Characterization of Maize Elongation Factor 1A and Its Relationship to Protein Quality in the Endosperm

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The protein synthesis elongation factor 1A (eEF1A) is a multifunctional protein in eukaryotic cells. In maize (Zea mays L.) endosperm eEF1A co-localizes with actin around protein bodies, and its accumulation is highly correlated with the protein-bound lysine (Lys) content. We purified eEF1A from maize kernels by ammonium sulfate precipitation, ion-exchange, and chromatofocusing. The identity of the purified protein was confirmed by microsequencing of an endoproteinase glutamic acid-C fragment and by its ability to bundle actin. Using purified eEF1A as a standard, we found that this protein contributes 0.4% of the total protein in W64A+ endosperm and approximately 1% of the protein in W64Ao2. Because eEF1A contains 10% Lys, it accounts for 2.2% of the total Lys in W64A+ and 2.3% of the Lys in W64Ao2. However, its concentration predicts 90% of the Lys found in endosperm proteins of both genotypes, indicating that eEF1A is a key component of the group of proteins that determines the nutritional quality of the grain. This notion is further supported by the fact that in floury2, another high-Lys mutant, the content of eEF1A increases with the dosage of the floury2 gene. These data provide the biochemical basis for further investigation of the relationship between eEF1A content and the nutritional quality of cereals.

Protein synthesis factor eEF1A is composed of four subunits: A, Bα, Bβ, and Bγ (Merrick and Hershey, 1996). Subunit eEF1A, formerly referred to as EF-1α (Browning, 1996), binds aminoacyl-tRNA s to the acceptor (A) site of ribosomes during peptide chain elongation (Merrick and Hershey, 1996). In addition to its role in protein synthesis, eEF1A appears to be involved in multiple cellular processes. It was reported that eEF1A interacts with a number of proteins, including the valyl-tRNA synthase complex (Motorin et al., 1988), actin (Yang et al., 1990, 1993), microtubules (Duroso and Cyr, 1994), and calmodulin (Kaur and Ruben, 1994). eEF1A has also been implicated in the ubiquitin-dependent proteolysis of N-acylated proteins (Gonen et al., 1994) and in the activation of the phosphatidylinositol 4-kinase that resides on the plasma membrane of carrot suspension-cultured cells (Yang et al., 1993). At the subcellular level, eEF1A was found to be associated with the centrosphere (Kuriyama et al., 1990) and mitotic apparatus (Ohta et al., 1990) of sea urchin eggs, the ER membranes in Chinese hamster fibroblast cells (Hayashi et al., 1989), and protein bodies in maize (Zea mays L.) endosperm (Clore et al., 1996). In Dictyostelium sp. bundling of actin by eEF1A was reported to be pH-dependent in a physiological range that coincides with the well-documented dependence of protein synthesis on pH (Edmonds et al., 1995). Although the biological significance of these interactions is not fully understood, there is evidence that eEF1A acts as a "bridge," linking protein synthesis with the cytoskeleton network (Condeelis, 1995; Liu et al., 1996).

The concentration of eEF1A appears to provide a useful index of the protein quality of cereal grains. The predominant proteins in these grains are storage proteins, such as zeins in maize, which are devoid of Lys, an essential amino acid for monogastric animals. As a consequence, cereal protein contains on average about 2% Lys, which is less than one-half of the concentration recommended for human nutrition by the Food and Agriculture Organization of the United Nations (FAO/WHO/UN Expert Consultation, 1985). Two naturally occurring maize mutants, opaque2 (o2) and floury2 (fl2) were found to contain higher levels of Lys in the endosperm (Mertz et al., 1964; Nelson et al., 1965). o2 encodes a transcription factor that regulates zein mRNA synthesis (Schmidt et al., 1990), whereas fl2 is a mutant α-zein, the abnormal processing of which affects storage protein synthesis and protein body formation (Lending and Larkins, 1992; Lopes et al., 1994; Coleman et al., 1995). We showed that the increase in Lys content in o2 coincides with a significant increase in eEF1A (Habben et al., 1993). Furthermore, the eEF1A content in maize endosperm, as well as that in barley and sorghum, is highly correlated with Lys content (Habben et al., 1995). A survey of 93 maize genotypes revealed a more than 3-fold range in the content of Lys/eEF1A, suggesting significant phenotypic variation and the potential to use eEF1A content as an index of the Lys content for maize breeding (Moro et al., 1996).

We have begun to investigate the basis of the correlation between eEF1A and endosperm Lys content, including the direct contribution of this protein to total Lys level. As an initial step, we developed a simple procedure that allows the purification of large quantities of eEF1A to homogene-

Abbreviations: DAP, days after pollination; eEF1A, elongation factor 1A; 2-ME, p-mercaptoethanol; PBE 118, polybuffer exchanger 118.
ity. We confirmed the identity of the purified protein by microsequencing a proteolytic fragment and comparing it with a full-length cDNA clone. Our quantitative analysis suggests that the Lys content of maize endosperm is not determined by eEF1A alone, but rather is determined by proteins, the concentration of which have a stoichiometric relationship with eEF1A.

MATERIALS AND METHODS

Chemicals
SP Sepharose, PBE 118, and Pharmalyte 10.5-8 were purchased from Pharmacia. Endoproteinase Glu-C and other chemicals were obtained from Sigma.

Plant Materials
The maize (Zea mays L.) genotypes W64A+, W64Aa2, and W64Af2 were grown at the University of Arizona Farm (Tucson). For the developmental analysis, kernels were harvested at 10, 15, 20, and 25 DAP, frozen with liquid nitrogen, and stored at -80°C until use. Mature kernels were harvested and air-dried.

Purification of eEF1A

Extraction
One-hundred grams of maize kernels (16 DAP, W64Aa2) was ground with a mortar and pestle and then homogenized for 30 s in 200 mL of buffer A (20 mM KPi, pH 7.2, containing 25% glycerol, 10 mM 2-ME, 0.1 mM PMSF, and 1 mM iodoacetic acid) using a polytron. The homogenate was centrifuged at 7000 rpm with a GSA rotor (Sorvall), filtered through two layers of Miracloth (Calbiochem), and adjusted for 40, 60, and 80% ammonium sulfate.

Ion-Exchange Chromatography
The pellet precipitated by 60 to 80% ammonium sulfate was collected by centrifugation at 15,000 rpm for 30 min with an SS-34 rotor (Sorvall), resuspended in 50 mL of buffer B (50 mM Tris-HCl, pH 8.0, containing 10% glycerol, 10 mM 2-ME, and 0.1 mM PMSF) and loaded onto an SP Sepharose column (1.5 x 10 cm), which was preequilibrated with the same buffer. The column was washed with 25 mL of buffer B and subsequently eluted with a linear gradient of 0 to 1.0 M NaCl in buffer B, with a total volume of 100 mL. eEF1A eluted from the column was analyzed by immunoblotting using antibodies against maize eEF1A (Habben et al., 1995).

Chromatofocusing
eEF1A purified by ion-exchange chromatography was dialyzed against buffer C (25 mM triethylamine, pH 10.5, containing 10% glycerol, 10 mM 2-ME, and 0.1 mM PMSF) overnight and loaded onto a PBE 118 column (0.8 x 10 cm) that was preequilibrated with the same buffer. After washing the column with 10 mL of buffer C, eEF1A was eluted with Pharmalyte 10.5-8 containing 10% glycerol, 10 mM 2-ME, and 0.1 mM PMSF at pH 8.0. Immunoblotting was performed to monitor eEF1A eluted from the column.

Isolation and Characterization of eEF1A cDNA Clones
An eEF1A cDNA fragment corresponding to the carboxyl domain of the protein (Shen et al., 1994) was labeled with digoxigenin and used to screen a maize endosperm cDNA library (Habben et al., 1993). Plaque lifting and membrane treatment were performed as described previously (Shen et al., 1994). Filter hybridization and washing were done according to McCreery and Helenjaris (1994). A full-length eEF1A cDNA clone was plaque-purified and sequenced using the dideoxy-sequencing method (Sanger et al., 1977).

Microsequencing of Maize eEF1A
Three-hundred micrograms of purified eEF1A was incubated with 5 µg of endoproteinase Glu-C in 500 µL of 0.1 M NH4HCO3, pH 7.8, for 10 min at 35°C. The reaction was stopped by adding 500 µL of 10% TCA. Proteins were pelleted by centrifugation with a microfuge for 30 min at 4°C, washed twice with acetone, and resuspended in SDS-PAGE loading buffer. The digested proteins were separated by electrophoresis in a 17% SDS-polyacrylamide gel, transferred to an Immobilon P membrane (Millipore), and stained with Coomassie blue. A band of 14.7 kD was excised and sequenced by Edman degradation at the microsequencing facility at the University of Arizona.

Actin Purification and Bundling Assay
Actin was purified from budding yeast (Saccharomyces cerevisiae) according to Honts et al. (1994). The bundling assay was performed in a total volume of 50 µL. One-hundred-fifty picomoles of purified yeast actin was mixed with 15 pmol of eEF1A or Cyt c in 5 mM Hepes-KOH, pH 7.5, 0.2 mM CaCl2, 0.2 mM ATP, and 0.5 mM DTT. Actin polymerization and bundling was initiated by adding 10 µL of polymerization buffer (112.5 mM Hepes, pH 7.5, 250 mM KCl, 20 mM EGTA, and 80 mM MgCl2). The mixture was incubated at 22°C for 2 h. Five microliters of the mixture was then placed on carbon-coated grids, negatively stained with 2% uranyl acetate, and visualized by transmission electron microscopy.

Quantitative Analysis of Total Protein, Total Lys, and eEF1A
Maize kernels were degermed, lyophilized, and ground into fine powder using a Wig-L-Bug amalgamator (Crescent Dental Mfg. Co., Lyons, IL). Total protein and nonzein protein were extracted and separated as described previously (Moro et al., 1996). eEF1A content was determined by quantitative ELISA (Habben et al., 1995) using purified eEF1A as a standard. Protein content was determined by micro-Kjedahl analysis, and the N value was converted to
protein by multiplying a factor of 5.7 (Mossé, 1990). Total Lys was analyzed with an amino acid analyzer (model 6300, Beckman) (Habben et al., 1995).

**SDS-PAGE, Immunoblotting, and Protein Assay**

Protein samples were analyzed by SDS-PAGE in 12.5% polyacrylamide gels (Laemmli, 1970) and stained with Coomassie blue. Immunoblotting was carried out as described by Towbin et al. (1979) using polyclonal antibodies against eEF1A fusion proteins (Habben et al., 1995). During eEF1A purification protein concentration in the fractions was determined with protein assay reagent (Bio-Rad), and the A$_{540}$ was determined by a microplate reader (MR700, Dynatech, Chantilly, VA).

**RESULTS**

**Accumulation of eEF1A during Endosperm Development**

As a first step to purify eEF1A from maize endosperm, we compared the level of the protein in developing endosperms of W64A$^+$ and W64Ao2 at 10, 15, 20, 25, and 45 DAP (maturity). Based on an ELISA assay (Habben et al., 1995; Moro et al., 1996), the relative concentration of eEF1A decreased during endosperm development in both o2 and the wild type (Fig. 1). This reflects the increased accumulation of carbohydrates and storage proteins throughout this period in both genotypes. Figure 1 shows that the difference in the content of eEF1A between the two genotypes is statistically significant after 25 DAP, but not between 10 and 20 DAP. At 25 DAP the o2 genotype contains about 2-fold more eEF1A than the wild type and this difference persists until maturation. Because the 15 DAP endosperm contains a high level of eEF1A and a relatively large mass of tissue, we selected kernels at this stage for eEF1A purification.

**Figure 1.** Relative content of eEF1A in developing W64A$^+$ and W64Ao2 endosperm. The content of eEF1A was determined with an ELISA and values are expressed based on flour weight. All values were normalized against that of the wild-type endosperm at maturity, which was arbitrarily set at 1. Bars equal the SE (n = 3).

**Figure 2.** Purification of eEF1A by SP Sepharose and PBE 118 column chromatography. eEF1A separated through ammonium sulfate precipitation was further purified by SP Sepharose (A and B) and PBE 118 (C and D) chromatography. A and C, Proteins eluted by NaCl gradient (plain line) or Pharmalyte (•) from the columns were monitored by the Bio-Rad assay (•). B and D, Aliquots of 10 μL from each fraction were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies against maize eEF1A.

**Purification of eEF1A**

eEF1A was purified to homogeneity from maize kernels by combining ammonium sulfate precipitation, ion-exchange chromatography with SP Sepharose, and chromatofocusing on a PBE 118 column. Significant enrichment was obtained by cation-exchange chromatography on SP Sepharose (Fig. 2, A and B). As shown in Figure 3A, a 54-kD protein constituted the majority of protein eluted from the SP Sepharose column. This observation is in agreement with a report that in yeast the majority of positively charged proteins correspond to eEF1A (Thiele et al.,
Figure 3. SDS-PAGE and immunoblotting of eEF1A during steps of purification. eEF1A from the crude extract (lanes 1) was purified through ammonium sulfate precipitation (lanes 2), ion-exchange on SP Sepharose (lanes 3), and chromatofocusing on PBE 118 (lanes 4). Two micrograms of proteins from each step was separated by SDS-PAGE and stained with Coomassie blue (A) or transferred to nitrocellulose membrane and probed with polyclonal antibodies against eEF1A (B). Lane 5 contains prestained protein markers.

Sequence Analysis of an eEF1A cDNA Clone and the Purified eEF1A Protein

Once eEF1A was purified to homogeneity, we compared the amino acid sequence with that encoded by an endosperm mRNA. A partial eEF1A cDNA clone of 1.2 kb has been isolated from a maize endosperm cDNA library (Shen et al., 1994), and we used this clone as a probe to isolate a full-length cDNA. eEF1AZME16 was found to have an identical nucleotide sequence to that of the probe. As shown in Figure 4, this clone is 1.6 kb in length and has an open reading frame of 1341 bp, encoding a protein of 447 amino acid residues. The deduced amino acid sequence is more than 96% identical to the eEF1A from barley (p34824), tomato (p17786), and Arabidopsis (p13905) (data not shown). A recently reported partial eEF1A cDNA clone from maize seedlings (Berberich et al., 1995) shares 98% identity to eEF1AZME16.

To obtain the amino acid sequence of the purified eEF1A protein, we tried to sequence both the N terminus and its proteolytic degradation fragments. Direct sequencing of the N terminus failed to provide any sequence information, presumably because it is blocked. However, when a 14.7-kD fragment generated from endoproteinase Glu-C was microsequenced, 12 amino acid residues were obtained. As shown in Figure 4, the polypeptide sequence is identical to the deduced amino acid sequence of eEF1A from H-136 to Q-147. Database searching with the BLAST program revealed that these amino acids are highly conserved among eEF1A proteins in higher eukaryotes.

Actin-Bundling Activities of Purified eEF1A

It has been shown that eEF1A is a multifunctional protein that is able to cross-link and bundle actin (Yang et al., 1993). To assay this property of endosperm eEF1A, we analyzed the actin-bundling activity of the purified protein by mixing it with yeast actin in vitro in a molar ratio of 10 to 1. In the appropriate buffer yeast actin polymerized to form F-actin, as shown by the presence of filaments in the negatively stained preparation examined by electron microscopy (Fig. 5A). When Cyt c, a highly basic protein, was added, no changes in the organization of F-actin were observed (Fig. 5B). In contrast, when purified eEF1A was added to yeast F-actin, the filaments became organized into bundles (Fig. 5C).

Table I. Purification of eEF1A from maize endosperm

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Volume</th>
<th>Protein</th>
<th>mg/mL</th>
<th>mg</th>
<th>mg/mL</th>
<th>mg</th>
<th>%</th>
<th>-fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenization</td>
<td>230</td>
<td>9.10</td>
<td>2093</td>
<td>0.59</td>
<td>135.7</td>
<td>100</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>50</td>
<td>2.90</td>
<td>145</td>
<td>0.37</td>
<td>18.50</td>
<td>13.6</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>SP Sepharose</td>
<td>7.0</td>
<td>0.84</td>
<td>5.89</td>
<td>0.69</td>
<td>4.83</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBE 118</td>
<td>13.5</td>
<td>0.23</td>
<td>3.08</td>
<td>0.23</td>
<td>3.08</td>
<td>2.3</td>
<td>15.4</td>
<td></td>
</tr>
</tbody>
</table>

It was observed that further purification of the protein was obtained by chromatofocusing on a PBE 118 column. Maize eEF1A was eluted from this column at a pH of 9.3 using Pharmalyte 10.5-8 (Fig. 2, C and D). As shown in Figure 3A, chromatofocusing on PBE 118 allowed purification of eEF1A to homogeneity, as judged by SDS-PAGE. Figure 3B shows that the purified protein has a molecular mass of 54 kD and reacts strongly with polyclonal antibodies against maize eEF1A (Habben et al., 1995).

The method for purification of maize eEF1A is summarized in Table I. This three-step procedure enriched eEF1A 15.4-fold and yielded 3.1 mg of eEF1A from 100 g of W64Ao2 kernels, with a final recovery of 2.3%.
Dosage Effect of fl2 on the Relative Content of eEF1A

fl2 is the second “high-Lys” mutant identified by Mertz and Nelson (Nelson et al., 1965). To determine if this mutation also affects the level of eEF1A in the endosperm, we analyzed the relative content of eEF1A in maize genotypes. Taking advantage of the triploid nature of maize endosperm, we generated W64A crosses containing genotypes. Using the purified eEF1A as a standard, we were able to estimate the concentration of eEF1A in mature endosperm flour. This was accomplished with an ELISA, using the procedure described in our previous reports (Habben et al., 1995; Moro et al., 1996). As shown in Table II, eEF1A accounts for 0.39% of the total protein in mature W64A+ endosperm, whereas in W64Ao2 it accounts for 0.95% of the total protein. Based on these protein concentrations, the mass of eEF1A accounts for 2.2 and 2.3%, respectively, of the Lys content of the flour.

Figure 5. Maize eEF1A bundles actin from yeast in vitro. Actin was incubated with 22.5 mM Hepes-KOH, pH 7.5, containing 50 mM KCl, 1 mM EGTA, and 4 mM MgCl2 for 120 min at 22°C either alone (A), in the presence of Cyt c (B), or with eEF1A (C). Aliquots were placed on carbon-coated grids, negatively stained with 2% uranyl acetate, and visualized using transmission electron microscopy. Bar = 0.5 μm.

Table II. eEF1A, Lys, and protein content in maize endosperm

<table>
<thead>
<tr>
<th>Content</th>
<th>W64A+</th>
<th>W64Ao2</th>
</tr>
</thead>
<tbody>
<tr>
<td>eEF1A (mg/100 mg flour)</td>
<td>0.046</td>
<td>0.088</td>
</tr>
<tr>
<td>Total protein (mg/100 mg flour)</td>
<td>11.6</td>
<td>9.30</td>
</tr>
<tr>
<td>Nonzein protein (mg/100 mg flour)</td>
<td>3.60</td>
<td>3.16</td>
</tr>
<tr>
<td>Lys (mg/100 mg flour)</td>
<td>0.21</td>
<td>0.38</td>
</tr>
<tr>
<td>eEF1A in total protein (%)</td>
<td>0.39</td>
<td>0.95</td>
</tr>
<tr>
<td>eEF1A in nonzein protein (%)</td>
<td>2.88</td>
<td>2.78</td>
</tr>
<tr>
<td>Lys contributed by eEFIA (%)</td>
<td>3.10</td>
<td>9.20</td>
</tr>
</tbody>
</table>

Figure 4. Nucleotide and deduced amino acid sequence of maize eEF1A. A full-length eEF1A cDNA clone (eEF1AZME16) was isolated from maize (cv W64A+ endosperm. Microsequencing of a proteolytic fragment of the purified eEF1A showed that 12 amino acid residues (underlined) are identical to the amino acid sequence deduced from the cDNA clone.

Quantitative Analysis of eEF1A in Maize Endosperm

Using the purified eEF1A as a standard, we were able to estimate the concentration of eEF1A in mature endosperm flour. This was accomplished with an ELISA, using the procedure described in our previous reports (Habben et al., 1995; Moro et al., 1996). As shown in Table II, eEF1A accounts for 0.39% of the total protein in mature W64A+ endosperm, whereas in W64Ao2 it accounts for 0.95% of the total protein. Based on these protein concentrations, the mass of eEF1A accounts for 2.2 and 2.3%, respectively, of the Lys content of the flour.

 Dosage Effect of fl2 on the Relative Content of eEF1A

fl2 is the second “high-Lys” mutant identified by Mertz and Nelson (Nelson et al., 1965). To determine if this mutation also affects the level of eEF1A in the endosperm, we analyzed the relative content of eEF1A.
DISCUSSION

We previously reported that a 54-kD protein in maize endosperm, which reacts with eEF1A antisera, is highly correlated with the Lys content (Habben et al., 1995). Here we present definitive evidence that the 54-kD protein is eEF1A. First, the purified 54-kD protein shares a peptide with the same amino acid sequence as eEF1A from other eukaryotes and with the amino acid sequence encoded by eEF1A. Because eEF1A has been shown to be associated with the actin cytoskeleton both in situ (Clore et al., 1996) and in vitro (Fig. 5), we speculate that the other major proteins contributing to the Lys content are components of this cytoskeletal network.

In previous reports we showed that the eEF1A content of a2 mutants is generally increased and that their Lys content is highly correlated with the eEF1A concentration (Habben et al., 1995). Here we show that in f2, another naturally occurring high-Lys mutant, the content of eEF1A is also elevated (Fig. 6), and that the increase in eEF1A content is proportional to the dosage of f2. This observation further supports the notion that eEF1A is a key component of the set of proteins responsible for the Lys content of maize endosperm.

In addition to their elevated Lys and eEF1A levels, a common feature of a2 and f2 is that both mutations affect the morphology of protein bodies. Compared with the wild type, the protein bodies in a2 are much smaller, whereas in f2 they are both smaller and irregularly shaped, with convoluted morphology (Lending and Larkins, 1992; Lopes et al., 1994). We speculate that eEF1A is increased in these mutants as a consequence of increased protein body surface area and, consequently, an associated increase in the cytoskeletal network surrounding the rough ER. This hypothesis is supported by the findings that eEF1A in maize

![Figure 6. Effect of floury2 dosage on the relative content of eEF1A in W64A mature endosperm. 0 doses, W64A+; 1 dose, W64A+ x W64Afl2; 2 doses, W64Afl2 x W64A+; and 3 doses, W64Afl2 x W64Afl2. The content of eEF1A from flour extracts was determined with an ELISA. All values were normalized against that of W64A+, which was arbitrarily set at 1. Bars equal the SE (n = 3).](chart.png)
endosperm cells is localized around protein bodies (Clore et al., 1996), and that the content of Cyt c reductase, a marker of the ER membrane, is increased in the o2 genotypes (J.E. Habben and B.A. Larkins, unpublished data). Experiments are in progress to examine this hypothesis by isolating components of the actin cytoskeleton and determining if their concentrations share the same relationship with eEF1A to the Lys content of the endosperm.

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