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.

The hormone abscisic acid mediates a number of important physiological processes in plants (1, 2). However, the mode of action of the hormone remains unclear. It is well documented that plant tissues respond to abscisic acid by expressing specific genes (2) encoding proteins of unknown function. Current molecular studies aim to delineate the transduction pathway mediating abscisic acid-responsive gene expression. Ion channels and active transport seem to be involved in osmoregulation and signaling in plant cells (3), and abscisic acid induces a rapid increase in intracellular ion channels and active transport seem to be involved in osmoregulation and signaling in plant cells (3), and abscisic acid induces a rapid increase in intracellular CA++ levels in guard cells (4) and in roots following water stress (5). Evidence from studies on maize indicate that protein phosphorylation is also involved (6).

Earlier studies on the regulation of gene expression during embryogenesis in Zea mays L. have shown a set of basic polyamines of 23 kDa that are rapidly induced in young embryos by exogenous abscisic acid treatment. These polyamines also appear in normal embryogenesis during maturation, coinciding with the period when endogenous abscisic acid levels reach a maximum (7). Further studies showed that the more acidic forms of this group of 23-kDa polypeptides are due to post-translational phosphorylation (6) of a basic protein encoded by the gene responsive to abscisic acid RAB-17 (8).

Sequence analysis of the RAB-17 gene showed that it encodes a glycine-rich protein containing a cluster of serine residues and contains conserved tracts of amino acids with abscisic acid-responsive proteins from other plant species (9, 10). Phosphoamino acid analysis of isolated RAB-17 protein indicates that only serine residues are phosphorylated in vivo (8).

Interestingly, a putative casein kinase 2-phosphorylatable sequence (11, 12) was identified in RAB-17 following the serine cluster (8). Moreover, protein kinase activities have been detected in a number of higher plants (13), and casein kinase 2-type enzymes have been isolated from maize seedlings (14) and other plants (15).

As a first step to elucidate the molecular mechanisms involved in the abscisic acid action in the expression of RAB-17, we have studied the in vitro phosphorylation of isolated RAB-17 protein by casein kinase 2 and by a casein kinase 2-type enzyme isolated from maize embryos.

**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/HCl (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 with 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/HCl (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/HCl (pH 8.0), and the column was washed with the same buffer. The protein fraction was eluted with 1 M NaCl.

**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/HCl (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 with 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/HCl (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/HCl (pH 8.0), and the column was washed with the same buffer. The protein fraction was eluted with 1 M NaCl.
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 nl) in the same buffer.

In Vivo Protein Labeling and Protein Analysis—In vivo labeling of isolated embryos was performed by incubating them simultaneously with 0.25 mM P-[γ-32P]ATP (40 Ci/mM) and L-[35S]methionine (200 Ci/mM) for 2–4 h. Total proteins were separated by 50 mM 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 acetate 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 following 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 rephosphorylation studies were made phosphatase-free by application to a CM-Sepharose column equilibrated with 50 mM Tris/HCl (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 32P-labeled RAB-17 polypeptides were digested with cyanogen bromide in 80% (v/v) formic acid (18, 19) 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 × 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.

Purification of Casein Kinase 2—Casein kinase 2 from rat liver cytosol was purified according to Martos 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% (v/v) glycerol, 1 mM PMSF, and 0.25 mM sucrose. The homogenate was centrifuged at 10,000 × g for 30 min. After being filtered through glass wool, the supernatant was centrifuged at 140,000 × 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 × g for 30 min. This pellet was resuspended in 10 ml of 50 mM Tris/HCl (pH 7.5) containing 1 mM dithiothreitol, 5% (v/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 × g for 30 min and applied batch-wise to 2 ml of phosphocellulose equilibrated with Buffer A. After 2 h, the resin was poured off into a new 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 Vivo 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 × 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 caseine/min of incubation at 30 °C.

In Vitro Phosphorylation of RAB-17—Phosphorylation of RAB-17 in vitro 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 phosphatase 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 subsequence...
purified RAB-17 polypeptides were dephosphorylated with calf intestinal alkaline phosphatase and then incubated with amounts of casein kinase 2 from maize embryos. Protein content was followed by measuring the absorbance at 280 nm. Fractions were assayed for casein kinase activity under the standard assay conditions in the presence of [γ-32P]ATP (A) or [γ-32P]GTP (V).

strate site of casein kinase 2. To ascertain whether RAB-17 polypeptides could be phosphorylated by casein kinase 2, the purified RAB-17 polypeptides were dephosphorylated with calf intestinal alkaline phosphatase and then incubated with casein kinase 2. As shown in Fig. 3, RAB-17 is phosphorylated by casein kinase 2 in the presence of [γ-32P]GTP.

The stoichiometry of 32P incorporation catalyzed by maize embryo casein kinase 2 was ~0.3 mol/mol of RAB-17 protein. This low level of phosphorylation could be due to the low amounts of casein kinase 2 that were used. A similar value of 32P incorporation into RAB-17 was observed with casein kinase 2 from rat liver (Fig. 3, lane 4) when present at concentrations similar to those used with casein kinase 2 from maize embryos 35 days after pollination (Fig. 3, lane 2). The accessibility of purified preparations of rat liver casein kinase 2 allowed us to study the potential number of phosphorylation sites present in RAB-17 protein for this class of enzymes. As observed with other protein substrates (19), the stoichiometry of 32P incorporation into dephosphorylated RAB-17 protein depended on the amounts of casein kinase 2 present in the reaction mixture. A plateau corresponding to 4 mol of phosphate/mol of protein (Fig. 4) was observed at high concentrations of casein kinase 2. 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).

Phosphorylation of Maize RAB-17

**Fig. 2.** Isolation of casein kinase 2 from maize embryos. Shown is the phosphocellulose chromatography of maize embryo extracts. Protein content was followed by measuring the absorbance at 280 nm (●). Fractions were assayed for casein kinase activity under the standard assay conditions in the presence of [γ-32P]ATP (▲) or [γ-32P]GTP (▼).

**Fig. 3.** Phosphorylation of RAB-17 protein by casein kinase 2. Samples of RAB-17 purified protein were incubated for 30 min at 30°C with [γ-32P]GTP alone (lane 1) or with 0.04 unit/ml casein kinase 2 from maize embryos (lane 2) or from rat liver cytosol (lane 4). Lanes 3 and 5 correspond to casein kinase 2 from maize embryos and from rat liver cytosol, respectively, in the absence of RAB-17 protein. Samples were applied to an SDS-polyacrylamide gel (12.5% acrylamide). A, Coomassie staining of the gel; B, autoradiograph. Lane Mr corresponds to molecular mass markers.

**Fig. 4.** Time course of phosphorylation of RAB-17 with casein kinase 2 from rat liver cytosol. RAB-17 was incubated under the standard conditions with 2 units/ml (●) or 4 units/ml (▲) casein kinase 2 from rat liver cytosol in the presence of [γ-32P]GTP. At the indicated times, 20-μl samples were removed and measured for 32P incorporation. Shown are moles of 32P incorporated per mole of RAB-17 protein.

**Fig. 5.** Two-dimensional gel electrophoresis of RAB-17 polypeptides. Excised mature embryos were labeled as described under "Experimental Procedures." A, fluorography of [35S]methionine-labeled RAB-17 polypeptides; B, in vivo phosphorylation of RAB-17 proteins from mature embryos; C, autoradiograph of two-dimensional electrophoresis of a sample of RAB-17 phosphorylated in vitro with 2 units/ml casein kinase 2 from rat liver cytosol.
Amino acid sequence of RAB-17 polypeptide containing potential phosphorylated peptide. Shown is the deduced primary sequence of RAB-17. Arrowheads indicate the predicted positions for CNBr cleavage. Underlined residues correspond to the sequence analysis data of the purified radiolabel-containing peptide from the in vitro labeled RAB-17 polypeptide determined by automated protein sequence analysis. +, amino acid identities; ?, possibility of identity; -, no clear result; *, positions of serine residues in the polypeptide sequence.

**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 IIIB) 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 seryl residues in the central region of the RAB-17 predicted sequence. This region (residues 56–80) 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
Phosphorylation of Maize RAB-17

(25), and differentiation of 3T3-L1 cells (26) and in cancer cells (27). Furthermore, the activity of the enzyme increases in several lines of culture after stimulation by insulin and growth factors (28). 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 (8). 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.

REFERENCES