Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 37-41

SYNTHESIS OF PHOSPHOLIPID TRANSFER PROTEINS FROM MAIZE SEEDLINGS

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Received October 6, 1988

The synthesis of phospholipid transfer proteins has been studied *in vitro* after isolation of poly(A)+RNAs from maize seedlings and by *in vivo* labelling of coleoptiles. After immunoprecipitation of translation products in wheat germ or in reticulocyte lysate systems, the analysis by electrophoresis revealed two bands of molecular mass 9 kDa and 12 kDa. The *in vitro* synthesized 12 kDa protein is a precursor of the 9 kDa purified protein from maize seedlings as suggested by competition experiments with the pure protein. After immunoprecipitation of *in vivo* labelled proteins, two bands were detected. One of them, having a molecular mass of 7 kDa, could be related to the *in vitro* synthesized 9 kDa protein, the other corresponding to the purified protein. Furthermore, biosynthesis of both precursors occurs on membrane-bound polysomes. Presumably a post translational process occurs, yielding to the mature forms.

Phospholipid transfer proteins (PLTPs) have been isolated from animal (1-2) and plant (3) tissues. It has been demonstrated that these proteins facilitate *in vitro* the transfer of phospholipids between two membranes (4). In plant tissues, PLTPs have been isolated from maize seedlings (5), spinach leaves (6) and castor bean endosperm (7). The biochemical properties of these proteins are now well known. In plants they have a molecular mass close to 9 kDa (3) and they are mainly basic (pI around 9). Furthermore the primary structure has been established for spinach (8) and castor bean endosperm proteins (9). Plant PLTPs have a broad specificity for transferring phospholipids; they transfer phosphatidylcholine, as well as phosphatidylethanolamine or phosphatidylinositol (3); in addition some experiments showed that PLTPs also bind fatty acids (10). Polyclonal antibodies against maize PLTP have been obtained (11). They have been used to show that PLTPs are partly bound to the membranes (12), although PLTPs are isolated from cytosolic extracts. In spite of the fact that most of the biochemical aspects are well known, the physiological function of PLTPs is not elucidated.

In order to know the *in vivo* function of PLTPs, studies on their biosynthesis have been done. We have isolated poly(A)+RNAs from maize coleoptile and subsequently chromatographed the *in vitro* translation products by HPLC (13). Among the synthesized proteins, a 9 kDa polypeptide was detected. This first result opened new perspectives for the studies of PLTPs.

Abbreviations: PLTP: phospholipid transfer protein, HPLC: high performance liquid chromatography.

0006-291X/88 $1.50

In the present paper, we go further in the study of the biosynthesis of maize PLTPs using antiPLTP antibodies. We have evidenced from *in vitro* translation experiments two forms of maize PLTPs. We also showed that PLTPs are synthesized as precursors on membrane-bound polysomes.

MATERIALS AND METHODS

Plant material and preparation of maize PLTP

Maize seeds (*Zea mays* L., c.v. Mona) were germinated on moist vermiculite at 30°C in the dark for 3 days.

Highly purified maize PLTP was prepared and radiolabelled as previously described (5,13).

mRNA and polysome preparations

Extraction of RNAs was carried out as previously described (13). Poly(A)+RNAs were purified by oligo(dT) cellulose affinity chromatography according to Laroche-Raynal et al (14).

Free cytoplasmic and membrane-bound polysomes were isolated as described by Tercé-Laforgue and Pernollet (15).

In vitro protein synthesis

Translation assays were carried out using a rabbit reticulocyte lysate kit (N150 from Amersham, England) or a wheat germ system prepared as described by Morch et al (16). (^{35}S) cysteine ($7.4\ 10^2$ kBq) (Amersham) were used for each assay. Various amounts of poly(A)+RNAs or polysomes were added to each translation mixture. An aliquot (2%) was taken for determining total incorporation of (^{35}S) cysteine in proteins; about 15% were taken for electrophoresis to obtain a total protein pattern as described previously (13) and the remainder was used for immunoprecipitation.

In vivo labelling and protein extraction

(^{35}S) Cysteine ($5.6\ 10^3$ kBq) was deposited on coleoptiles . After 5h, coleoptiles were washed with distilled water and treated as described by Meza-Basso et al (17).

Immunoprecipitation

Immunoprecipitation of labelled proteins was performed using antiPLTP antibodies prepared as described previously (11).

In vitro translation mixtures or *in vivo* radiolabelled protein extracts were diluted ten fold with a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 10 mM L. cysteine, 100 µM phenyl methyl sulfonyl fluoride (PMSF), 1% Tween. Preimmune serum (5 µl) was added and the mixture was incubated 1h at room temperature. Protein A Sepharose CL 4B (18) was then added and the incubation was continued for 1 h. The mixture was centrifuged at 10 000 *g* for 10 min. The supernatant was mixed with antiPLTP antibodies and shaken 1h at room temperature, then protein A Sepharose was added. Incubation was continued for 1h at the same temperature and then overnight at 4°C. The mixture was then deposited in yellow tip plugged with glass fiber. The column was rinsed three times with 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 1 M NaCl, 1% Triton X100, 100 µM PMSF, and then dried. The antibody-antigene complex was removed from protein A Sepharose with 50 µl of 30 mM Tris-HCl (pH 6.8), 1% SDS, 2.5% 2 mercaptoethanol, 5% glycerol and 10 µg/ml of bromophenol blue. The immunoprecipitation products were analyzed by electrophoresis on 15% polyacrylamide-SDS gels and detected by fluorography (13).

RESULTS

Poly(A)+RNAs isolated from maize coleoptiles were translated in wheat germ and reticulocyte lysate systems with (^{35}S) cysteine (Fig. 1). The products of translation were immunoprecipitated with antiPLTP antibodies, then analyzed by SDS-PAGE and fluorography (Fig. 2 lane 2 and Fig. 3 lane 1). Surprisingly, in both systems, two radioactive bands were detected corresponding to a molecular mass of 9 kDa and 12 kDa. To determine which

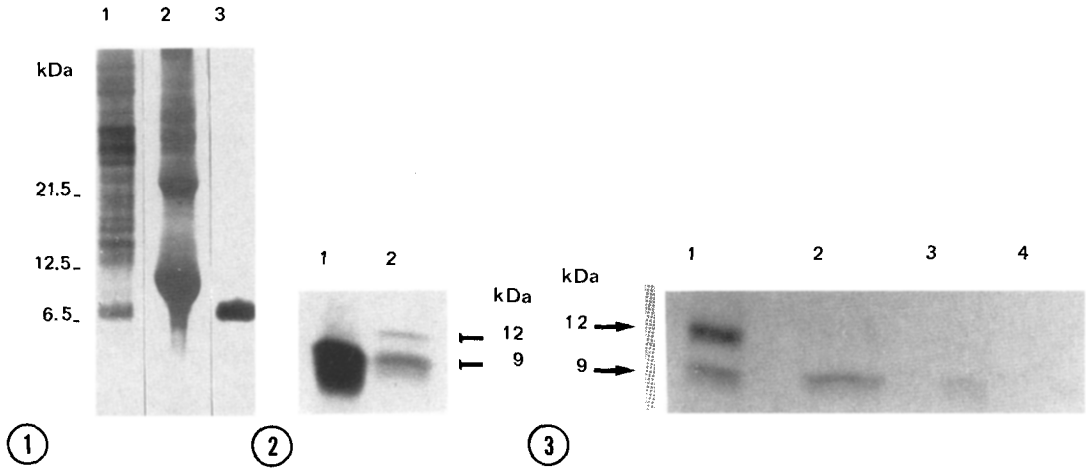

Fig. 1: SDS-PAGE of in vitro translation products of maize poly(A)+RNAs. Poly(A)+RNAs were translated in wheat germ (Lane 1) or reticulocyte lysate (Lane 2) systems in presence of (^{35}S) cysteine. Lane 3: (^{14}C) purified PLTP. The molecular masses are indicated on the left side of the figure.

Fig. 2: SDS-PAGE of immunoprecipitated proteins from in vitro translation products in wheat germ system.
Lane 1: (^{14}C) purified PLTP; Lane 2: Poly(A)+RNAs were translated in wheat germ system and immunoprecipitated with antiPLTP antibodies.

Fig. 3:SDS-PAGE electrophoresis of immunoprecipitated proteins from in vitro translation products in rabbit reticulocyte lysate system in presence of unlabelled purified PLTP.
Poly(A)+RNAs were translated and the products were immunoprecipitated with antiPLTP antibodies. 0 µg (Lane 1), 0.2 µg (Lane 2), 2 µg (Lane 3), 4 µg (Lane 4) of purified PLTP were added before the immunoprecipitation.

band corresponds to purified PLTP, various amounts of unlabelled PLTP were added prior to the immunoprecipitation. For low amounts (0.2 µg) of purified PLTP, the 12 kDa band disappears while the 9 kDa is unchanged. It is necessary to add a higher amount of purified PLTP (2-4 µg) to eliminate the 9 kDa band (Fig. 3).

These results show that the 12 kDa band obtained *in vitro* is related to the purified PLTP.

The synthesis of PLTP was also studied by *in vivo* labelling of maize coleoptiles (Fig. 4 lane 1). Synthesized proteins were immunoprecipitated and two labelled polypeptides were obtained, corresponding to 7 kDa and 9 kDa (Fig. 4 lane 2). When unlabelled purified PLTP was added before immunoprecipitation, a decrease in the intensity was observed only in the 9 kDa band which is the mature form of purified PLTP (Fig. 4 lane 3 and 4). Concerning the 7 kDa polypeptide it can be supposed that it is the mature form of the 9 kDa polypeptide obtained by immunoprecipitation of *in vitro* translation products.

These experiments indicate that PLTPs are synthesized as precursors with higher molecular masses which probably undergo a post translational maturation.

Preliminary experiments have been done on the maturation of the PLTPs. Membrane-bound and free polysomes were isolated and translated in wheat germ system (Fig. 5 lanes 1 and 2); the translation products were immunoprecipitated with antiPLTP antibodies. The two polypeptides evidenced by translation of poly(A)+RNAs were found to be synthesized exclusively on membrane-bound polysomes (Fig. 5 lane 4).

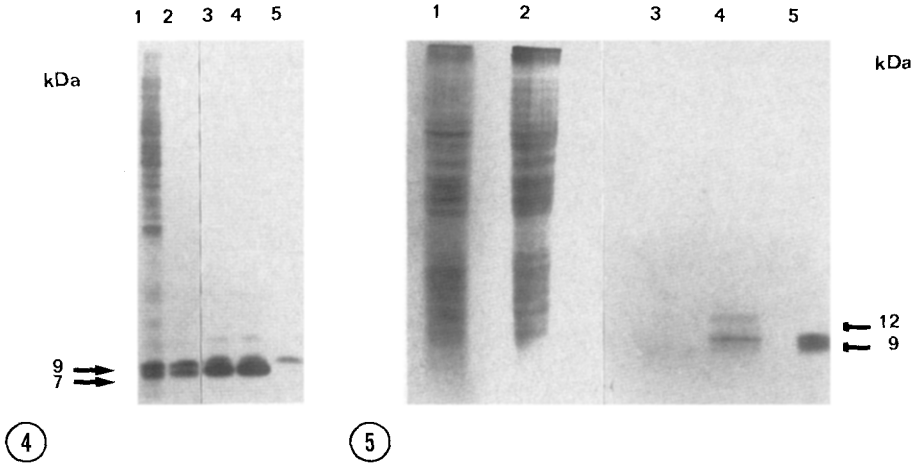

Fig. 4: SDS-PAGE of in vivo labelled proteins from maize coleoptile: total pattern and immunoprecipitated proteins.
Coleoptiles of 3-day old seedlings were labelled with (^{35}S) cysteine and total proteins were analyzed in SDS-PAGE (Lane 1). The proteins were immunoprecipitated with antiPLTP antibodies in the absence (Lane 2) or in the presence of unlabelled purified PLTP: 10 µg (Lane 3), 20 µg (Lane 4). Lane 5: (^{14}C) purified PLTP.

Fig. 5: SDS-PAGE electrophoresis of immunoprecipitated polypeptides synthesized on membrane-bound and free polysomes.
Free (Lane 1) and membrane-bound (Lane 2) polysomes were translated in wheat germ system in presence of (^{35}S) cysteine . The products were immunoprecipitated with antiPLTP antibodies corresponding to free polysomes (Lane 3) and to membrane-bound polysomes (Lane 4). Lane 5: (^{14}C) purified PLTP.

DISCUSSION AND CONCLUSION

For the first time in plant cells, we have demonstrated the presence of two forms of PLTP which are synthesized as larger precursors on membrane-bound polysomes. The two forms have molecular masses of 7 kDa and 9 kDa which correspond respectively to the 9 kDa and 12 kDa precursors. In a previous paper (13), *in vitro* translation products were separated by chromatography and only the 9 kDa polypeptide was detected. Our present results suggest the presence of signal peptides of respectively 2 kDa and 3 kDa, which are post translationally removed.

In animal tissues it has been recently shown that the nonspecific transfer protein, also called sterol carrier protein 2 (SCP2) is synthesized as a precursor larger than the mature form (19).

The existence of PLTP isoforms has been noticed in other tissues. In human liver (20), three different forms of SCP2 have been detected. In a preliminary study , the authors showed that one form was located in peroxisomes. In plant tissues, the major part of the transfer activity is associated with basic PLTPs; no isoform of these proteins has been demonstrated. Nevertheless, in castor bean endosperm, Tanaka and Yamada (21) have separated different acidic fractions which have a transfer activity.

The 7 kDa protein could be related with an observation made by Douady et al (12). These authors have immunoblotted protein fractions from different steps of purification of PLTP and from solubilized organelle membranes. All the fractions contained a 9 kDa protein but a lower band was also noticed in the cytosolic fractions.

The existence of two forms of PLTPs synthesized as precursor on the reticulum endoplasmic opens new perspectives for the study of the biosynthesis of PLTPs and their function. The next step will be the study of the maturation process of PLTP as well as the intracellular and tissular localization of the different forms of PLTPs.

ACKNOWLEDGMENT

The authors are much indebted to Drs. P. Puigdomenech and M. Delseny for their advice and helpful discussion. We also thank J. Thioust for her technical help.

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