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Characterization of a Low Molecular Mass Autophosphorylating Protein in Cultured Sugarcane Cells and Its Identification as a Nucleoside Diphosphate Kinase[1]

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A low molecular mass (18 kD) phosphoprotein (pp18) was characterized and purified from cultured sugarcane (*Saccharum officinarum* L.) cell line H50-7209. Autophosphorylation assays were used to detect pp18 after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Only pp18 was detected by a brief in situ phosphorylation method, whereas additional putative protein kinases were detected by an extended method. pp18 was present in both microsomal membrane and soluble fractions and exhibited anomalous turnover of ^{32}P label during in vitro phosphorylation experiments with highest levels present at shorter incubation times. Two major isoforms of the protein were identified in two-dimensional isoelectric focusing/SDS-PAGE of crude extracts and microsomal fractions. The levels of pp18 were enhanced approximately 4-fold by heat shock at 36°C and the elevated pp18 decayed after heat shock was discontinued. pp18 was purified to apparent homogeneity, could be phosphorylated on serine residues, and also exhibited kinase-like activity toward histone H1. The amino acid sequence obtained from a cyanogen bromide digest was greater than 80% identical to nucleoside diphosphate (NDP) kinases from a variety of organisms. Biochemical analysis of the purified protein confirmed the identity as NDP kinase. Thus, NDP kinase appears to be modulated by heat shock in plants.

Signal transduction events are likely to occur in response to environmental stresses in cells, in particular, the heat-shock response. Within minutes after the onset of heat-shock conditions, altered transcription, translation, and metabolism are observed (Lindquist, 1986). These changes may involve well-known signal transduction mechanisms such as alterations in phosphatidylinositol metabolism, in cellular Ca^{2+} concentration (Calderwood et al., 1988), and in protein phosphorylation (Schlesinger, 1988). In sugarcane (*Saccharum officinarum* L.) cells, the induction of the events leading to the development of thermotolerance has a temperature optimum of 36 to 38°C with a minimum induction period of about 30 min at 36°C. Temperatures below the optimum fail to induce all of the HSPs, particularly in the low molecular mass range, whereas treatments above the optimum disrupt protein synthesis in general (Moisyadi and Harrington, 1989). Although much research has been conducted on the heat-shock response, heat-shock genes, and proteins in plants (Vierling, 1991), little information is available on the specific signal transduction mechanisms that may function during sensing of and attenuation to elevated temperature. To this end, we are interested in the potential expression or alteration of protein kinase activities in response to heat shock in cultured plant cells.

Previous research by Trewavas and co-workers characterized an autophosphorylating low molecular mass protein (pp18) from pea as a catalytic subunit of a protein kinase. Major properties of this protein are membrane association, autophosphorylation on Ser residues, kinase activity toward both histone H1 and phosphoinositolphosphate, Ca^{2+} dependence, regulation of protein kinase activity by autophosphorylation, and a ratio of autophosphorylation to protein kinase activity of approximately 10:1 (Blowers and Trewavas, 1987, 1988, 1989). The small size of the enzyme (18 kD) is in contrast to the current understanding of the size limitations for Ser protein kinases. Typically, such enzymes have minimum sizes in the 43- to 45-kD range to include all of the conserved domains known for this class of enzymes (Hanks et al., 1988). Neither the biochemical characterization nor the potential roles of pp18 from pea have been clarified.

In this report, we characterize a sugarcane pp18 homologous to the pea pp18 as described by Trewavas and coworkers, we demonstrate that the activity of this enzyme is enhanced by heat shock, and we provide evidence that pp18 is, in fact, an NDP kinase. NDP kinases have been characterized from a variety of organisms, including bacteria, animals, and plants (Parks and Agarwal, 1973). This enzyme provides the majority of GTP, CTP, and UTP in cells (Ingraham and Ginther, 1978). Whereas the primary role of this enzyme may be to affect this interconversion of nucleoside triphosphates, the many isoforms of NDP kinase as well as developmental regulation (Rosengard et al., 1989; Biggs et

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Abbreviations: CNBr, cyanogen bromide; HSP, heat shock protein; MS, millisiemens; NDP, nucleoside diphosphate; pI, isoelectric point; PVDF, polyvinylidene difluoride.

al., 1990; Berges and Harrison, 1993), the interaction with signal transduction elements (Randazzo et al., 1991, 1992), and the potential to be modulated during heat shock suggest additional important roles.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Sugarcane cells (*Saccharum officinarum* L.), cell line H50-7209, were grown in the dark as suspension cultures on a rotary shaker at 25°C as described previously (Moisyadi and Harrington, 1989).

Protein Extraction, Gel Electrophoresis, and in Situ Phosphorylation of Proteins

Sugarcane cells (0.1 g in 5 mL) from mid-log phase cultures were incubated for the desired times and temperatures in shaking water baths, harvested by filtration, and washed with 50 mL of fresh growth medium. To obtain crude extracts, cells were quickly weighed, frozen in liquid N_2, homogenized under liquid N_2, and extracted with 0.5 mL of boiling 2× Laemmli (1970) sample buffer containing 4% SDS and 1 mM PMSF. The entire harvest procedure to this point was completed within 2 min to minimize degradation of protein extracts. This mixture was immediately placed in a boiling water bath for 5 min and then centrifuged at 15,000*g* for 15 min at 20°C. Protein estimation and separation by single-dimension SDS-PAGE (Laemmli, 1970) or two-dimensional IEF/SDS-PAGE using minigels were accomplished as before (Moisyadi and Harrington, 1989).

The two in situ phosphorylation procedures based on the method of Geahlen et al. (1986) were adapted for minigels. In a "rapid" procedure (method A) developed by Blowers and Trewavas (1987, 1989), proteins were separated by SDS-PAGE in gels with or without 20 μg mL^{-1} histone H1 (Sigma, type III-S) cast in the matrix. The gels were then treated as follows: three washes, 45 min each in 50 mM Hepes, pH 7.4; and two 30-min washes in buffer A consisting of 55 mM Hepes, pH 7.4, 11 mM $MgCl_2$, 0.55 mM $CaCl_2$, and 0.45 mM Na_2EGTA. The gels were labeled for 1 h in buffer A with the addition of 50 μCi [γ-^{32}P]ATP (New England Nuclear, 3000 Ci mmol^{-1}). Gels were then quickly rinsed three times with deionized H_2O, incubated for 3 h in 40 mM Hepes, pH 7.4, containing 5 g of Amberlite No. CG-400, and then washed twice (30 min each) in 40 mM Hepes, pH 7.4, containing 2% sodium pyrophosphate. All the incubation volumes were 100 mL.

In the second, "long" procedure (method B) as described by Klimczak and Hind (1990), proteins were separated as above in gels with or without 100 μg mL^{-1} histone H1 cast in the matrix. The gels were subjected to the following: four washes, 15 min each in 50 mM Tris, pH 8.0, containing 5 mM β-mercaptoethanol; a 4-h wash, and a subsequent 1-h wash in buffer B consisting of 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM DTT, 200 mg L^{-1} BSA, and 20% glycerol. The gels were in situ labeled in fresh buffer B for 8 h with the addition of 100 μCi [γ-^{32}P]ATP (New England Nuclear, 3000 Ci mmol^{-1}). The gels were washed four times with deionized H_2O for 20 min, incubated for 3 h with 50 mM Tris, pH 8.0, containing 5 g Amberlite No. CG-400, and then washed two times for 30 min with 10% TCA containing 2% sodium pyrophosphate. All the incubation volumes were 100 mL except the in situ phosphorylation medium, which was 50 mL. After the above treatments, the gels from either method were incubated for 30 min in Coomassie stain containing 2% sodium pyrophosphate, destained, and rapidly washed three times with deionized H_2O. The inclusion of sodium pyrophosphate in the gel stain significantly lowered nonspecific binding to the gel matrix. The gels were dried and the labeled proteins were visualized by autoradiography on preflashed film. Unless otherwise indicated, labeling using either of the above methods was accomplished using gels containing histone H1 and 20 μg of extracted cell protein was loaded in each lane.

Microsome Preparation

Microsomes were prepared from 23°C control and 36°C heat-shocked sugarcane cell cultures, dissolved in microsomal resuspension buffer C (0.3 M sorbitol, 50 mM Hepes, pH 7.6, 1 mM Na_2EDTA, 0.1 mM PMSF) and in vitro phosphorylated exactly as described by Hetherington and Trewavas (1984) in an ice bath or a 23°C water bath, in reaction buffer A consisting of 55 mM Hepes, pH 7.4, 11 mM $MgCl_2$, 0.55 mM $CaCl_2$, 0.45 mM Na_2EGTA (final volume 15–100 μL). The reaction was started by the addition of 20 μCi of [γ-^{32}P]ATP (New England Nuclear, 3000 Ci mmol^{-1}) and terminated by boiling in an equal volume of 2× Laemmli sample buffer for 5 min.

Purification of Sugarcane pp18

Mid-log phase sugarcane cells heat shocked at 36°C for 2 h were filtered under vacuum and washed with 5 times the culture volume of fresh culture media. The cells were frozen in liquid N_2 and ground to a fine powder in a chilled mortar and pestle on ice. The cell homogenate was washed with ice-cold acetone (10:1, v/w) and stirred for 2 h at 1°C. The resulting acetone powder was dried by vacuum filtration. The powder was extracted for 5 h at 1°C with twice the cell mass of buffer D (10 mM Tris buffer, pH 8, 2 mM EDTA, 2 mM Na_2EGTA, 1% β-mercaptoethanol, 0.1% Chaps, and 1 mM PMSF) and filtered through two layers of Miracloth. The filtrate was clarified at 2,500*g* for 10 min and subjected to step-wise ammonium sulfate precipitation. After stirring for 45 min the precipitated proteins were collected by centrifugation (20,000*g*, 45 min). All pellets were resuspended in a minimal volume of buffer E (10 mM Tris, pH 8, 1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM PMSF), dialyzed extensively against 20 mM ammonium bicarbonate, and lyophilized. The lyophilized powder was resuspended in buffer D and loaded on a DEAE-Sepharose column (2.5 × 25 cm) at 0.25 mL min^{-1}. The column was eluted (0.25 mL min^{-1}) with a NaCl gradient (0.05–0.8 M) in buffer D and 5-mL fractions (conductivity 8.9–18.8 mS cm^{-1}) containing pp18 were collected, dialyzed, and lyophilized as above. The residue was then resuspended in buffer F (0.3 mM sorbitol, 6 mM Tris, pH 7.2, 6 mM Mes, 0.2 mM Na_2EDTA, 20 mM NaCl, 0.1 mM DTT) containing 10 mM $MgCl_2$.

The pp18 was chromatographed on an ATP-agarose (Sigma No. A-2767) column (6 mL) at 6 mL h^{-1}. The column was sequentially eluted with 30 mL each of buffer F containing 0.3 M NaCl, buffer F with 20 mM EDTA, buffer F with 1 M NaCl at pH 9.0, and buffer F with 1 M NaCl at pH 4. The final elution was with 30 mL of buffer F containing 1 mM GTP. All samples were dialyzed, lyophilized as above, and resuspended in deionized H_2O for further analysis. All extraction procedures were done in a cold room (1°C) using ice baths, and all centrifugations were performed at 4°C. The presence of pp18 in various fractions was monitored using in situ phosphorylation method B after SDS-PAGE.

Electroelution

Excised gel pieces containing proteins were placed in the cathode chamber of a microelutor (ISCO, Lincoln, NE) and soaked for 4 h in 0.4 M NH_4HCO_3, 2% SDS, 0.1% DTT. The outer electrode chamber contained 50 mM NH_4HCO_3, 1% SDS, and the inner electrode chamber held 10 mM NH_4HCO_3, 0.1% SDS. The proteins were electroeluted overnight at 4 W, 150 V, and 10 mA, dialyzed for 24 h against 10 mM NH_4HCO_3, lyophilized, dissolved in deionized H_2O, and stored at −70°C.

Protein Sequence Analysis

The purified protein was separated by SDS-PAGE and blotted to PVDF membrane. Bands containing the pp18 were excised and subjected to automated Edman degradation on an Applied Biosystems 475A protein sequencer. No sequential release of phenylthiohydantoin amino acids was observed on two different preparations of the protein. The blotted protein was then subjected to cleavage by CNBr (10 mg mL^{-1}) in 70% formic acid for 20 h at room temperature. The cleavage solution was removed and the PVDF membranes were washed twice with deionized H_2O. The membranes were then subjected to automated Edman degradation as described above. Combined washings and the CNBr supernatant solution were evaporated to dryness in a Speed Vac concentrator. The residue was resuspended in 30 μL of 0.1 M ammonium bicarbonate solution. Portions of the PVDF membranes and an aliquot of the CNBr supernatant were subjected to amino acid analysis. Bands subjected to automated Edman degradation contained 13 to 26 pmol of protein. The recovery of amino acids from the CNBr supernatant was <2 pmol, indicating that most of the protein was retained on the PVDF membrane. The CNBr cleavage procedure was repeated on three different samples so that amino acid sequence data could be compared and possible ambiguities resolved. Initial yield of phenylthiohydantoin amino acids from the membranes ranged from 20 to 30% based on quantitation of the protein on the membrane by amino acid analysis. Repetitive yields averaged 90%. Amino acid analysis of the protein on PVDF membranes was done on hydrolysates (6 N HCl, 115°C for 20 h) using a Waters Picotag system as previously described (Timerman et al., 1992).

NDP Kinase Assay

Enzyme activity was measured in an assay mixture (10 μL total) containing 50 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM ATP, 2 mM GDP, 5 μg BSA, and 10 μCi [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$) (Munoz-Dorado et al., 1990). Purified enzyme (10 ng μL^{-1}) was added to start the reaction and incubated at 32°C for 10 min unless specified. The reaction was stopped with the addition of 5 volumes of 10 mM EDTA, pH 3.65. Aliquots (1 μL) were loaded onto polyethyleneimine-cellulose TLC plates and separated by ascending chromatography with a solvent of 0.75 M KH_2PO_4, pH 3.65. The plate was dried and autoradiographed on preflashed x-ray film for 30 min. The radioactive spots corresponding to GTP were removed and quantified in a Beckman liquid scintillation counter. For time-course studies, the NDP kinase assay was carried out as described above except that the volume of the reaction mixture was 20 μL. The NDP kinase assay was performed with enzyme concentrations ranging from 0 to 20 ng μL^{-1} in a 10-μL reaction mixture.

RESULTS

General Properties of Sugarcane pp18

Crude extracts of sugarcane cells contained an 18-kD peptide that exhibited properties similar to those of the pea pp18. Using in situ phosphorylation method A, only peptides in the 18-kD region were labeled (Fig. 1, lanes 1 and 2). The omission of histone H1 from the gel matrix resulted in reduced label incorporation into the 18-kD region (Fig. 1, lane 2), suggesting pp18 autophosphorylation and histone H1 phosphorylation. An alternative explanation is that there may be 18-kD substrate peptides present in the crude extract; however, labeling experiments using two-dimensional IEF/SDS-PAGE gels greatly reduces this possibility (see below).

With in situ phosphorylation method B, the overall level of ^{32}P incorporation into pp18 was much greater and several

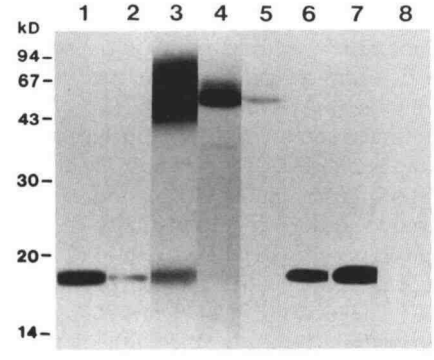

Figure 1. Analysis of sugarcane pp18 under different conditions. Crude extracts were separated by SDS-PAGE and phosphorylated in the gel matrix using protocol A (lanes 1 and 2) or protocol B (remaining lanes) and visualized by autoradiography. Equal amounts of protein (20 μg) were loaded in each lane. Gels were without (lanes 2 and 4) or with 20 μg mL^{-1} (lane 1) or 100 μg mL^{-1} (lanes 3, 5, and 8) histone H1 cast in the matrix. Lane 5, Labeled in the presence of EGTA (5 mM). Substrates were [γ-^{32}P]ATP (lanes 1–6), [γ-^{32}P]GTP (lane 7), [α-^{32}P]ATP (lane 8).

additional putative kinases were detected (Fig. 1, lane 3), including heavily labeled peptides in the 50- to 70-kD range and a 36-kD peptide. Inclusion of EGTA (5 mM) in the labeling buffer completely inhibited labeling of pp18 (Fig. 1, lane 5). Labeling was greater with GTP than ATP at equimolar levels (Fig. 1, lanes 6 and 7), suggesting a somewhat higher affinity for GTP. No labeling was detected for any peptide when [α-^{32}P]ATP was used as the substrate (Fig. 1, lane 8).

Comparisons of the two in situ phosphorylation methods used here suggest that caution be used when interpreting results of such experiments. In method A, only pp18 was labeled in crude extracts, whereas the longer method B detected several additional putative kinases. However, many additional protein kinases must go undetected. Method B employs greater concentrations of ATP (4×), more histone H1 (5×), and a longer labeling period (8×), and may detect putative kinases with different renaturation or kinetic properties that are not active in the short protocol. When method A was modified to include a longer labeling period, additional putative protein kinases were visualized (data not shown). The long protocol, although detecting additional putative kinases, was subject to diffusion of proteins during the extended incubation periods, especially when histone H1 was reduced or omitted from the gel matrix (Fig. 1, lane 4).

Analysis of Sugarcane pp18 in Microsomes

The sugarcane pp18 was present in microsomes (Fig. 2). The protein was most heavily labeled in short incubation times (15 s) followed by rapid losses as the time course proceeded (Fig. 2A), in contrast to label accumulation into larger peptides (e.g. 43- to 67-kD range). Similar but much slower dephosphorylation kinetics were obtained when the time course was carried out at 0°C (Fig. 2B). These results are identical to the labeling patterns for pp18 in pea microsomes, where the turnover of label was proposed to be due to the presence of a specific phosphatase (Blowers and Trewavas, 1987). If such a phosphatase is present in sugarcane cells, it had little effect on higher molecular mass proteins. The sugarcane pp18 was also present in the post-microsomal soluble fraction and exhibited similar anomalous label patterns (Harrington et al., 1990).

Sugarcane microsomes were solubilized in buffer D and chromatographed on DEAE-Sephadex. The activity eluted at 8.9 to 18.8 mS cm^{-1}, similar to pp18 purified from whole cells (see below). Autoradiography of two-dimensional IEF/SDS-PAGE gels of samples labeled in vitro for different times indicated the presence of two labeled peptides (pI 5.4 and 5.8) (Fig. 2C). The level of ^{32}P label in these proteins declined with incubation time, indicating that both peptides comprised the pp18 activity observed in SDS-PAGE. This partially purified DEAE fraction phosphorylated histone H1 in vitro (Moisyadi, 1991).

Effect of Heat Shock on pp18 Activity

Extractable pp18 activity increased with time during heat shock (Fig. 3, A and B). Measurements of radioactivity in pp18 indicated approximately a 4-fold increase during a 2-h

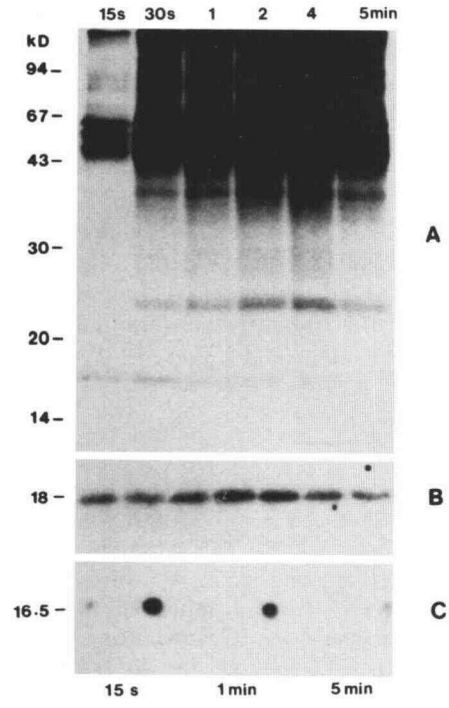

Figure 2. Analysis of sugarcane pp18 in microsomes. Microsomes were in vitro phosphorylated for the indicated times, separated by SDS-PAGE, and visualized by autoradiography. A, Labeled at 23°C; B, labeled at 0°C; C, two-dimensional IEF/SDS-PAGE of partially purified microsomal pp18 labeled at 0°C. A and B, 1.4 μg protein lane^{-1}; C, 10 μg protein gel^{-1}.

heat shock (Fig. 3C). Elevated levels of pp18 were not present in cells heat shocked for 2 h at 32°C (Moisyadi, 1991) or in cells heat shocked for periods less than 20 min at 36°C (Fig. 3). These data correlate well with the requirements for the induction of 18-kD HSPs in sugarcane cells (Moisyadi and Harrington, 1989). Omission of histone H1 from the gel matrix resulted in reduced labeling of the 18-kD region, suggesting both autophosphorylation and histone phosphorylation in the gel matrix (Fig. 3B). In situ phosphorylation of two-dimensional IEF/SDS-PAGE gels indicated increased labeling of two 18-kD peptides in response to heat shock (Fig. 3D). Each of these peptides (pI 5.6 and 6.0) was slightly more basic than the in vitro phosphorylated peptides from microsomes (Fig. 2).

In extended heat-shock experiments, pp18 behaved as a low molecular mass HSP. Elevated levels of pp18 activity were present only in the extracts from heat-shocked cells (Fig. 4). As the time course progressed the elevated levels of pp18 returned to lower, constitutive levels. Further, the rate of decay was slower in cells incubated continuously at 36°C (Fig. 4C). These patterns are similar to the turnover of 18-kD HSPs under these conditions (Moisyadi, 1991) and suggest that pp18 activity increases in response to heat shock. This may be due to one (or a combination) of the following possibilities: the level of pp18 is elevated by heat shock, pp18 is activated during heat shock, or other 18-kD HSPs are phosphorylation substrates for pp18, whose concentration

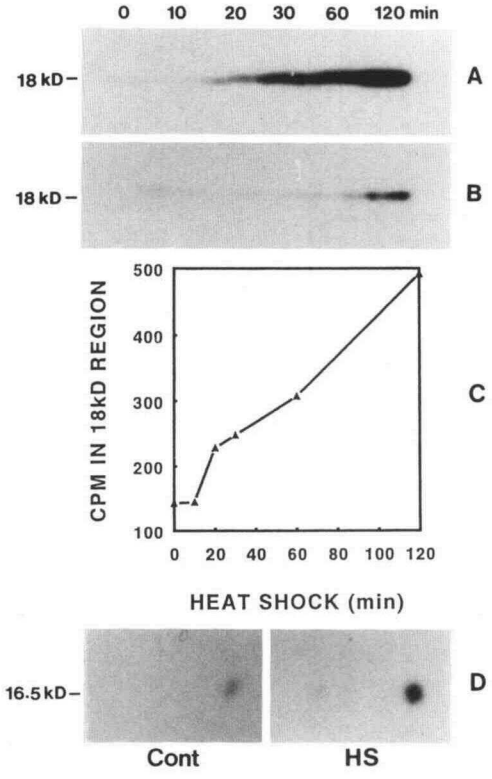

Figure 3. Effect of heat shock on the level of pp18. Crude extracts were obtained from cells heat shocked for the indicated times and phosphorylated after SDS-PAGE using in situ protocol A. A, B, D, Autoradiographs of labeled gels; C, analysis of gel A 18-kD region by liquid scintillation counting; B, without histone H1; D, analysis of pp18 activity by two-dimensional IEF/SDS-PAGE (10 μg protein gel^{-1}).

remains constant during heat shock. The latter possibility is greatly reduced in the in situ phosphorylation experiments with two-dimensional gels and by the presence of 100 μg mL^{-1} histone H1 in SDS-PAGE gels, which would presumably act as a saturating substrate.

Klimczak and Hind (1990) suggested that the pp18 as described by Trewavas and his co-workers is either not active or is a proteolytic fragment in barley leaf extracts. Proteolysis during extraction was apparently not a contributing factor in the appearance of pp18 here. Phosphorylation patterns identical to those in Figure 3 were obtained from control and heat-shocked cells using extracts prepared with 2× Laemmli sample buffer (1970) containing 1 mM PMSF, 1 mM 2-2′ dithiodipyridine, 1 μg mL^{-1} leupeptin, 1 μg mL^{-1} pepstatin A, 1.5 μg mL^{-1} aprotinin. Antibodies against the catalytic domain of the Ca^{2+}-dependent protein kinase (Harper et al., 1991) recognized 50- to 60-kD peptides in sugarcane cell extracts but not pp18 (data not shown).

Purification of pp18

Sugarcane pp18 activity was obtained in the 45 to 60% ammonium sulfate pellet (Moisyadi, 1991). This result may explain the failure of Klimczak and Hind (1990) to detect pp18 in 40% ammonium sulfate fractions obtained from barley. Chromatography of this fraction on DEAE-Sepharose (or -Sephadex) yielded a peak of pp18 at conductivity 8.9 to 18.8 mS cm^{-1}, indicating similarity to the microsomal pp18. No other putative kinases were found in fractions containing pp18 using in situ method B; however, several additional putative kinases were detected in other DEAE fractions (data not shown).

Pooled pp18 from the DEAE step were chromatographed on ATP-agarose. The majority of silver-stainable protein and a small amount of pp18 activity were observed in the initial column washes (Fig. 5, A and B, lanes 2 and 3). Little or no pp18 activity was eluted by buffer washes with 20 mM Na_2EDTA or 1 M NaCl, pH 9.0 (lanes 4–6). Elution of the column with 1 M NaCl buffer at pH 4.0 released small amounts of pp18 (lanes 7) with the majority of the activity appearing in a GTP elution (lanes 8). This fraction contained peptides only in the 18-kD region as evidenced by silver staining (Fig. 5A). A different ATP-agarose chromatography method (Blowers and Trewavas, 1989) lacking DTT and 20 mM NaCl in the column buffers resulted in the elution of small amounts of pp18 activity in the EDTA fraction (Moisyadi, 1991). Phosphorylation of this fraction on gels by either in situ method occurred only if the incubation buffer contained additional $MgCl_2$ (20 mM). This pp18 fraction was not active in vitro even after extensive dialysis when eluted by this method. However, a 6 M urea strip of the column contained the majority of the pp18 in situ activity, and this fraction phosphorylated in vitro (Moisyadi, 1991).

The purified pp18 fraction contained three to five isoforms that migrated at approximately 16.5 kD (pI 5.2, 5.5–5.6, and 5.9–6.0) and two at 18 kD (pI 5.6 and 6.0) in two-dimensional IEF/SDS-PAGE (Fig. 5, C and D). The silver-stained peptides and the in situ phosphorylation signals migrated to identical positions. The two major isoforms (pI 5.6 and 6.0) correspond to the major pp18 isoforms found in crude extracts. Taken together, these results suggest the presence of multiple isoforms of the same enzyme. Purification may result in the

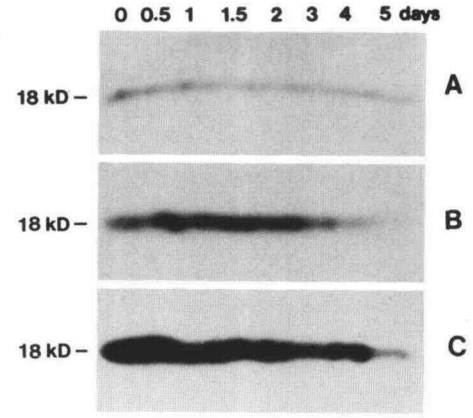

Figure 4. Turnover of elevated pp18 during heat shock. Crude extracts were obtained from cells at the indicated times and labeled as in Figure 3. A, 23°C control; B, 36°C heat shock (2 h) followed by incubation at 23°C; C, 36°C continuous heat shock.

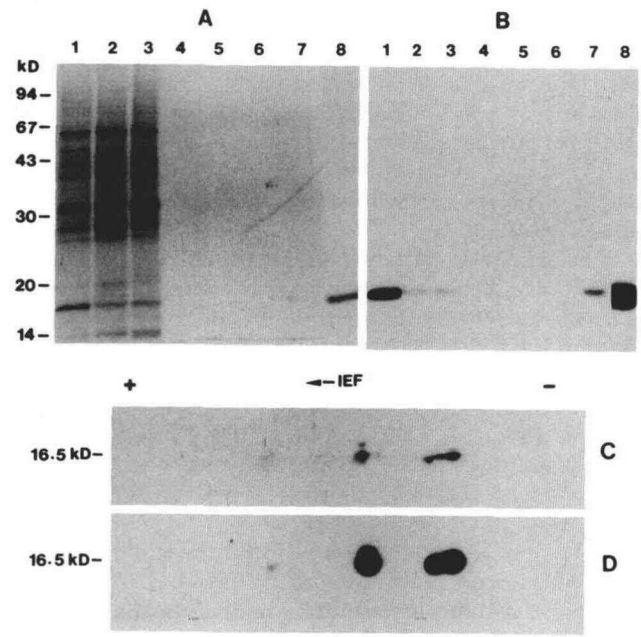

Figure 5. Purification of pp18. A and C, Silver-stained gels; B and D, autoradiograms of A fractions labeled as in Figure 3; C and D, two-dimensional IEF/SDS-PAGE of purified pp18 from lane 8. Lanes 1, DEAE peak; lanes 2 to 8, ATP agarose elution fractions. Lanes 2, Flow through; lanes 3, 0.3 M NaCl; lanes 4 and 5, sequential 20 mM EDTA; lanes 6, 1 M NaCl, pH 9.0; lanes 7, 1 M NaCl, pH 4.0; lanes 8, 1 mM GTP. Lanes 1 to 6, Two micrograms of protein; lanes 7, 0.2 μg of protein; lanes 8, 0.66 μg of protein. C, 0.66 μg of protein gel^{-1}; D, 0.4 μg of protein gel^{-1}.

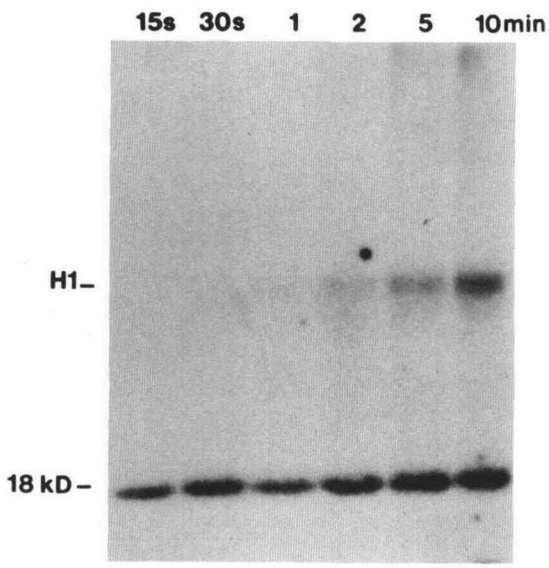

Figure 6. Time course of histone H1 phosphorylation with purified pp18. Reaction was performed as in "Materials and Methods" and separated by SDS-PAGE. Each lane contained 11 μg of pp18 and 250 μg of histone H1. Autoradiogram of dried gel.

concentration of minor isoforms, which were below the level of detection in total cell extracts.

Due to the presence of additional protein kinases and phosphatases that might have been active in vitro, the purification of pp18 was estimated using in situ phosphorylation protocol B and determining the radioactivity in the 18-kD region. The specific activity values reflect the level of phosphorylation achieved during an 8-h period. A summary of the purification for the pp18 (Table I) indicates a 450-fold purification of the enzyme activity based on the autophosphorylation assay.

Time Course of Histone H1 Phosphorylation

The ATP-agarose-purified pp18 phosphorylated histone H1 in vitro (Fig. 6). In agreement with earlier results in peas, the majority of label was found in pp18 rather than in histone H1. A similar result was obtained with pp18 purified from microsomes by DEAE chromatography. Reactions in which the enzyme was omitted showed no labeling of histone H1 (Moisyadi, 1991).

Table I. *Purification of pp18*

Activity of pp18 was determined by measuring the radioactivity incorporated into 18-kD peptides during in situ phosphorylation method B.

Purification Step	Protein	Specific Activity	Purification
	mg	*pmol ^{32}P mg^{-1} protein 8 h^{-1}*	*fold*
Total extract	761	1.1	
40% ammonium sulfate pellet	190		
70% ammonium sulfate pellet	240	1.3	1.1
70% ammonium sulfate supernatant	132		
DEAE active pool	21.9	37.2	32.6
ATP-agarose			
Flowthrough	5.7	10.9	9.6
0.3 M NaCl	1.4	8.9	7.8
20 mM EDTA (1)	0.4		
20 mM EDTA (2)	0.3		
1 M NaCl, pH 9.0	0.4		
1 M NaCl, pH 4.0	0.005	456	414
1 mM GTP	0.207	495	450

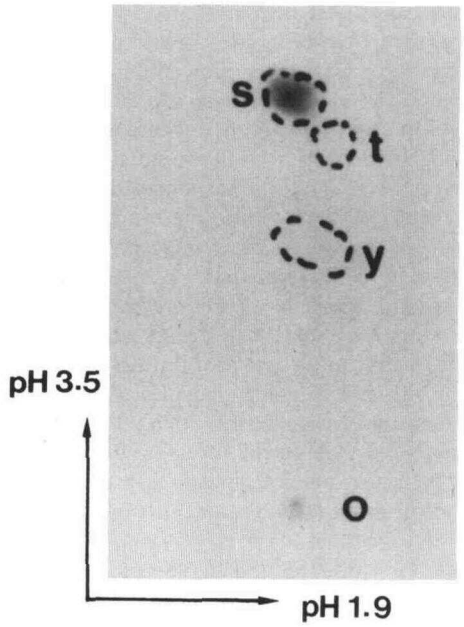

Figure 7. Analysis of phosphorylated amino acids in pp18. Autoradiogram of two-dimensional TLC. o, Origin; s, Ser; t, Thr; y, Tyr.

Analysis of Phosphorylated Residue

The purified pp18 was phosphorylated in situ in the presence of histone H1 (method B) and the 18-kD region was cut from the gel and separated on a second SDS gel. Phosamino acid analysis (Crow et al., 1990) showed that the labeled species in pp18 was phosphoserine (Fig. 7). Longer exposure of this and other TLC separations failed to detect any label incorporation into either Tyr or Thr residues.

Protein Sequence Analysis

Table II summarizes the amino acid sequence analysis data of the pp18 protein CNBr fragment. Even though the same major sequence was present in each of three independent sequence runs, there was always a persistent background of amino acids in each cycle. The disparity of amino acids in particular cycles (e.g. 4, 6, and 14) suggests the presence of isoforms. A search of the Protein Information Resource data base (Release 33) using FASTA (Pearson and Lipman, 1988) always resulted in NDP kinase from various organisms as the identified homologs. With the first amino acid assumed from the CNBr cleavage specificity, the sugarcane pp18 exhibited a high degree of homology (up to 85%) with conserved N-terminal sequences from other NDP kinases and homologs (Fig. 8). The presence of multiple isoforms of NDP kinases in many organisms may explain the sequence disparity observed here.

Table II. *Summary of automated Edman degradation of the pp18 protein*

Amino acids in parentheses were amino acids also identified in the Edman cycle at a level of at least 50% of the major amino acid.

Edman Cycle	Run 1 pmol	Run 2 pmol	Run 3 pmol
1	Val 3.0	Val 3.7	Val 2.7
2	Lys 1.8	Lys 0.6	Lys 0.9
3	Pro 3.2	Pro 1.2	Pro 1.7
4	Asp 2.8	Asp 0.9 (Gly)	Asp 1.9 (Gly)
5	Gly 1.3	Gly 1.8	Gly 0.8
6	Val 2.2	Ser 1.3	Val 1.7 (Ser)
7	Gln 1.7 (Val)	Gln 0.6 (Val)	Gln 1.2 (Val)
8	Arg 2.7	Arg 0.3	Arg 0.3
9	Gly 1.1 (Pro)	Gly 0.3	Gly 0.5
10	Leu 1.0	Leu 0.9	Leu 0.3
11	Xaa[a]	Xaa	Xaa
12	Gly 0.9	Gly 0.9	Xaa
13	Glu 0.8	Glu 0.3	Xaa
14	Ile 0.6	Val 0.3	Xaa

[a] Xaa, Unknown amino acid.

```
Sugarcane pp18                    M-V-K-P-D-G-V-Q-R-G-L-x-G-E-I
Human Nm 23-H1              1.....A-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Human Nm 23 H2              1.....A-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Mouse Nm 23                 1.....A-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Dictyostelium NDPK       1.....A-:-:-:-:-:-:-A-:-:-:-V-:-:-:
Drosophila Awd              1.....:-:-:-:-:-:-:-:-:-:-:-V-:-K-:
Spinach NDPK I                 1.....:-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Spinach NDPK II             1.....:-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Rat NDPK α                  1.....A-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Rat NDPK β                  1.....A-I-:-:-:-:-:-:-:-:-:-V-:-:-:
E. coli NDPK                1.....I-I-:-:-N-A-:-A-K-N-V-I-:-N-:
M. xanthus NDPK             1.....I-I-:-:-:-:-L-E-K-:-V-I-:-K-:
```

Figure 8. Sequence comparison of sugarcane pp18, NDP kinases, and homologs. All known sequences aligned beginning with the amino terminus designated 1.

Biochemical Characterization of Sugarcane NDP Kinase Activity

The purified pp18 exhibited NDP kinase activity as determined by the transfer of label from [γ-^{32}P]ATP to GDP to form GTP (Fig. 9). Depending on the age of the preparation, the addition of BSA to the reaction mixture enhanced the activity. The reaction was linear with time for at least 15 min and with amount of protein up to 10 ng μL^{-1} (Fig. 9) with a pH optimum of 7.4 (S. Dharmasiri and H.M. Harrington, unpublished results). Preliminary kinetic analysis indicates apparent K_m values of 2.3 mM and 0.2 mM for ATP and GDP, respectively (S. Dharmasiri and H.M. Harrington, unpublished results). These data agree with the kinetic constants of

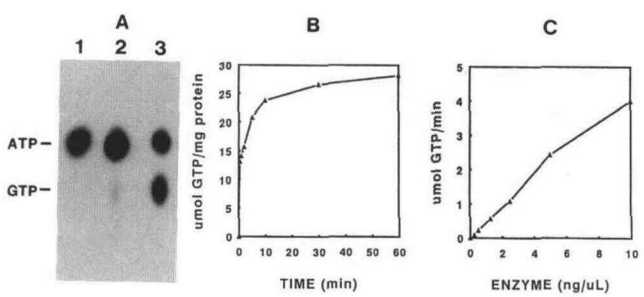

Figure 9. Analysis of NDP kinase activity in pp18. NDP kinase activity was assayed as in "Materials and Methods." A, Autoradiogram of TLC. Lane 1, no pp18; lane 2, plus purified pp18; lane 3, plus pp18 and BSA. B, NDP kinase reaction time course. C, Effect of protein concentration on NDP kinase reaction.

spinach NDP kinase I, which has K_m values 2.0 and 0.091 for ATP and GTP, respectively, and a molecular mass of 16 kD (Nomura et al., 1991). The spinach type II NDP kinase is typically larger (18 kD) and differs in kinetics constants, with K_m values for ATP and GDP of 0.89 and 0.10 mM, respectively (Nomura et al., 1991). Based on the analysis of the purified pp18 on two-dimensional gels and the sequence data, our preparation likely contains both isoforms.

DISCUSSION

The results presented here indicate that the sugarcane pp18 is NDP kinase based on both sequence and biochemical analysis. The sequence of an internal fragment of the sugarcane pp18 is greater than 80% identical to a conserved region of NDP kinases from a variety of sources. Our data indicate that sugarcane pp18/NDP kinase is homologous to the pea pp18 extensively characterized by Trewavas and co-workers. Similarities to pea pp18 include a "ladder-like" in situ phosphorylation pattern on gels, autophosphorylation on Ser residues, anomalous in vitro labeling patterns, kinase activity toward histone H1, apparent Ca^{2+} dependence, and association with microsomes. The purified sugarcane protein consists of both 16.5- and 18-kD isoforms.

NDP kinases have been characterized from a variety of organisms including bacteria, animals, and plants. This ubiquitous enzyme (EC 2.7.4.6.) catalyzes the reaction

$$N_ITP + N_{II}DP \rightarrow N_IDP + N_{II}TP$$

through a classical ping-pong mechanism in which a high-energy phosphoenzyme intermediate is formed (Parks and Agarwal, 1973). Gene and amino acid sequences indicate a high degree of conservation in a variety of organisms (Kimura and Shimada, 1988; Biggs et al., 1990; Kimura et al., 1990; Lacombe et al., 1990; Nomura et al., 1992; Postel et al., 1993), in addition to providing the majority of GTP, CTP, and UTP in cells (Ingraham and Ginther, 1978). NDP kinase may play a role in G-protein-regulated processes either directly or indirectly by maintaining available GTP levels in the vicinity of G proteins (Randazzo et al., 1991, 1992). The proteins controlling tumor metastasis (Nm23) in mammals (Rosengard et al., 1989) and normal development (Awd) in *Drosophila* (Biggs et al., 1990) are NDP kinase homologs. Recently, the human c-*myc* transcription factor, PuF, was identified as NDP kinase (Postel et al., 1993). In *Thalassiosira pseudonana* the level of NDP kinase was correlated with both high and low growth rate, with the enzyme being low at intermediate growth rates (Berges and Harrison, 1993).

The ping-pong reaction mechanism of NDP kinase may explain some of the properties for both the sugarcane and pea pp18s. The ability to remain bound to ATP agarose under a variety of conditions may reflect the need for a second substrate. The majority of the protein was released by GTP and we have since found that GDP is very efficient at releasing pp18 from ATP agarose (S. Dharmasiri and H.M. Harrington, unpublished results). NDP kinase may be phosphorylated in two ways by [γ-^{32}P]ATP. One species, the reaction intermediate, is usually a phosphohistidyl residue (Munoz-Dorado et al., 1993). The presence of a reactive phosphohistidine may account for the opportunistic phosphotransferase activity as represented by low-protein kinase activity of pp18 from both sources and phosphatidylinositol kinase activity of pea pp18 (Blowers and Trewavas, 1988). The reaction mechanism may also explain the rapid turnover of ^{32}P label in in vitro experiments here and by Blowers and Trewavas (1987, 1989) as ADP stimulated the turnover of ^{32}Pi in pea pp18. Prior autophosphorylation of the pea pp18 results in the loss of histone kinase activity (Blowers and Trewavas, 1987). The experimental protocol would have generated ADP in the initial autophosphorylation step, providing a second substrate for the favored NDP kinase reaction. Ser autophosphorylation occurs at low stoichiometry (<0.01 mol^{-1} PO_4 mol^{-1} protein) through an intermolecular event rather than a true autophosphorylation within an individual molecule per se (Hemmerich and Pecht, 1992). The enzyme phosphorylates other proteins such as histone H1 or casein at high enzyme concentration, but the physiological significance of these activities and the mechanisms involved are currently uncertain.

Our data indicated that the activity of the sugarcane NDP kinase is enhanced by heat shock; however, measurement of NDP kinase activity in crude extracts is complicated by the presence of many reactions utilizing nucleoside triphosphates. We have measured increased NDP kinase activity in crude extracts of heat-shocked cell, although not as high as the 4-fold elevation detected by in situ phosphorylation on gels (S. Dharmasiri and H.M. Harrington, unpublished results). Apparent heat-shock induction is likely due to increased synthesis of the enzyme during heat shock based on co-migration in two-dimensional IEF/SDS-PAGE of both unphosphorylated and phosphorylated NDP kinase isoforms with newly synthesized HSPs (Moisyadi, 1991). Western analysis of protein levels will shed further light on this question. The heat-shock response involves major alterations in gene transcription, mRNA translation, protein synthesis, as well as other metabolic events leading to the development of thermotolerance. Of particular interest are requirements for nucleoside triphosphates in mRNA transcription, in protein synthesis, and in signal transduction during the generation of second messenger responses. These central activities may dictate the need for increased ability to modulate nucleoside triphosphate pools through elevated levels of NDP kinase. The maintenance of nucleoside triphosphate pools would be a crucial activity essential for survival and would be expected to be highly conserved.

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