

Low temperature signal transduction during cold acclimation: protein phosphatase 2A as an early target for cold-inactivation

Antonio F. Monroy, Veena Sangwan and Rajinder S. Dhindsa*

Department of Biology, McGill University, 1205 Avenue Docteur Penfield, Montréal, Québec H3A 1B1, Canada

Summary

The authors have previously shown that cold acclimation and cold acclimation-specific (*cas*) gene expression in alfalfa require cold-triggered calcium influx and phosphorylation of specific pre-existing proteins. In this study, the authors used the expression of *cas15* gene as an end-point marker to examine the role of protein phosphorylation in low temperature signal transduction in alfalfa cells. Whereas the protein kinase inhibitor staurosporine prevented the cold induction of *cas15*, the protein phosphatase inhibitor okadaic acid induced the *cas15* at 25°C. Upon exposure of cells to cold, total cellular protein phosphatase activity rapidly declined by 30% but all of this decline could be attributed to an almost complete inhibition of protein phosphatase 2A (PP2A). This cold-inactivation of PP2A was found to be mediated by calcium influx and could be reproduced at 25°C by treating the cells with the calcium ionophore A23187 or with the calcium channel agonist Bay K8644. On exposure of cells to cold, the transcript and protein levels of the PP2A catalytic subunit (PP2Ac) did not decline, but the binding of anti-PP2Ac antibody to native PP2Ac increased, indicating a demasking of the PP2Ac epitope. The possible mechanisms by which cold might inhibit PP2A are discussed.

Introduction

Exposure of seedlings (Mohapatra *et al.*, 1989) or cell suspension cultures (Wolfrain *et al.*, 1993) of alfalfa to low but non-freezing temperatures (cold acclimation) induces the expression of *cas* (cold acclimation-specific) genes and the development of freezing tolerance. Cold acclimation is associated with rapid and reversible changes in the phosphorylation status of specific pre-existing proteins (Monroy *et al.*, 1993a), and is triggered by cold-induced

influx of cell wall calcium into the cytosol (Monroy and Dhindsa, 1995). Inhibition of the calcium influx or of protein kinase activity prevents the cold-induced protein phosphorylation, *cas* gene expression, and the development of freezing tolerance (Monroy *et al.*, 1993a; Monroy and Dhindsa, 1995). Moreover, cells display differential regulation of members of a calcium-dependent protein kinase gene family during the early stages of cold acclimation (Monroy and Dhindsa, 1995). Thus, it appears that a calcium-dependent, protein phosphorylation-mediated pathway participates in low temperature signal transduction. However, it is not clear how cold-induced changes in protein phosphorylation are brought about. It has been shown that the phosphorylation level of a protein is determined by the dynamic equilibrium between the activities of the related protein kinase and protein phosphatase (Jaquet *et al.*, 1995). Furthermore, small changes in this equilibrium can have dramatic consequences for metabolic processes (Cohen, 1992).

Therefore, in order to better trace transduction pathways leading to cold acclimation, it is necessary to understand the relative roles of protein kinases and protein phosphatases. In the present study, we have used the expression of *cas15* gene of alfalfa as an end-point marker for the progress of the low temperature signal transduction cascade. Since cold induction of this gene relies solely on pre-existing proteins (Monroy *et al.*, 1993b), its expression serves as an ideal marker. We have probed the roles of protein phosphatases 1 (PP1) and 2A (PP2A), which together account for more than 85% of the total protein phosphatase activity in plant cells (Cohen *et al.*, 1990; MacKintosh and Cohen, 1989; Smith and Walker, 1996). We report here that PP2A is inactivated by cold early during cold acclimation. While the precise mechanism underlying this inactivation of PP2A is presently unclear, we present evidence for the involvement of calcium and a cold-induced demasking of the epitope of its catalytic subunit (PP2Ac).

Results

Activity of protein kinase(s) and inhibition of protein phosphatase(s) are required for cold induction of cas15 gene expression

We first studied the effects of the protein kinase inhibitor staurosporine and the protein phosphatase inhibitor okadaic acid on cold-induced *cas15* transcript accumulation

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*For correspondence (fax +1 514 398 5069; e-mail: rdhindsa@bio1.lan.mcgill.c).

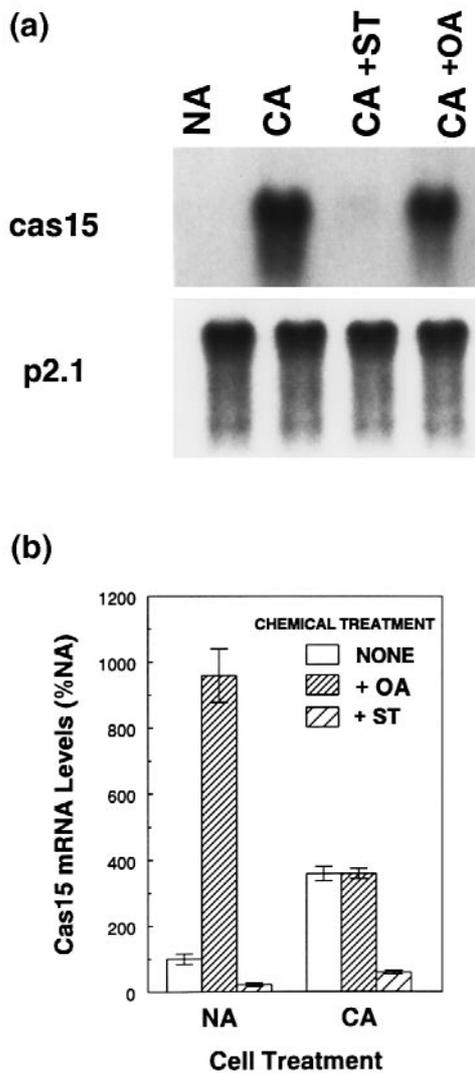


Figure 1. Expression of the *cas15* gene requires protein kinase action and protein phosphatase inhibition.

(a) Prevention of cold induction of *cas15* by the protein kinase inhibitor staurosporine but not by the protein phosphatase inhibitor okadaic acid. Effects of 5 μM staurosporine or 0.25 μM okadaic acid on accumulation of *cas15* or p2.1 (a constitutively expressed gene used as a control) mRNAs during cold acclimation. Aliquots of non-acclimated (NA) cell suspension cultures were cold acclimated for 24 h in the absence (CA) or presence of staurosporine (CA + ST) or okadaic acid (CA + OA). Total RNA was extracted and analysed by RNA gel blot hybridization to the indicated probes.

(b) Induction of *cas15* transcript accumulation at 25°C by the protein phosphatase inhibitor, okadaic acid (OA), but not by the protein kinase inhibitor, staurosporine (ST). Aliquots of cell suspension cultures were first incubated for 1 h at 25°C with or without okadaic acid or staurosporine, and then incubated for 7 h at either 25°C or 4°C. Total RNA was extracted and analysed by RNA gel blot hybridization. The data presented were obtained by densitometry of the autoradiographic signals.

and calcium influx. The data presented in Figure 1(a) show that in cells acclimated to 4°C for 24 h, staurosporine inhibited *cas15* transcript accumulation whereas okadaic acid had little effect. Thus, the activity of a staurosporine-sensitive protein kinase appears to be required for low

temperature signal transduction leading to *cas15* expression. Cold-induced calcium influx was little affected by either inhibitor (data not show), indicating that the staurosporine-sensitive protein kinase acts downstream from the calcium signal.

The observation that *cas15* expression was unaffected by okadaic acid at 4°C suggested the possibility that phosphatase activity might be already inhibited at 4°C to allow the *cas15* induction. We reasoned that in such a case, okadaic acid should induce *cas15* at 25°C. To test this prediction, cells were treated for 7 h at 25°C with either okadaic acid or staurosporine. A period of 7 h was found to be sufficiently long for the detectable accumulation of *cas15* transcripts. The data in Figure 1(b) show that treatment with okadaic acid, but not with staurosporine, induced *cas15* expression at 25°C to levels even higher than those induced by cold during the same time. The effects of calyculin A, another phosphatase inhibitor, were similar to those of okadaic acid described above (data not shown). It was concluded that cold-mediated down-regulation of protein phosphatases plays an important role in low temperature signal transduction.

Effects of okadaic acid in cell-free extracts implicate PP2A as a principal target for cold inactivation

To further examine the down-regulation of okadaic acid-sensitive phosphatases by cold, we studied the effects of cold on total protein phosphatase activity (Figure 2a) by the phosphorylase phosphatase assay. This assay has been previously used to determine protein phosphatase activity in plant extracts (MacKintosh and Cohen, 1989). Since cold-induced gene expression could be detected within 3 h of cold acclimation (Monroy and Dhindsa, 1995), we reasoned that a relevant down-regulation of protein phosphatases would have to occur within this time. The data presented in Figure 2(a) show that a 30% inhibition of the total activity of cellular protein phosphatases could be detected within 30 min of cold acclimation and that this inhibition persisted throughout the experimental period. Thus, the inhibition of protein phosphatase activity precedes the *cas15* expression during cold acclimation.

We then asked whether this inhibition could be attributed to a specific protein phosphatase. PP1 and PP2A are the most prevalent protein phosphatases in plants and are responsible for more than 85% of the total cellular phosphatase activity (MacKintosh and Cohen, 1989). The activities of these two phosphatases can be discriminated *in vitro* by their differential sensitivity to okadaic acid. Whereas the IC_{50} (concentration causing 50% inhibition) of okadaic acid for PP1 is 10–15 nM, it is at least 100 times lower for PP2A (Cohen *et al.*, 1990). The data presented in Figure 2(b) show that in extracts from cells exposed to 4°C, phosphatase activity was only slightly affected by 0.5 nM okadaic

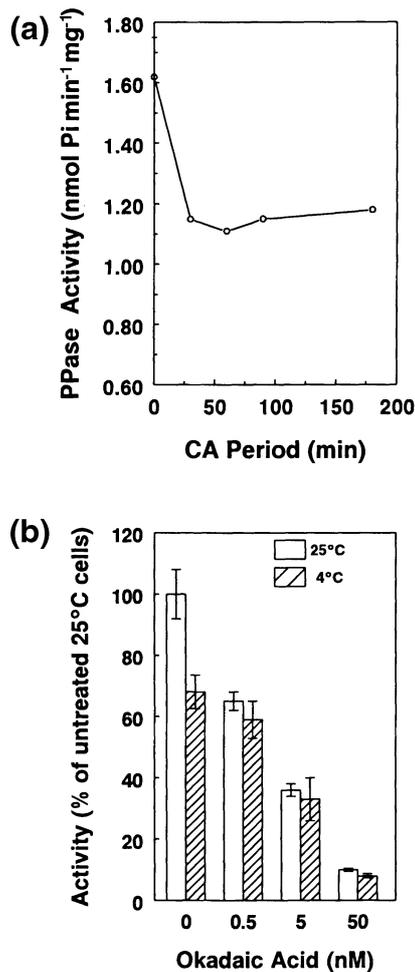


Figure 2. Cold-inhibition of protein phosphatase 2A. (a) Effect of cold acclimation on the combined total cellular activity of protein phosphatases as determined by the phosphorylase phosphatase assay. Homogenates of cell suspensions, acclimated to 4°C for the indicated periods, were prepared with Triton X-100 and supernatants obtained from them were assayed for dephosphorylation of ³²P-labeled glycogen phosphorylase (S.A. 4.4×10^5 dpm/nmole phosphate). Phosphatase specific activity was calculated after 10 min of reaction at 25°C. Each datum point is the average of activity obtained with three different sample dilutions (S.D., 12%), 8, 16, and 32 μ g protein ml⁻¹. (b) Effects of okadaic acid on cold-modulated protein phosphatase activity. Homogenates prepared from cells maintained at 25°C or incubated at 4°C for 1 h were incubated with the indicated concentrations of okadaic acid and assayed for phosphatase activity as above. It should be noted that at or below 0.5 nM okadaic acid, only PP2A is known to be inhibited, and above 0.5 nM, both PP1 and PP2A are inhibited.

acid, which is the concentration known to inhibit PP2A completely but having little effect on PP1 (Cohen *et al.*, 1990). In extracts from cells maintained at 25°C, the activity of extracts treated with 0.5 nM okadaic acid is almost equal to that of the extracts of cold acclimated cells. Thus, the cold-inhibition of protein phosphatase activity could be entirely attributed to PP2A, and the inhibition by okadaic acid at concentrations greater than 0.5 nM is largely attributable to PP1 in both non-acclimated and cold acclimated cells. It was concluded that PP2A is likely to be a principal

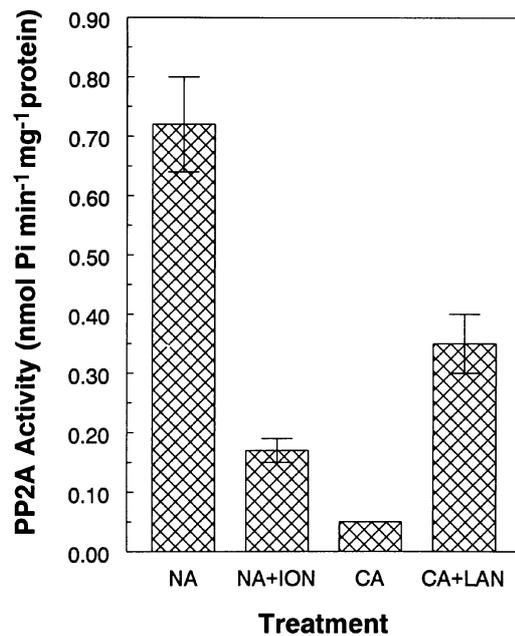


Figure 3. Involvement of calcium in cold-inhibition of PP2A activity. Aliquots of cell suspension cultures were incubated for 1 h at 25°C with or without 50 μ M A23187 (ION) or 10 mM lanthanum (LAN), then either maintained at 25°C (NA) or incubated for 1 h at 4°C (CA). Supernatants of cell homogenates obtained from each treatment were then assayed for the dephosphorylation of ³²P-labeled phosphoenolpyruvate carboxylase from *Sorghum* which is known to be specifically dephosphorylated by PP2A. Each value is a mean of three replicates \pm SD.

and early target for cold-inactivation during low temperature signal transduction.

Down-regulation of PP2A is mediated by cold-triggered calcium influx

We have previously demonstrated that the calcium channel blocker, lanthanum, prevents calcium influx and the expression of *cas15* at 4°C, whereas the calcium ionophore A23187 induces calcium influx and *cas15* expression at 25°C (Monroy and Dhindsa, 1995). To determine if PP2A inactivation is a step in the calcium-mediated low temperature signal transduction cascade, we investigated whether a chemical modulation of calcium influx would affect PP2A activity. In order to specifically measure PP2A activity, we used *Sorghum* phosphoenolpyruvate carboxylase as a substrate (Duff *et al.*, 1995), which has been shown to be dephosphorylated by PP2A but not PP1 (Carter *et al.*, 1990; McNaughton *et al.*, 1991) because Inhibitor-2, a specific inhibitor of PP1, has no effect on dephosphorylation of phosphoenolpyruvate carboxylase by plant extracts (Carter *et al.*, 1990). The data presented in Figure 3 show that the activity of PP2A declined by 85% within 1 h of cold acclimation. Moreover, it can be seen that a treatment of non-acclimated cells with the calcium ionophore A23187 causes an inhibition of PP2A activity at 25°C. Conversely, a

pretreatment of cells with lanthanum substantially prevents the inhibition of PP2A by cold. These results suggest that cold-inhibition of PP2A is mediated by cold-triggered calcium influx.

Down-regulation of PP2A by low temperature is post-translational

To study the mechanism of cold-inactivation of PP2A, we first considered cold-dependent activation of an inhibitor. Natural inhibitors are known to regulate protein phosphatases in animal cells (Cohen *et al.*, 1990; Jaquet *et al.*, 1995; Li *et al.*, 1995). The activity of a putative cold-inducible inhibitor of PP2A was assessed by measuring phosphorylase phosphatase activity in the presence or absence of 1 nM okadaic acid in a 1:1 mixture of extracts containing equal amounts of total protein, prepared from cells that were incubated at 4°C or maintained at 25°C. We reasoned that an inhibitor in the cold-acclimated cells would result in an inhibition of PP2A activity in this mixture of cellular extracts. PP2A activity was 0.43 nmol P_i min⁻¹ mg⁻¹ protein in mixed extracts, whereas it was 0.415 nmol P_i min⁻¹ mg⁻¹ protein in extracts of cells maintained at 25°C. These results indicate that either a specific inhibitor is absent in extracts from cold-acclimated cells or, if present, it is tightly bound to PP2A.

Next, we considered low temperature regulation of the expression of *pp2Ac*, the gene for PP2A catalytic subunit (PP2Ac), at transcript and protein levels. Regulation of *pp2Ac* expression at the transcript level was first examined by RNA gel-blot hybridization using the *Brassica napus pp2Ac* cDNA (MacKintosh *et al.*, 1990) as a probe. We found that *pp2Ac* transcript levels did not decrease during cold acclimation, while PP2A activity declined (data not shown). However, in light of reports of multiple genes for PP2A in *Arabidopsis* (Arino *et al.*, 1993), we used a modified version of the restriction fragment length polymorphism (RFLP)-coupled domain-directed differential display (RC4D) protocol, a combination of RT-PCR and RFLP (Fischer *et al.*, 1995), to evaluate changes in *pp2Ac* transcript levels. The data in Figure 4(a) show that RC4D displayed at least eight polymorphic *pp2Ac* transcripts in cells maintained at 25°C. In cold-exposed cells, transcript levels of some PP2A genes increased, but in no case was a decrease detected coincident in time with the observed decline in PP2A activity. Thus, the observed cold-inhibition of PP2A activity was not due to a decreased transcript level.

To examine post-transcriptional regulation, relative levels of PP2Ac protein were determined by immunoblotting. Polyclonal antibodies raised against the 13 highly conserved C-terminal amino acids of PP2Ac recognized the presumptive antigen, a 36 kDa polypeptide, and two additional polypeptides in cells maintained at 25°C. Levels of all three antigenically related proteins slightly increased

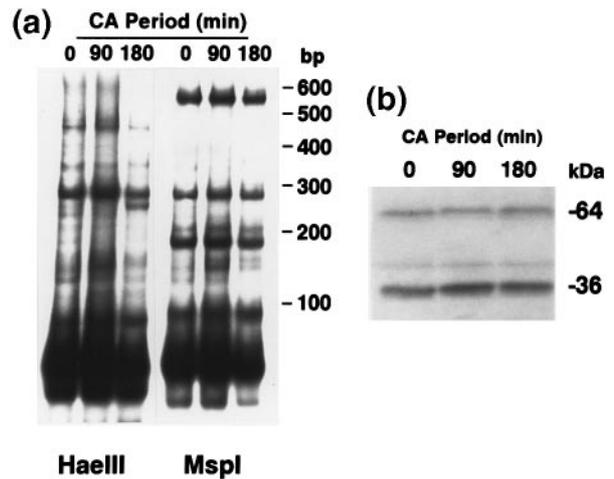


Figure 4. Expression of genes encoding the PP2A catalytic subunit (PP2Ac) in early stages of cold acclimation.

(a) Display of transcripts of the *pp2Ac* gene family by RC4D. Total RNA, extracted from cells acclimated to 4°C for the indicated periods, was first subjected to RT-PCR using degenerate oligonucleotide primers designed to amplify residues 86–272 of alfalfa PP2Ac (Pirck *et al.*, 1993). Resulting amplicons, representing *pp2Ac* family members, were further resolved by RC4D. Restriction enzymes used are indicated at the bottom. ³²P-labeled amplicons were separated on native polyacrylamide gels and visualized by autoradiography.

(b) Immunoblot analysis of protein levels of PP2Ac. Total proteins from alfalfa cells acclimated to 4°C for the indicated periods were separated by SDS-PAGE, blotted onto a PVDF membrane, and probed with rabbit anti-PP2Ac polyclonal antibodies. Immunoreacted protein was then visualized by chemiluminescence generated by goat antirabbit IgG-conjugated horseradish peroxidase.

or remained unaltered during the first 3 h of cold acclimation (Figure 4b). Thus, the cold-induced decline in PP2A activity could not be due to a decrease in the level of PP2Ac protein.

Since PP2A is known to be regulated by a variety of regulatory subunits (Cohen *et al.*, 1990; McCright and Virshup, 1995; Smith and Walker, 1996), we then analysed protein complexes associated with PP2Ac. Proteins labeled *in vivo* with ³⁵S-amino acids were immunoprecipitated with anti-PP2Ac antibodies and analysed by SDS-PAGE coupled with autoradiography. At least five polypeptides could be detected in the immunoprecipitated complex. However, the pattern of ³⁵S-polypeptides that coprecipitated with PP2Ac was qualitatively and quantitatively the same in cells maintained at 25°C or exposed to 4°C (data not shown). During these studies we noticed that protein extracts denatured by SDS exhibited higher immunoreactivity to anti-PP2Ac antibodies than native protein extracts. To confirm this observation, parallel immunoprecipitations were performed on both native and SDS-denatured extracts using anti-PP2Ac antibodies. Immunoprecipitated proteins were then processed for immuno-blotting and probed with anti-PP2Ac antibodies. The data presented in Figure 5 show that when proteins were denatured, the level of immunoprecipitated PP2Ac in extracts from cells main-

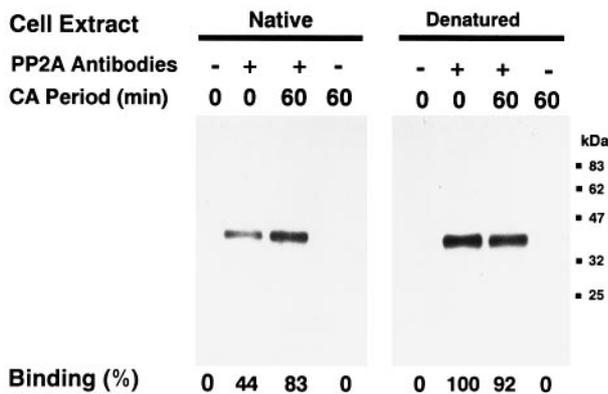


Figure 5. Cold-modulated changes in PP2Ac epitope exposure. Native or SDS-denatured cell-free extracts from cells acclimated to 4°C for the periods indicated were incubated without (–) or with (+) anti-PP2Ac antibodies. The immunoprecipitated proteins were separated by SDS-PAGE, blotted onto a PVDF membrane, and probed with anti-PP2Ac antibodies. Immunoreacted protein was visualized by chemiluminescence. The amount of antibody binding was quantified by densitometry of the immunoreacted protein and is represented by numbers shown at the bottom of the figure. The amount of antibody binding to denatured extracts of non-acclimated cells was considered to be 100%. Each value is an average of two replicates deviating by no more than 12%.

tained at 25°C was slightly higher than that from cells exposed to 4°C. On the other hand, when proteins were not denatured, levels of immunoprecipitated PP2Ac were considerably higher in extracts from cells exposed to 4°C than in extracts of cells maintained at 25°C. Therefore, the cold-inactivation of PP2A appears to be associated with a demasking of the PP2Ac epitope.

Discussion

We have previously shown that cold-induced influx of calcium from the cell wall into the cytosol, and changes in the phosphorylation of specific pre-existing proteins, are required for the cold acclimation of alfalfa cells (Monroy and Dhindsa, 1995; Monroy *et al.*, 1993a). How low temperature brings about these changes in protein phosphorylation is presently unknown. Here, we provide evidence for the cold-inactivation of a specific protein phosphatase, PP2A, and, more interestingly, for a link between its inactivation, calcium influx, and *cas* gene expression. Thus, our finding that PP2A is a target for cold inactivation provides an important clue to understanding the modulation of protein phosphorylation by low temperature and the elucidation of low temperature signal transduction pathways.

The results of the present study show that PP2A is a principal target for cold-inactivation. This is shown by the observation that concentrations of okadaic acid known to inhibit PP2A, but not PP1, have no effect on phosphatase activity of extracts from cold acclimated cells, indicating that PP2A is already inhibited in this extract. The same concentration of okadaic acid causes a decline in phosphat-

ase activity of extracts from non-acclimated cells to the level found in the cold acclimated cells (Figure 2b). Furthermore, the results of experiments using [³²P]-labeled phosphoenolpyruvate carboxylase as substrate for dephosphorylation by cell extracts (Figure 3) also demonstrate that PP2A is a specific target for inactivation by cold. It is known that the dephosphorylation of P-pyruvate carboxylase by plant extracts is not inhibited by Inhibitor-2, a specific inhibitor of PP1 (Carter *et al.*, 1990). Other protein phosphatases such as PP2B or PP2C are unlikely to affect dephosphorylation of phosphorylase or phosphoenolpyruvate carboxylase in the presence of chelators of divalent cations because these phosphatases absolutely require divalent cations for their activity (Cohen *et al.*, 1989). It should be noted, however, that concentrations of okadaic acid which are expected to inhibit both PP1 and PP2A (Figure 1b), induce *cas15* expression to levels higher than those induced by cold during the same time, suggesting that PP1 may also play a role in low temperature signal transduction. PP1 is known to dephosphorylate signal transducing proteins and nuclear factors, sometimes in conjunction with PP2A (DeFranco *et al.*, 1991; Hagiwara *et al.*, 1992; Lai *et al.*, 1993; Mermoud *et al.*, 1992).

How does low temperature bring about the inactivation of PP2A? Although much information is available on how this versatile phosphatase participates in the regulation of a variety of cellular functions (including signal transduction, metabolism, cell division, and gene expression (Cohen *et al.*, 1990; Turowski *et al.*, 1995)), the precise mechanism of the regulation of its activity is presently unclear. The possible mechanisms include differential gene expression (Cohen *et al.*, 1990), post-translational modifications (Chen *et al.*, 1992; Guo and Damuni, 1993; Turowski *et al.*, 1995), differential association with regulatory subunits (Hubbard and Cohen, 1993; McCright and Virshup, 1995), and interaction with protein inhibitors (Li *et al.*, 1995). The results of the present study appear to rule out a cold-induced modulation of the mRNA, or protein levels of PP2Ac. However, the PP2Ac from cold acclimated and non-acclimated cells shows similar immunoreactivity in the denatured state, but different in the native state, this reactivity being higher in PP2Ac from the cold acclimated cells. This suggests a cold-induced demasking of the PP2Ac epitope. How does cold bring about the demasking of the PP2Ac epitope? Methylation of mammalian PP2Ac has been shown to decrease its reactivity to antibodies through epitope masking (Turowski *et al.*, 1995). The antibodies used in the latter and the present studies were raised against homologous peptides. However, since our immunoblot analysis of SDS-denatured proteins (Figure 4) revealed no significant change in antibody-binding with cold acclimation, methylation appears to be unlikely to play a role in cold-induced demasking of PP2Ac epitope. The most likely cause of demasking of epitope is a cold-induced change in the

interaction of PP2Ac with other proteins. Although a cold-induced dissociation of PP2Ac from other subunits might demask its epitope, it would not account for the observed decline in PP2A activity because PP2Ac is active when not associated with another protein (Chen *et al.*, 1992), although its substrate specificity may change. Yet another possibility is that epitope demasking represents a reassociation of PP2Ac with different subunits at low temperature. In many organisms, including plants, PP2A is a heterotrimer, consisting of a conserved dimer of a catalytic and core regulatory subunit, and a variable member of a family of regulatory subunits (Cohen *et al.*, 1990) which are believed to confer spatial and substrate specificity on PP2A (McCright and Virshup, 1995; McCright *et al.*, 1996). Furthermore, protein phosphatases, as well as kinases, have recently been found to reversibly associate with scaffold (Klauck *et al.*, 1996) or anchoring (Coghlan *et al.*, 1995) proteins. Whether such an association alters their activity is unclear. Also, protein inhibitors with high specificity for PP2A have recently been isolated (Li *et al.*, 1995). Thus, any of these protein-protein interactions could result in demasking of the PP2Ac epitope. However, our examination of the radiolabeled proteins complexed with PP2Ac obtained by immunoprecipitation has not revealed any protein showing cold-specific presence in the complex (data not presented). It appears, therefore, that further understanding of the cold-regulation of PP2A activity would require the probes for regulatory subunits and must await the isolation of homologues of the regulatory subunits from alfalfa.

An intriguing observation made in the present study is that the down-regulation of PP2A activity by cold may be mediated by the cold-triggered calcium influx. Since divalent cations need to be chelated in order to measure the activity specific to PP1 and PP2A, it is unlikely that calcium directly interacts with PP2A to inactivate it. Therefore, the calcium effect on PP2A activity is possibly through calcium modification of another regulator of signal transduction which then brings about an inhibition of PP2A. It is tempting to propose that a cold-modulated and calcium-dependent protein phosphorylation event mediates PP2A inactivation. Mammalian PP2A activity has been shown to be down-regulated by phosphorylation of PP2Ac (Chen *et al.*, 1992; Guo and Damuni, 1993). The phosphorylatable residues are invariably present in predicted polypeptides of all reported PP2A sequences from plants, suggesting their involvement in PP2A regulation (Smith and Walker, 1996). Our preliminary experiments indicate that the alfalfa PP2Ac is phosphorylated but there is no net change in its phosphorylation levels during the cold-inactivation of PP2A. In addition, recent evidence suggests that phosphorylation of PP2A regulatory subunits can also modulate its activity (McCright *et al.*, 1996). Since protein phosphorylation is extensively modulated during cold acclimation

(Monroy *et al.*, 1993a), the possibility that cold-inactivation of PP2A is mediated by protein phosphorylation deserves to be studied upon availability of cloned PP2A regulatory subunits.

Although the mechanism of PP2A inactivation, and thus the upstream events of low temperature signal transduction, are presently unclear, the consequences of PP2A inactivation for downstream events can be speculated upon, based on its known substrates and intracellular distribution. The core regulatory subunit of PP2A is found in both the cytoplasm and nucleus of mammalian cells, but is more concentrated in the nucleus (Turowski *et al.*, 1995). The cytoplasmic PP2A has been shown to inactivate MAP kinases, receptor kinases, and second messenger-dependent kinases including calcium-dependent kinases (Barnes *et al.*, 1995; Chen *et al.*, 1992; Cohen *et al.*, 1990). Thus, the cytoplasmic PP2A could repress the activity of one or more key kinases which are components of a calcium-dependent pathway (Monroy and Dhindsa, 1995) and/or a central signal integrating pathway (Jonak *et al.*, 1996). Inactivation of PP2A by low temperature could result in increased kinase activity, thus facilitating signal flow to the nucleus. In the nucleus of animal cells, PP2A has been shown to prevent transcription by inactivating transcription factors (Wheat *et al.*, 1994). Although a similar role of PP2A has not been identified in plants, dephosphorylation of nuclear factors has been shown to regulate the expression of plant stress genes (Després *et al.*, 1995). Thus, inactivation of PP2A could promote low temperature signal transduction, either indirectly by de-repressing signal transducing protein kinases, or directly, by regulating the activity of transacting factors.

Experimental procedures

Plant material

Conditions for growth and cold acclimation of alfalfa (*Medicago sativa* ssp. *falcata* cv Anik) cell suspension cultures, protoplast isolation, and analysis of calcium influx were undertaken as described previously (Monroy *et al.*, 1993a; Monroy and Dhindsa, 1995).

Phosphorylase phosphatase assay of protein phosphatase activity

Aliquots (20 ml) of cell suspension cultures, acclimated to 4°C for 0–180 min, were washed by filtration as described (Monroy *et al.*, 1993a), and then frozen in liquid nitrogen and stored at –80°C. Extracts were prepared essentially as described (MacKintosh and Cohen, 1989). Briefly, frozen cells were ground at 2 ml g⁻¹ f.wt. in extraction buffer (50 mM HEPES, pH 7.2, 4 mM EDTA, 25 mM sucrose, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1.5 mM L-cysteine, 0.1% v/v 2-mercaptoethanol), the homogenates mixed with Triton X-100 (2% v/v), shaken at 4°C for 10 min, and cleared by centrifugation at 20 000 g for 15 min. The

resulting supernatants (3–5 mg protein ml⁻¹) were filtered through glasswool and immediately assayed for activity. Protocols for the preparation of [³²P]-phosphorylase and phosphatase assays were as described in the Protein Phosphatase Assay System (Life Technologies, Gaithersburg, MD, USA). Reactions were carried out for 10 min at 25°C in buffer supplemented with 0.2 mM EGTA. Since the bivalent cations required for the activity of other phosphatases were chelated, the activity recorded was that of PP1 and PP2A. To specifically measure PP2A activity, [³²P]-labeled phosphoenolpyruvate carboxylase, prepared according to Duff *et al.* (1995), was used as a substrate for the phosphatase assay because it is dephosphorylated by PP2A, but not by PP1 (Carter *et al.*, 1990). Dephosphorylation was carried out in the presence of chelators of divalent cations to avoid the effects of protein phosphatases PP2B and PP2C (Smith and Walker, 1996). Results of preliminary experiments indicated that protein phosphatase activity was linear as a function of dilution of the cell homogenate in the range of 4–40 µg protein ml⁻¹ reaction volume. Thus, assays were carried out at 125–500-fold dilution of each extract. Under these conditions, maximum dephosphorylation of the substrate never exceeded 25%.

Immunoblotting

Proteins resolved by SDS-PAGE (Laemmli, 1970) were transferred onto PolyScreen (DuPont/NEN, Boston, MA, USA) PVDF membranes. Blots were saturated for 1 h with phosphate-buffered saline (PBS) containing 0.2% Tween-20, and then incubated with antibodies in the same buffer for 30 min at 37°C. The anti-PP2A polyclonal antibodies (Upstate Biotechnology Inc., Lake Placid, NY, USA) were diluted 1:1000. Membranes were washed with PBS containing 0.5% Tween-20 for 30 min at 37°C, and then incubated for 30 min at 37°C with peroxidase-conjugated anti-IgG antibodies (Transduction Laboratories) and antigen-antibody complexes were visualized using the Renaissance chemiluminescence reagent (DuPont/NEN).

Immunoprecipitation

Protein extracts were prepared as described for the phosphorylase phosphatase assay. Aliquots of the protein extracts were either kept in native form or were denatured with 1% SDS followed by incubation for 10 min at 80°C. For immunoprecipitation, an aliquot (150–250 µg) of protein extract was mixed with concentrated PBS to give 1 × PBS, and 2 µg of anti-PP2A antibodies in a total volume of 250 µl. Antibody-antigen complexes were allowed to form for 1 h at 4°C. To capture immunocomplexes, Protein A sepharose was added to give 1.5% suspension, and the mixture was agitated for 30 min at 4°C. Sepharose beads were washed three times at 4°C with PBS. Protein was then released from the beads by boiling for 5 min in SDS-PAGE sample buffer (Laemmli, 1970), followed by centrifugation for 4 min. Equal volumes of the supernatants were subjected to electrophoresis on 10% or 12% denaturing gels.

RNA extraction and RNA gel-blot analysis

Procedures for RNA extraction and RNA gel-blot analysis with *cas15* cDNA and the constitutively expressed *p2.1* cDNA, the latter used as a control, as probes have been described previously (Monroy *et al.*, 1993a, 1993b). Quantitation of autoradiograph signals was obtained by densitometry using a Model SM3 scanner

interfaced with Image Master 1-D software (Pharmacia/PDI Inc., Huntington Station, NY, USA).

Restriction fragment length polymorphism-coupled domain-directed differential display (RC4D) protocol

To minimize manipulations and to maximize amplification of PP2A sequences to be displayed, modifications were introduced to the original RC4D protocol of Fischer *et al.* (1995). First-strand cDNA pools were produced with Superscript II Reverse Transcriptase (Life Technologies) using 25 µg total RNA and the previously described KSXT primer (Monroy and Dhindsa, 1995). Codon sequences corresponding to amino acid residues 86–272 of PP2A (Pirck *et al.*, 1993) were then amplified by AmpliTaq Polymerase (Perkin Elmer, Foster City, CA, USA). The upstream primer, encoding FMGDYVDR, was 5'-TT (T/C)ATGGG (A/T)GA (T/C)TA (T/C)GTNGA (T/C) (A/C)G-3', whereas the downstream primer, complementing the region encoding APNYCYR, was 5'-GGGGTACC (G/T) (G/A)TAACA (G/A)TA (G/A)TTNTGTGC-3'. Amplification was for 25 cycles, consisting of denaturation at 94°C for 30 sec, annealing at 58°C for 90 sec, and extension at 72°C for 90 sec, using 0.2 µM unlabelled upstream primer and 0.2 µM ³²P-labeled downstream primer. For primer labeling, 11.5 pmol [³²P]ATP, and 10 U of T4 polynucleotide kinase for 45 min at 37°C. Unpolymerized primers were removed by Quiaquick spin columns (Qiagen Inc., Chatsworth, CA, USA), and amplicons of the predicted length, 576 bp, were purified by agarose-gel electrophoresis. Equal portions of each recovered amplicon were separately treated with restriction enzyme *Hae*III or *Msp*I in 25 µl of reaction buffer for 1 h at 37°C. For analysis, each sample was mixed with non-denaturing loading buffer, and a 5–10 µl aliquot was subjected to electrophoresis on non-denaturing polyacrylamide gels (8% T, 0.66% C), for 2–2.5 h at 10 V cm⁻¹. For autoradiography, gels were dried without prior fixation, and exposed to BioMAX MR (Eastman Kodak, Rochester, NY, USA) film for 8–16 h.

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