Phosphorylation of Maize RAB-17 Protein by Casein Kinase 2*

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The maize gene RAB-17, which is responsive to abscisic acid, encodes a basic glycine-rich protein containing, in the middle part of its sequence, a cluster of 8 serine residues followed by a putative casein kinase 2-type substrate consensus sequence. This protein was found to be highly phosphorylated in vivo. Here, we show that RAB-17 protein is a real substrate for casein kinase 2. RAB-17 protein is phosphorylated in vitro by casein kinase 2 isolated from rat liver cytosol and from maize embryos. A maximum of 4 mol of phosphate were incorporated per mol of RAB-17 protein following incubation with casein kinase 2.

Phosphopeptide mapping experiments show that the peptide phosphorylated by casein kinase 2 in vitro is identical to that derived from the protein phosphorylated in vivo. Purification by high performance liquid chromatography and partial sequencing of the phosphopeptide indicate that it corresponds to the region of the protein (residues 56–89) containing the cluster of serine residues. Our results indicate that RAB-17 is phosphorylated by casein kinase 2 or a kinase with a similar specificity and that phosphorylation takes place in the serine cluster region of the protein both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—Plants of Z. mays L. inbred line W64 A were used. Mature embryos (60 days after pollination) were manually dissected. [32p]Phosphate was purchased from Amersham Corp. Calf intestinal alkaline phosphatase (1 unit/ml) was supplied by Boehringer Mannheim.

Purification of RAB-17 Polypeptides—Mature embryos (14 g) were homogenized with a mortar and pestle under liquid nitrogen and resuspended in 140 ml of buffer containing 50 mM Tris/HC1 (pH 8.0), 10 mM NaCl, 1 mM PMSF, 20 mM sodium molybdate. The homogenate was centrifuged at 10,000 × g for 30 min. The supernatant was collected and ultracentrifuged for 30 min at 141,000 × g. The clear supernatant was precipitated by ammonium sulfate at 65% saturation. The protein precipitate was collected by centrifugation at 10,000 × g for 45 min; dissolved in buffer containing 50 mM phosphate (pH 6.0), 1 mM PMSF; and dialyzed overnight against the same buffer. After dialysis, the protein solution was ultracentrifuged for 30 min at 141,000 × g, and the supernatant was applied to a 75-ml column (1.6 × 37 cm) of CM-Sepharose (Pharmacia LKB Biotechnology Inc.) equilibrated with 50 mM phosphate (pH 6.0). The column was then washed with the same buffer until no protein could be detected in the eluate. RAB-17 protein was not retained on this column.

The CM-Sepharose RAB-17 protein fraction was precipitated by ammonium sulfate at 65% saturation, collected by centrifugation, resuspended in 50 mM Tris/HC1 (pH 8.0), and dialyzed overnight against the same buffer. The protein solution was then applied to a 9.5-ml column (0.9 × 15 cm) of DEAE-Sepharose (Pharmacia) equilibrated with 50 mM Tris/HC1 (pH 8.0), and the column was washed with the same buffer.

The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylene-nitritol)]tetraacetic acid; HPLC, high performance liquid chromatography.

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washed until no protein could be detected in the eluate. RAB-17 protein was eluted by using an 80-ml linear gradient of 0–0.5 M NaCl (40 ml/40 ml) in the same buffer.

In Vitro Protein Labeling and Protein Analysis — In vitro labeling of isolated embryos was performed by incubating them simultaneously with 

(2)P-labeled [γ-32P]ATP (300 μCi/ml) and (2)P-labeled [γ-32P]GTP (200 μCi/ml) for 2–4 h. Total protein was isolated from 50 ml Tris/HCl (pH 8.0) containing 10 mM NaCl, 10 mM sodium molybdate, 1 mM PMSF, precipitated in 20% (w/v) trichloroacetic acid, and washed with 0.003% (w/v) ammonium acetic acid in methanol. One-dimensional SDS-polyacrylamide gel electrophoresis was performed as described (16). Two-dimensional gel electrophoresis was performed as previously described (7) with the following modifications. (a) In the first dimension, we used 0.8% (w/v) Ampholine (Pharmacia LKB Biotechnology Inc., pH range 3.5–10) plus 0.8% (w/v) Ampholine (pH range 5–8). (b) The second dimension was performed at 18% (w/v) acrylamide, 0.4% (w/v) bisacrylamide. Exposure of dried gels was done using two films as described (6). Protein concentration was determined by the method of Bradford (17) using crystalline bovine serum albumin as standard.

Dephosphorylation of RAB-17 Protein Phosphorylated Forms—Pure RAB-17 protein (0.8 ml; A280 = 1.203) was incubated with 80 units of calf intestinal alkaline phosphatase at 37°C for 30 min in buffer containing 50 mM Tris/HCl (pH 8.0). The reaction was stopped by addition of an equal volume of electrophoresis buffer when the samples were to be analyzed by electrophoresis. The samples to be used for in vitro repholphorylation studies were made phosphorysase-free by application to a CM-Sepharose column equilibrated with 50 mM NaCl (pH 6.0) after dialysis against this buffer. Under these conditions, the phosphatase was not retained into the column. Dephosphorylated RAB-17 protein was eluted from the column by step elution with 0.3 M NaCl in the same chromatography buffer.

Phosphopeptide Mapping—In vitro (2)P-labeled RAB-17 polypeptides were digested with cyanogen bromide in 80% (v/v) formic acid for 18 h in the dark (CNBr/protein molar ratio of 10:1). The mixture was diluted 10 times with water and then evaporated to dryness. The dry residue was dissolved with water and then lyophilized. Peptides were separated by reverse-phase HPLC using a Waters Pico-Tag analytical column (0.5 x 20 cm). Absorbance of the eluate was monitored at 214 nm. Solvent A was 0.1% (v/v) trifluoroacetic acid in water, and solvent B was 0.1% (v/v) trifluoroacetic acid in 70% (v/v) acetonitrile. Peptides were separated on a 40-ml linear gradient of 0–60% solvent B with a 1 ml/min constant flow rate. Phosphopeptide-containing peaks were detected by radioactivity counting in a Beckman β-counter.

Phosphoamino Acid Analysis—(2)P-labeled RAB-17 polypeptides were excised from unfixed SDS-polyacrylamide gels (12.5% acrylamide). Protein was then electrophoresed from the gel at 150 V for 6 h and recovered by precipitation with 20% (w/v) trichloroacetic acid using 50 μg of bovine serum albumin as carrier.

Purification of Casein Kinase 2—Casein kinase 2 from rat liver cytosol (18), according to Martins et al. (18). Casein kinase 2 was also isolated from maize embryos (35 days after pollination) as follows. Maize embryos (25 g) were homogenized with a mortar and pestle under liquid nitrogen and resuspended in 250 ml of buffer containing 50 mM Tris/HCl (pH 7.5) plus 10 mM NaCl, 1 mM dithiothreitol, 5% (w/v) glycerol, 1 mM PMSF, and 0.25 M sucrose. The homogenate was centrifuged at 10,000 X g for 30 min. After being filtered through glass wool, the supernatant was centrifuged at 140,000 X g for 60 min. Solid ammonium sulfate was added to the supernatant to reach 60% saturation. After stirring for 30 min at 4°C, the precipitate was collected by centrifugation at 10,000 X g for 30 min. This pellet was resuspended in 10 ml of 50 mM Tris/HCl (pH 7.5) containing 1 mM dithiothreitol, 5% (w/v) glycerol, and 1 mM PMSF (Buffer A) and dialyzed extensively against the same buffer. After dialysis, the protein solution was clarified by centrifugation at 10,000 X g for 30 min and applied batch-wise to 2 ml of phosphocellulose equilibrated with Buffer A. After 2 h, the resin was poured into a column and washed with increasing concentrations of KCl in Buffer A. Fractions with casein kinase activity were pooled, diluted with Buffer A, and applied to a 1 ml column of heparin-agarose equilibrated with the same buffer. The casein kinase activity was eluted with Buffer A plus 0.5 M KCl. Fractions with casein kinase activity were pooled and dialyzed against Buffer A.

In Vitro Protein Phosphorylation—Casein kinase assays were done at 30°C. The incubation mixture contained 25 mM sodium β-glycerophosphate (pH 7.0), 1 mM dithiothreitol, 8 mM MgCl₂, 0.5 mM EDTA, 0.48 mM EGTA, 0.125 mM (100–500 cpm/pmol) [γ-32P]ATP or [γ-32P]GTP, and 4 mg/ml casein. In some experiments, casein was substituted by phosvitin. The reaction was initiated by addition of casein kinase. After 10 min of incubation, the reaction was stopped, and a sample was spotted on 2 x 2-cm Whatman No. 3MM paper and washed with 10% (w/v) trichloroacetic acid. After several washes, the paper was dried and counted for 32P in a scintillation counter. One unit of casein kinase activity is defined as the amount that incorporates 1 nmol of 32P into casein/min of incubation at 30°C.

In Vitro Phosphorylation of RAB-17 Protein—In vitro phosphorylation of RAB-17 protein was done under conditions similar to those standard for casein kinase assays except that RAB-17 protein was present instead of casein. The amounts of casein kinase 2 used in the different experiments are indicated in the text, and the incubation time was 30–60 min. When the samples were analyzed by SDS-polyacrylamide gel electrophoresis, the reaction was stopped by addition of electrophoresis sample buffer and was boiled for 3 min.

Phosphoamino Acid Analysis—Phosphoamino acid analysis of RAB-17 phosphorylated by casein kinase 2 in vitro was done as described previously (8). N-terminal amino acid analyses were performed in an Applied Biosystems Protein Sequencer.

RESULTS

Purification and Phosphatase Treatment of RAB-17 Protein—RAB-17 protein present in maize embryos exhibits different degrees of phosphorylation, as has been demonstrated previously (6). Using the procedure described under "Experimental Procedures," we have isolated the protein in a highly purified form (Fig. 1A). Enzymatic hydrolysis of the phosphoester bonds of the purified RAB-17 polypeptide forms by treatment with calf intestinal alkaline phosphatase prior to one-dimensional SDS-polyacrylamide gel electrophoresis resulted in a polypeptide with a migration slightly faster than that of the original RAB-17 protein (Fig. 1B).

Characteristics of Casein Kinase 2 from Maize Embryos—Casein kinase 2 was isolated from maize embryos (35 days after pollination). The elution profile is shown in Fig. 2. The purification procedure for casein kinase 2 from maize embryos described here gives a preparation with a specific activity of 4 units/mg of protein, which represents a purification factor of 1200 with a recovery of 40% with respect to the homogenate. Just as the rat liver enzyme, casein kinase 2 from maize embryos was able to use GTP as efficiently as ATP (GTP/ATP activity ratio of 0.85), and its activity on phosvitin and casein was totally inhibited by 5 μg/ml heparin.

In Vitro Phosphorylation of RAB-17 Polypeptides by Casein Kinase—The specificity of casein kinase 2 has been well established by studies using both protein and synthetic peptide substrates. Sequences phosphorylated by casein kinase 2 contain serines or threonines that are followed by acidic residues (11, 12). We have identified a cluster of serine residues in the predicted sequence of the RAB-17 polypeptide (8) whose amino acid environment contains the consensus sub-
purified RAB-17 polypeptides were dephosphorylated with calf intestinal alkaline phosphatase and then incubated with casein kinase from maize embryos or from rat liver cytosol. A plateau corresponding to 4 mol of phosphate/mol of protein (Fig. 4) was observed at high concentrations of casein kinase. By two-dimensional electrophoresis, it can be seen that rephosphorylation by casein kinase 2 yields three different phosphorylated forms that shift to acid pI values with respect to the dephosphorylated forms (Fig. 5).

Phosphamino acid analysis of RAB-17 protein rephosphorylated by rat liver casein kinase 2 in vitro shows that $^{32}$P incorporation is exclusively in serines (data not shown). We have previously shown that in vivo phosphorylation of RAB-17 takes place only in serines as well (8).

**Comparison of RAB-17 Phosphorylated in Vivo and in Vitro**—To determine whether the regions of RAB-17 that are phosphorylated in vitro by casein kinase 2 correspond to those phosphorylated in vivo, phosphopeptide maps of RAB-17 labeled with $^{32}$P in vivo and in vitro by casein kinase 2 from either rat liver or maize embryos were compared. Analysis by gradient electrophoresis of the radiolabeled peptides showed identical mobilities for the in vivo and in vitro labeled forms (Fig. 6).

Thus, RAB-17 labeled in vivo gave rise to the same phosphopeptide as that found in RAB-17 labeled by casein kinase 2 in vitro. These data indicate that the kinase that phosphorylates RAB-17 in intact cells transfer phosphate to the same region of RAB-17 protein as does casein kinase 2 in vitro.

![Fig. 2. Isolation of casein kinase 2 from maize embryos.](image)

![Fig. 3. Phosphorylation of RAB-17 protein by casein kinase 2.](image)

![Fig. 4. Time course of phosphorylation of RAB-17 with casein kinase 2 from rat liver cytosol.](image)

![Fig. 5. Two-dimensional gel electrophoresis of RAB-17 polypeptides.](image)
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FIG. 6. Electrophoretic analysis of radiolabel-containing peptides from RAB-17 phosphorylated in vitro and in vivo. A, the bands corresponding to RAB-17 phosphorylated with casein kinase 2 either from rat liver or maize embryos were excised by SDS-polyacrylamide gel electrophoresis and then treated with CNBr as described under "Experimental Procedures." The digests were applied to an SDS-polyacrylamide gel using a linear gradient (11-19%) of acrylamide. Bands were detected by autoradiography. Lane 1, radiolabel-containing peptide from RAB-17 phosphorylated in vitro by casein kinase 2 from maize embryos; lane 2, radiolabel-containing peptide from RAB-17 phosphorylated in vitro by casein kinase 2 from rat liver cytosol. B, radiolabel-containing peaks from HPLC were collected, lyophilized, and subjected to SDS-polyacrylamide gel electrophoresis using a linear gradient (11-19%) of acrylamide. Bands were detected by autoradiography. Lane 1, radiolabel-containing peptide from RAB-17 phosphorylated in vitro by casein kinase 2 from rat liver cytosol; lane 2, radiolabel-containing peptide from RAB-17 isolated from in vivo 32P-labeled embryos.

FIG. 7. HPLC profile of RAB-17 phosphopeptides. RAB-17 phosphorylated by casein kinase 2 in vitro was digested with CNBr, and the resulting peptides were resolved by HPLC. Upper trace, absorbance; and lower trace, radioactivity. Only one phosphopeptide was detected. The last peak corresponds to phosphorylated RAB-17 protein not digested by CNBr.

The N-terminal amino acid sequence for the single radioactive peptide isolated from RAB-17 phosphorylated in vitro with casein kinase 2 was then determined by sequence analysis. Fig. 7 shows a reverse-phase HPLC separation of radiolabeled peptides generated after CNBr digestion of RAB-17 phosphorylated by casein kinase 2 in vitro. The sequence data show that the phosphorylated peptide corresponds to the central part of the RAB-17 polypeptide in the region of the serine cluster (Fig. 8). We can conclude from these data that this represents the major region of the protein that is phosphorylated in vivo and in vitro. The different electrophoretic forms seen by two-dimensional electrophoresis probably come from sequential phosphate incorporation into the individual serine residues of the cluster.

DISCUSSION

It has been documented that RAB-17 polypeptides accumulate in maize embryos as a highly phosphorylated form(s) (6). Not much is known about the protein kinases present in higher plants. Studies on wheat germ (20-22) indicate the existence of casein kinases, some of which have kinetic properties similar to those of animal casein kinases 1 and 2. The presence of casein kinase 2-type enzymes in maize has been previously reported (13). Studies on the phosphorylation of short synthetic peptides indicated that the sequence requirements for maize casein kinase IIB are related, albeit not identical, to those of the rat liver enzyme. On the contrary, the other type of casein kinase 2 also present in maize, namely casein kinase IIA, shares all characteristics of rat liver casein kinase 2 (23). The data obtained in this work on the peptide map of RAB-17 phosphorylated by the maize enzyme preparations (which probably contain a mixture of casein kinases IIA and IIB) and by the rat liver enzyme provide further evidence for the presence in maize of a protein kinase similar to rat liver casein kinase 2.

Our results demonstrate that the site(s) for in vitro phosphorylation by casein kinase 2 lie on serinal residues in the central region of the RAB-17 predicted sequence. This region (residues 56-89) includes a large cluster of serines followed by acidic residues that were initially identified as a putative casein kinase 2 substrate consensus sequence (8). Phosphopeptide maps derived from RAB-17 phosphorylated in vitro and in vivo are identical. This suggests that the site for in vitro and in vivo phosphorylation lies within the same peptide. Nevertheless, the extent of in vitro phosphorylation by casein kinase 2 did not fully restore the complex pattern of RAB-17 protein forms observed in vivo (6). This result may be explained in part by the limitations of the in vitro reaction or could be due to the involvement of other types of kinases present in maize. Work is in progress to investigate whether other kinases might phosphorylate RAB-17 in vitro.

Animal casein kinases 2 are receiving increasing attention due to their possible involvement in the phosphorylation of proteins that are responsible for the regulation of important metabolic pathways and gene expression. The activity of animal casein kinase 2 has been shown to undergo dramatic changes during fetal development (24), hepatic regeneration...
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Furthermore, the activity of the enzyme increases in several lines of culture after stimulation by insulin and growth factors. Thus, it is conceivable that these changes in casein kinase 2 activity could affect the phosphorylation level of its target protein substrates and, in this way, affect the rate of cellular proliferation and/or differentiation.

The possible role of type 2 casein kinases in plants is still unknown. It is evident that the identification of endogenous substrates will help to understand its physiological function. RAB-17 polypeptides are of particular interest for several reasons. (a) They are specifically synthesized during embryogenesis; (b) their synthesis is induced by the hormone abscisic acid; (c) they can be induced also in leaves by water stress; and (d) phosphorylation of RAB-17 polypeptides is lacking when protein synthesis is induced in vegetative tissues. These findings raise the possibility that RAB-17 phosphorylation by casein kinase 2 could play a role in the cellular response accompanying the onset of the developmental program in maize seeds, which is mediated, to a great extent, by the hormone abscisic acid. Knowledge of the possible relationship between stimulation by abscisic acid and casein kinase 2 activation as well as the functional significance of casein kinase 2-induced phosphorylation of RAB-17 polypeptides will be required to assess the validity of this model.

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