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Immunogold Localization of Photosynthetic Fructose-1,6-Bisphosphatase in Pea Leaf Tissue[1]

Rosario Hermoso, María R. de Felipe, Amparo Vivó, Ana Chueca, Juan J. Lázaro, and Julio L. Gorge*

Department of Plant Biochemistry (R.H., A.Ch., J.J.L., J.L.G.), *Estación Experimental del Zaidín (CSIC), 18008 Granada, Spain; Department of Plant Biology* (M.R.F., A.V.), *Instituto de Edafología y Biología Vegetal (CSIC), 28006 Madrid, Spain*

ABSTRACT

An enriched IgG serum fraction obtained from rabbits immunized against pea chloroplast fructose-1,6-bisphosphatase (FBPase) was used, coupled to colloidal gold (15 nanometer particles) goat anti-rabbit IgG, to analyze by electron microscopy the location of photosynthetic FBPase in pea (*Pisum sativum* L.) leaf ultrathin sections. In accordance with earlier biochemical studies on distribution of FBPase activity, the enzyme was visualized both in the stromal space and bound to the chloroplast membranes. Some gold particles also appear in the cytoplasm, which can be related to the presence in the cytosol of a high molecular weight precursor of this nuclear coded enzyme.

Chloroplast FBPase[2] is a regulatory enzyme of the photosynthetic reductive pentoses-phosphate pathway. In this process its activity appears light increased through the rise in the pH and Mg^{2+} concentration which takes place in the dark-light transition (18), but also by reduction of essential -S-S- groups of the enzyme molecule via the ferredoxin-thioredoxin system (4).

This enzyme, as some others of the Calvin cycle and related processes, has been so far considered to exist in a soluble form in the stromal space. However, some evidence has shown the existence, under particular conditions, of membrane-bound P-ribulokinase (8), glucose-6-P dehydrogenase (3), P-fructokinase (22), and Rubisco (16). Moreover, Kow and Gibbs (14) have isolated under mild conditions chloroplast membranes with low, but conclusive, CO_2 assimilation capability. Concerning FBPase, low levels of thylakoid-bound enzyme have been described in soybean (1) and spinach (22), whereas Rodríguez Andrés *et al.* (20) found about 40% of the enzyme activity bound to the membranes when pea chloroplasts were quickly and gently isolated in the presence of 100 mM KCl or NaCl. A physiological explanation of a transitional binding to the membranes of photoregulated enzymes could be a more efficient reductive modulation via the photosynthetic electron transport chain. This can be related with the existence of a strong thioredoxin-FBPase hydrophobic interaction (17), and with the presence of thioredoxin-like molecules linked to the chloroplast membranes (2).

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[2] Abbreviations: FBPase, fructose-1,6-bisphosphatase; Rubisco, ribulose-1.5-bisphosphate carboxylase oxygenase; LHC, light harvesting complex; TBST, Tris buffer saline Tween 20.

In spite of the mild conditions used in the isolation of organelles and lamellar membranes, the appearance of artefactual FBPase bound to the membranes by an unspecific adsorption cannot be completely ruled out. On the contrary, the immunocytochemical techniques have the advantages of being nondestructive of the leaf structure, in addition to a high specificity and sensitivity when coupled to electron microscope visualization. In this work we study the intrachloroplast localization of photosynthetic FBPase by coloidal gold-coupled immunoreaction.

MATERIALS AND METHODS

Plant Material

We have used pea (*Pisum sativum* L. cv Lincoln) plants grown in vermiculite for 12 d, in a growth chamber under 70% RH and 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ quantum flux (Grolux Sylvania fluorescent tubes), with 14 h photoperiod and a light:dark temperature gradient of 25°C:20°C.

Immunocytochemistry and Tissue Preparation for Electron Microscopy

Three mm wide strips from the central part of young leaves were longitudinally cut into 1 mm sections. These small leaf pieces were fixed for 2 h at 0°C at 2.5% (v/v) glutaraldehyde in 0.05 M Na cacodylate buffer (pH 7.4) and washed three times, for 1 h each, in the above buffer. Some of the samples were postfixed for 2 h at 0°C with 1% (w/v) OsO_4 in the cacodylate buffer and washed as above. All the samples were then dehydrated by successive treatments with increased acetone solutions in the range 30 to 100%, and embedded in Spurr (30), Araldite (Durcupan ACM) or Lowicryl K4M resins at −40°C.

Ultrathin sections were obtained with a Reichert OM U2 ultramicrotome, and picked up on uncoated nickel grids. Prior to labeling, the OsO_4 postfixed sections were treated for 1 h with 0.56 M Na metaperiodate to overcome the masking of antigenic sites by osmium, and then were rinsed with distilled H_2O. Sections were incubated for 1 h at 37°C in 10 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl, 2% BSA, 0.02% Na azide, and 0.3% Tween 20 (2% BSA-TBST buffer), by flotation of the grids in a droplet supported on Parafilm. The grids were then transferred to a 20 μL droplet of an antibody solution containing 15 μg/mL of anti-FBPase IgG in 2% BSA-TBST buffer. After incubation at 37°C for 1 h, the sections were washed with the same buffer but containing

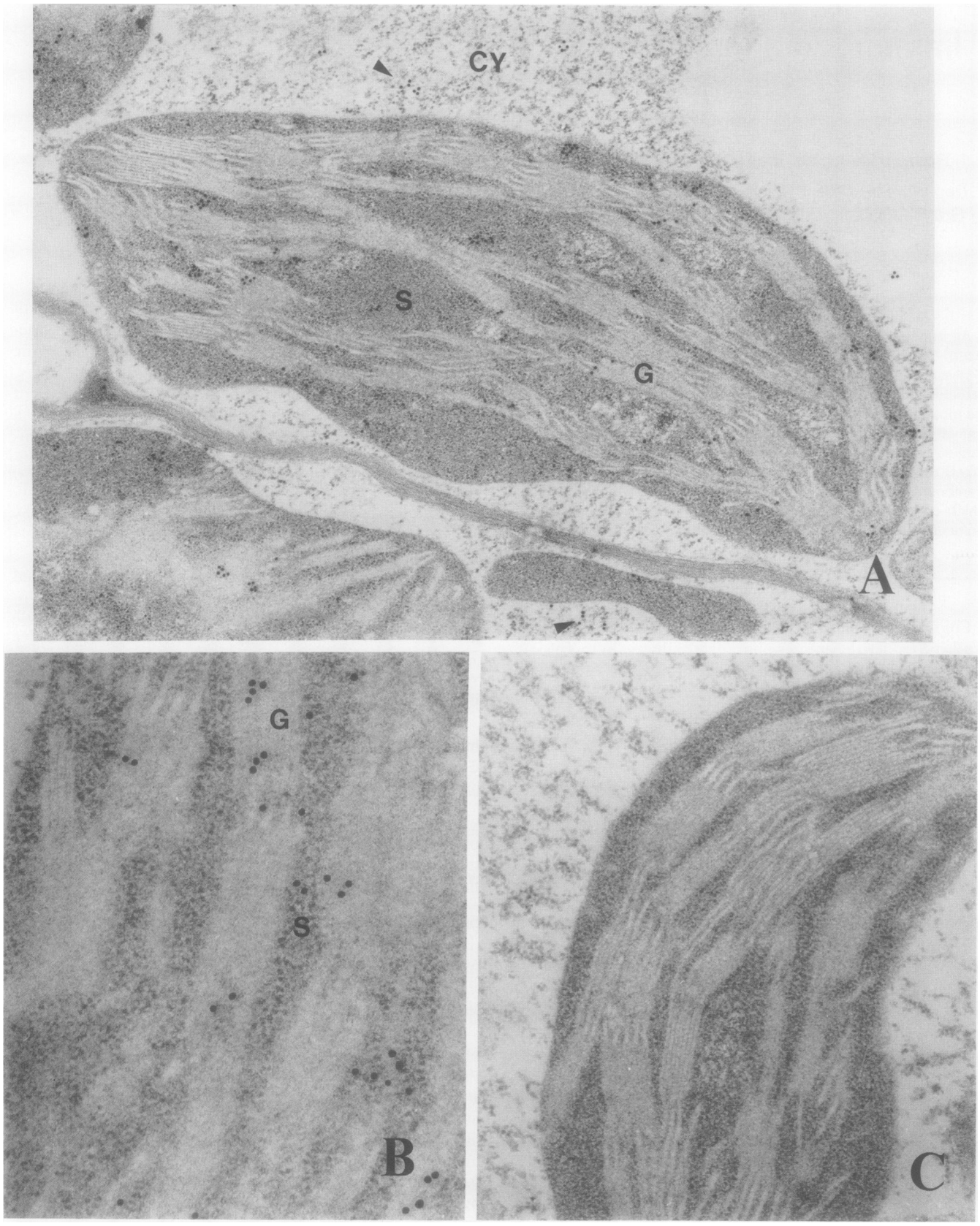

Figure 1. Thin section of pea mesophyll embedded in Spurr's resin. A, Immunogold labeling with anti-FBPase IgG (×30,000). Gold particles are concentrated over the stroma (S) and thylakoid membranes (G); some particles also appear in the cytosol (CY) compartment (arrow heads). B, Localization of the enzyme in the stromas (S) and membrane (G) at higher magnification (×78,000). C, Immunogold labeling with preimmune serum (×40,000).

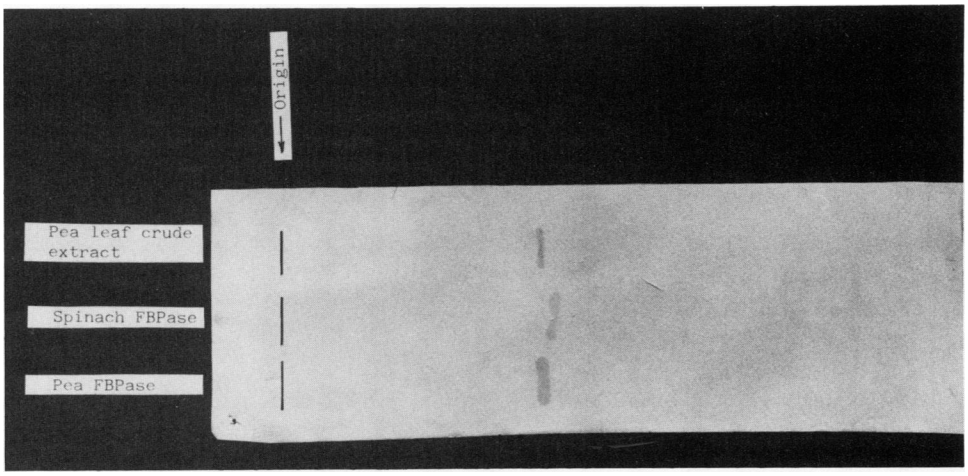

Figure 2. Western blotting in nitrocellulose sheets of pea leaf crude extract. Homogeneous spinach and pea FBPases were developed in parallel as standards.

0.2% BSA (0.2% BSA-TBST buffer), and now incubated for 1 h at 37°C in a 15 μL droplet of 15 nm colloidal gold conjugated to goat anti-rabbit IgG (GAR-G15 Janssen Life Sci.) diluted 1/10 with 0.2% BSA-TBST buffer. After a first set of being washed three times with the 0.2% BSA-TBST buffer, and a second one with the same buffer containing 0.1% Triton X-100, the sections were finally washed with twice-distilled H_2O, and then poststained for 10 min at room temperature with aqueous 2% uranyl acetate, and for additional 2 min with Pb citrate. Parallel controls were performed with preimmune serum, or without the anti-FBPase IgG. Electron microscopy observations were carried out on a Philips 300 electron microscope, at an accelerating voltage of 80kV.

Specific antiserum against pea photosynthetic FBPase was prepared from rabbits as described by Hermoso *et al.* (12). Enriched IgG fractions were obtained by precipitation of the serum with 40% saturation $(NH_4)_2SO_4$, followed by DEAE-cellulose chromatography and affinity chromatography on a FBPase-Sepharose column, according with Pharmacia protocols. The IgG concentration of the terminal product was determined as described by Hurn and Chantler (13).

Western blotting of leaf extracts and purified FBPases was performed after protein electrophoresis in 12% polyacrylamide gel as described by Laemmli (15). Proteins were then transferred to nitrocellulose sheets according to Towbin *et al.* (28) and, after saturation of additional protein binding sites with 1% serum albumin in 20 mM phosphate buffer (pH 7.3) made 0.5% in Tween 20, the sheets were soaked for 2 h at 37°C with the above anti-FBPase IgG solution. After washing with 20 mM phosphate buffer (pH 7.3), the specific protein-antibody complexes were visualized by coupling with peroxidase-conjugate goat anti-rabbit IgG (Sigma), followed by diaminobenzidine staining.

RESULTS AND DISCUSSION

Because of the low FBPase level in the chloroplast, and the high antigen preservation of Lowicryl K4M (21), we tried to use this resin for tissue inclusion. However, we found serious difficulties for getting suitable ultrathin cuts, in addition to the low resolution of thylakoid membranes earlier found with this resin (9). As it has been pointed out by other authors (9, 29), Spurr resin showed a better antigen preservation and resolution of the chloroplast membranes than did Araldite, and because of this it was regularly used for inclusion, without the OsO_4 postfixation. In spite of its successful application to other antigens, the osmium postfixation and the metaperiodate procedure for antigenic regeneration did not show good results concerning gold labeling. In addition, the immunogold technique on ultrathin sections shows the advantage on the immunofluorescent procedure of lack of any interference due to endogenous fluorescence.

Figure 1, A and B, shows the gold labeling pattern obtained with anti-FBPase antibodies. Gold particles appeared both in the stroma and thylakoid membranes (granal membranes, commonly), in accordance with the distribution of FBPase activity when intact chloroplasts and membranes were isolated in very mild conditions (20). The faint chloroplast labeling can be explained because the low FPBase concentration in the chloroplast, which is about 3 ng/mg Chl (our unpublished data) in comparison with the 0.6 to 6.0 mg of Rubisco/mg Chl, as can be deduced from the Rubisco concentration (7) and the Chl content (6) of pea leaves. Controls with preimmune serum, as well as without anti-FBPase IgG treatment, did not show any gold labeling in the stroma and membranes (Fig. 1C). The immunogold labeling has been earlier used for localization into the chloroplast of Cyt *f*, ATP synthase, and LHC proteins (25), some PSII-linked polypeptides (9), the NADPH-protochlorophyllide oxidoreductase (26), and Rubisco (29). An outstanding feature is the appearance of some gold particles in the cytosol (Fig. 1A). Besides the chloroplast enzyme, the photosynthetic cell contains a cytoplasmic FBPase engaged in gluconeogenesis. Even though some type of homology has been described between the photosynthetic FBPase and the gluconeogenic one from mammals (11), cross-antigenic reactions have not yet been detected. Western-blot experiments with pea leaf crude extracts against pea photosynthetic FBPase anti-IgG showed only one protein band, with an electrophoretic mobility in the range of those of homogeneous pea or spinach chloroplast enzyme (Fig. 2). It seems more reasonable to associate this cytosol labeling with the presence in the cytoplasm of the precursor of this nuclear coded enzyme (5). Even though Grossman *et al.* (10) were unable to detect the high mol wt precursor in a cell-free protein synthesizing system, but only the intrachloroplast mature functional protein when intact organelles were

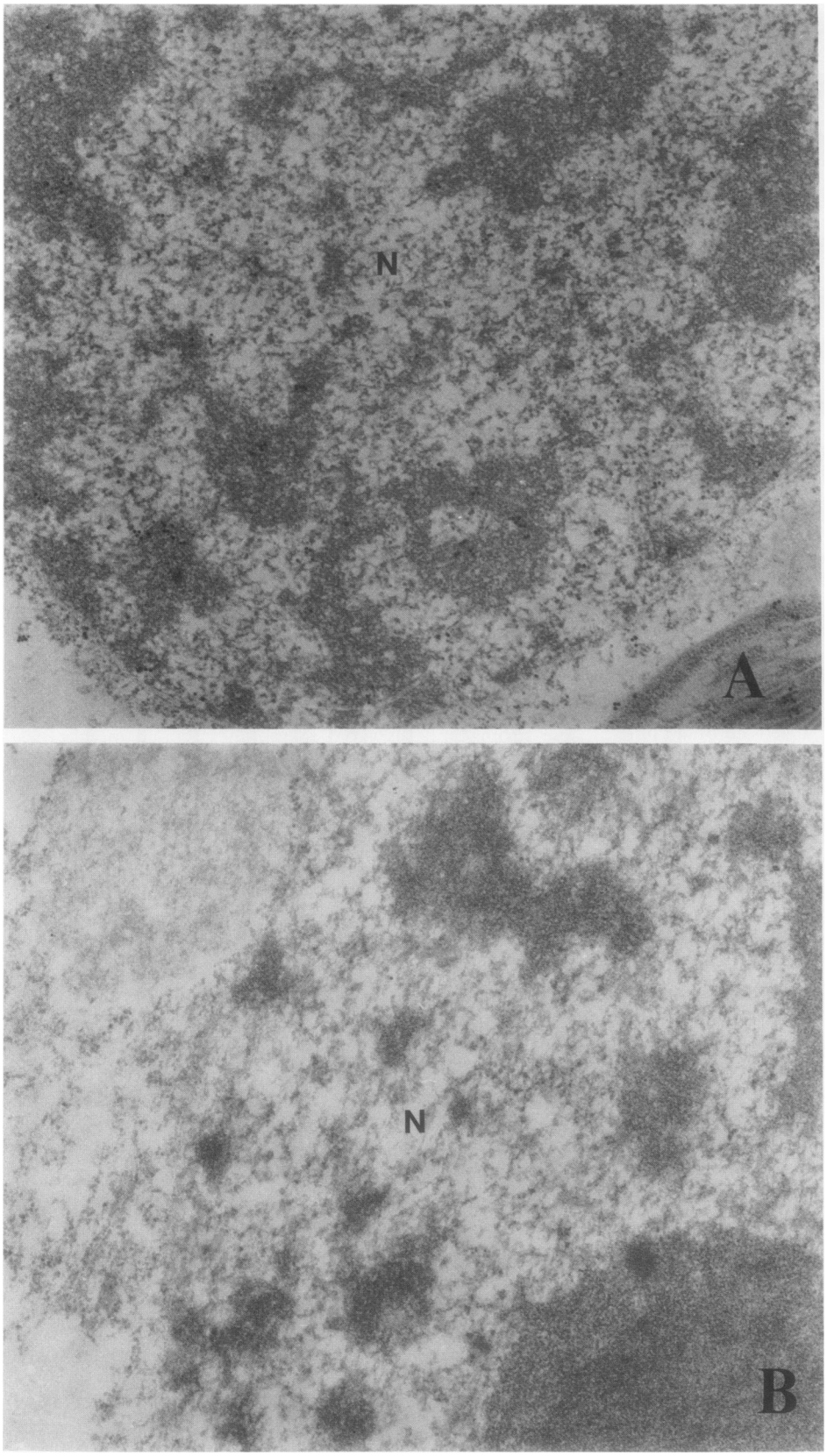

Figure 3. Thin section of pea mesophyll embedded in Spurr's resin. A, Immunogold labeling with anti-FBPase IgG (×30,000). Gold particles appear in the nucleus. B, Immunogold labeling in which the primary antibody was omitted.

added to the incubation mixture, we have identified this precursor by Western-blotting of a cell-free translation system with pea poly(A) mRNA and wheat-germ ribosomes, coupled to a pea chloroplast FBPase antiserum (23). Since the precursors of nuclear coded chloroplast proteins have only short additional sequences at their N terminus (24), it is feasible to think a conservation of the antigenic properties of the mature protein.

A remarkable result is the appearance of some gold labeling in the nucleus (Fig. 3A). This labeling is not due to a nonspecific adhesion, since no gold particles were observed when the primary antibody was omitted (Fig. 3B). In spite of a similar behavior found in the cellular immunolocalization of leghemoglobin (19) and abcisic acid (27), the significance of this nuclear labeling remains unclear.

In conclusion, as a complement of the earlier data found by biochemical analysis of suborganular components, we have now got direct evidence for the existence of a thylakoid-bound photosynthetic FPBase.

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