURANTON, J.-C. PERNOLLET & J.-C. KADER: Phospholipid transfer protein: full-length cDNA and amino acid sequence in maize. Amino acid sequence homologies between plant phospholipid transfer proteins. *J. Biol. Chem.* 263, 16849-16855 (1988)
9. VERGNOLLE, C., V. ARONDEL, F. TCHANG, M. GROSBOIS, F. GUERBETTE, A. JOLLIOT & J.-C. KADER: Synthesis of phospholipid transfer proteins from maize seedlings. *Biochem. Biophys. Res. Commun.* 157, 37-41 (1988)
10. WATANABE, S. & M. YAMADA: Purification and characterization of a non-specific lipid transfer protein from germinated castor bean endosperms which transfers phospholipids and galactolipids. *Biochim. Biophys. Acta* 876, 116-123 (1986)
11. WETTSTEIN-KNOWLES, P. VON: Facets of the barley genome. In: *Science for Plant Breeding. Vorträge für Pflanzenzüchtung*, vol 16, P. Parey Sci. Publ., Berlin (in press)

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