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Phosphorylation by a Cyclin-Dependent Kinase Modulates DNA Binding of the Arabidopsis Heat-Shock Transcription Factor HSF1 in Vitro[1]

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Phosphorylation is one of the mechanisms controlling the activity of heat-shock transcription factors in yeast and mammalian cells. Here we describe partial purification, identification, and characterization of a protein kinase that phosphorylates the Arabidopsis heat-shock factor AtHSF1 at multiple serine residues. The HSF1 kinase forms a stable complex with AtHSF1, which can be detected by kinase pull-down assays using a histidine-tagged AtHSF1 substrate. The HSF1 kinase interacts with the cell-cycle control protein Suc1p and is immunoprecipitated by an antibody specific for the Arabidopsis cyclin-dependent CDC2a kinase. Phosphorylation by CDC2a in vitro inhibits DNA binding of AtHSF1 to the cognate heat-shock elements, suggesting a possible regulatory interaction between heat-shock response and cell-cycle control in plants.

HSPs in plants, as well as in other organisms, play an essential role as molecular chaperones controlling protein folding, assembly, and degradation (Nover, 1991; for review, see Vierling, 1991). Expression of most *HSP* genes is induced by elevated temperatures, as well as by other environmental-stress stimuli (Parsell and Lindquist, 1993). Transcriptional activation of the eukaryotic *HSP* genes is mediated by binding of homotrimeric HSFs to palindromic repeats of HSEs within the *HSP* promoters (for review, see Scharf et al., 1994). HSFs carry conserved DNA-binding and trimerization domains in combination with diverse activation domains. The activity of HSFs is known to be regulated by intramolecular control elements, as well as by interaction with HSP70 (Hoj and Jakobsen, 1994; Scharf et al., 1994; Wu, 1995).

In spite of general structural and functional conservation of HSFs, marked differences exist between molecular mechanisms of HSF activation in budding yeast *(Saccharomyces cerevisiae)* and other eukaryotes (Sorger et al., 1987). In yeast HSF is encoded by a single essential gene and detected in a DNA-bound trimeric form in the nucleus of unstressed cells (Jacobsen and Pelham, 1988). Conformational change and temperature-dependent phosphorylation during heat shock are thought to release the C-terminal transactivation domain from an intramolecular interaction with the heptapeptide domain CE2 that negatively controls HSF activation in budding yeast (Sorger and Pelham, 1988; Jacobsen and Pelham, 1991). In higher eukaryotes, including plants (Scharf et al., 1990; Hübel and Schöffl, 1994), multiple, differentially regulated genes code for HSFs (for review, see Scharf et al., 1994; Wu, 1995), which are detected in association with HSP70 in the cytoplasm of unstressed cells (Abravaya et al., 1992; Baler et al., 1992). Mutations and experimental conditions reducing the activity or synthesis of HSP70s in mammals and plants result in the activation of HSFs, leading to constitutive *HSP* gene expression and acquired thermotolerance (Anderson et al., 1989; Mosser et al., 1993; Rabindran et al., 1994; Lee et al., 1995; Lee and Schöffl, 1996). These data support the model that HSP70 may feedback-regulate the induction of *HSP* gene expression by playing the role of cytoplasmic chaperon, regulating the heat-induced conformational change of HSFs required for their nuclear transport and trimerization (Craig and Gross, 1991; Westwood and Wu, 1993; Larson et al., 1995).

Transcriptional activation is mediated by DNA binding and phosphorylation of HSFs, which is unaffected by HSP70 (Hensold et al., 1990; Rabindran et al., 1994). Heat-shock-induced phosphorylation in budding yeast is reported to enhance the interaction of DNA-bound HSF with the transcriptional apparatus (Sorger and Pelham, 1988), whereas phosphorylation assists the deactivation of HSF in *Kluyveromyces lactis* (Hoj and Jakobsen, 1994). Human HSF1 is phosphorylated as an inactive monomer (Baler et al., 1993; Sarge et al., 1993) and activated by heat-shock-induced hyperphosphorylation following DNA binding (Baler et al., 1993; Westwood and Wu, 1993; Cotto et al.,

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Abbreviations: CKII, casein kinase II; DNA-PK, DNA-dependent protein kinase; HSE, heat-shock response element; HSF, heat-shock transcription factor; HSP, heat-shock protein; NTA, nitrilotriacetic acid.

1996). The fact that thermotolerant cells with altered HSP70 levels display defective regulation of stress-activated protein kinases (Zanke et al., 1996) suggests that HSF activation is linked to stress-signaling pathways controlling cell cycle and apoptosis (Adler et al., 1995; Cosulich and Clarke, 1996). A major target of these stress-activated protein kinase pathways is a DNA-PK, which is activated by interaction with HSF (Peterson et al., 1995). In fission yeast and mammals DNA-PK controls the DNA damage and S-phase checkpoints of the cell cycle by ultimately modulating the activity of cyclin-dependent cell-cycle kinases (McConnel and Dynan, 1996; Stewart and Enoch, 1996). The data described here indicate that further regulatory interactions may exist between cell-cycle control and HSF1 activation. By partial purification from *Arabidopsis thaliana* cell suspensions, we have identified an HSF1 kinase as cyclin-dependent protein kinase CDC2a, which phosphorylates HSF1 at multiple Ser residues thereby reducing its DNA-binding activity to cognate HSEs in vitro.

MATERIALS AND METHODS

Partial Purification of an HSF1 Kinase from an Arabidopsis Cell Suspension

A root-derived cell-suspension culture of *Arabidopsis thaliana* ecotype Col-1 was maintained in Murashige and Skoog medium containing 1 mg/L 2,4-D (Mathur et al., 1995) and was subcultured weekly by diluting 1 volume of culture with 4 volumes of fresh medium. To prepare protein extracts, Arabidopsis cells were collected 3 d after subculturing and grinding with quartz sand in a homogenization buffer (25 mM Tris-HCl [pH 7.6]; 15 mM $MgCl_2$; 15 mM EGTA; 75 mM NaCl; 5 mM *p*-nitrophenylphosphate; 60 mM β-glycerophosphate; 1 mM DTT; 0.1% Nonidet P-40; 0.1 mM Na_3VO_4; 1 mM NaF; 1 mM PMSF; 0.1 mM benzamidine; 50 μg/mL *N*-tosyl-L-Phe chloromethyl ketone; 10 μg/mL leupeptin, aprotinin, and soybean trypsin inhibitor; and 5 μg/mL antipain, chymostatin, and pepstatin). The crude extract was centrifuged at 40,000*g* for 1 h at 0°C, and then the supernatant was sedimented at 200,000*g* for 1 h. The extract was loaded onto a 25-mL DEAE-Sepharose Fast Flow column (Pharmacia) equilibrated with buffer D (20 mM Tris-HCl [pH 7.8], 5 mM $MgCl_2$, 5 mM EGTA, 75 mM NaCl, 5 mM β-glycerophosphate, 1 mM DTT, 0.01% Nonidet P-40, 0.1 mM Na_3VO_4, 1 mM NaF, 0.25 mM PMSF, 0.1 mM benzamidine, and 1 μg/mL leupeptin and aprotinin). Bound proteins were stepwise eluted from the column with buffer D containing 150, 300, and 600 mM NaCl.

The flow-through fraction, containing unbound proteins, was concentrated by the addition of solid ammonium sulfate to 60% saturation and loaded onto a Sephacryl S-300 sizing column (180 mL, Pharmacia) equilibrated in buffer S (20 mM Tris-HCl [pH 7.8], 2 mM $MgCl_2$, 2 mM EGTA, 100 mM NaCl, 5 mM β-glycerophosphate, 0.5 mM DTT, 0.1 mM Na_3VO_4, 1 mM NaF, 10% glycerol, and 1 μg/mL leupeptin and aprotinin). The Sephacryl S-300 column was calibrated using thyroglobulin (669 kD), ferritin (440 kD), aldolase (158 kD), transferrin (79.5 kD), BSA (66 kD), ovalbumin (44 kD), and soybean trypsin inhibitor (20.1 kD). Chromatography fractions were subjected to protein kinase assays, using the Arabidopsis HSF1 protein (AtHSF1) as the substrate, as well as to immunoblotting with anti-PSTAIRE, anti-CDC2a, and anti-CDC2b antibodies as described below. Starting from 120 mg of total proteins in the cleared cell extract the chromatography steps resulted in a 25-fold purification of the AtHSF1 kinase.

HSF1 Kinase Assay of Chromatography Fractions

An N-terminal His-tagged version of the AtHSF1 protein was expressed and purified using the Qiaexpress system (Qiagen, Chatsworth, CA) as described (Hübel and Schöffl, 1994). After purification on Ni^{2+}-Sepharose, the AtHSF1 protein was dialyzed in a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10% glycerol to eliminate the Ni^{2+} contamination released from the affinity column. Aliquots of the chromatography fractions (25 μL) were combined with 1.5 μg of purified AtHSF1 protein to assay for HSF1 kinase activity. The HSF1 phosphorylation assays were initiated by the addition of ATP, containing 5 μCi of [γ-^{32}P]ATP (Amersham), to a final concentration of 10 μM in a volume of 31 μL. After 20 min of incubation at 24°C, 50 μL of 50% (v/v) Ni^{2+}-Sepharose resin (Ni^{2+}-NTA) was added to quantitatively extract the phosphorylated His-tagged AtHSF1 substrate from the reaction mixture. The Ni^{2+}-NTA resin was washed three times, each with 200 μL of a kinase buffer (50 mM Tris-HCl [pH 7.8], 100 mM NaCl, 15 mM $MgCl_2$, 2 mM EGTA, and 0.5 mM DTT). The AtHSF1 protein was eluted from the resin by 25 μL of 0.3 M imidazole (pH 7.5). After addition of 6 μL of 5× SDS sample buffer, the samples were boiled and fractionated on 10% SDS-polyacrylamide gels, which were subsequently stained with Coomassie brilliant blue G-250, vacuum-dried, and subjected to autoradiography.

Antibodies

Polyclonal antibodies against the EGVPSTAIREISLLKE region and the C-terminal FKDLGGMP polypeptide of Arabidopsis cyclin-dependent CDC2a kinase, as well as against the C-terminal DSLDKSQF polypeptide of CDC2b protein, were raised in rabbits using synthetic polypeptides conjugated to keyhole limpet hemocyanin as immunogens. IgG fractions were prepared from the crude serums by ammonium sulfate precipitation, then were further purified on peptide-coupled affinity columns as described (Harlow and Lane, 1988). Affinity-purified polyclonal antibody against subdomain III polypeptide of the α-subunit of human casein kinase II was obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibody recognizing the MRSGHHHHHH peptide sequence was purchased from Qiagen.

Immunoprecipitation and p13^{suc1}-Sepharose Binding of HSF1 Kinase

One-hundred-microliter aliquots from the HSF1 kinase fractions were precleared for 30 min with 50 μL of 25% (v/v) suspension of protein A-Sepharose (Pharmacia)

equilibrated in a bead buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.1% Nonidet P-40, 0.1 mM benzamidine, and 10 μg/mL leupeptin and aprotinin) at 4°C. The matrix was removed by centrifugation, and the supernatant was transferred to another Eppendorf tube containing 3 μg of anti-CDC2a antibody. The mixture was incubated for 2 h at 4°C. Following the addition of 50 μL of 25% (v/v) suspension of protein A-Sepharose, the mixture was further incubated at 4°C for 1 h with gentle agitation. Subsequently, the protein A-Sepharose bead was washed three times each with 0.5 mL of the bead buffer and once with 0.5 mL of the kinase buffer (see above). The bead-precipitated immunocomplexes were tested for protein kinase activity by adding AtHSF1 substrate and [γ-^{32}P]ATP, as described above. To assay specific binding of the HSF1 protein kinase fractions to p13^{suc1}-Sepharose, the fission yeast Suc1p protein was expressed in bacteria, purified, and coupled to cyanogen bromide-activated Sepharose CL-4B (Pharmacia) at a concentration of 5 mg/mL as described (Meijer et al., 1989). Mock-coupled and BSA-coupled beads were prepared similarly and used as control matrices for kinase binding. One-hundred-microliter aliquots of the HSF1 kinase fractions were mixed with 60 μL of 25% (v/v) p13^{suc1}-Sepharose beads and incubated for 1 h at 4°C to allow binding. The p13^{suc1}-Sepharose beads were washed three times each with 0.5 mL of the bead buffer, then once with 0.5 mL of the kinase buffer, and the HSF1-bound kinase activity was tested as described above.

HSF1 Kinase Pull-Down Assays

Purified AtHSF1 was bound to the Ni^{2+}-NTA matrix at a concentration of 3 mg/mL. The HSF1 beads were pelleted and washed twice with 50 mM phosphate buffer (pH 7.0) containing 750 mM NaCl to remove unbound AtHSF1. Aliquots of HSF1 kinase fractions (100 μL) were added to 25 μL of HSF1-Ni^{2+}-NTA affinity resin and incubated for 1 h at 4°C. The beads were washed three times with 400 μL of a binding buffer (20 mM Tris-HCl [pH 7.8], 100 mM NaCl, 5 mM β-glycerophosphate, 5 mM $MgCl_2$, 1 mM NaF, 10% glycerol, 0.01% Nonidet P-40, and 5 μg/mL leupeptin, aprotinin, pepstatin, and chymostatin). Proteins binding to the HSF1 affinity matrix were eluted by boiling the beads with an SDS sample buffer. Aliquots of eluted proteins were resolved on 10% SDS-polyacrylamide gel and transferred onto PVDF membrane (Immobilon-P, Millipore), which was subsequently probed with anti-PSTAIRE, anti-CDC2a, and anti-CDC2b antibodies using enhanced chemiluminescence detection (ECL system, Amersham).

Tryptic Peptide Mapping and Phosphoamino Acid Analysis

Tryptic peptide mapping of phosphorylated AtHSF1 and analysis of phosphorylated amino acids were performed according to standard protocols (Boyle et al., 1991), except that phosphoamino acids were resolved by one-dimensional electrophoresis as described (Jelinek and Weber, 1993).

Electrophoretic Mobility-Shift Assay of HSF1 DNA Binding

Oligonucleotide probes carrying either consensus HSEs or mutated HSE sequences were annealed overnight at 25°C as described (Hübel and Schöffl, 1994). Double-stranded oligonucleotides were purified from 8% polyacrylamide gels and 5′ end-labeled with [γ-^{32}P]ATP using T4-polynucleotid kinase (Boehringer Mannheim). To prepare phosphorylated AtHSF1, 0, 5, 10, and 20 μg of proteins from the peak kinase fraction of 80 kD were mixed with streptavidin magnetic beads (Promega) coated with biotinylated p13^{suc1} protein. Biotinylation of p13^{suc1} was performed as described (Jerry, 1993). The beads were then divided in half. To one portion 0.2 μg of AtHSF1 and ATP (a final concentration of 100 μM) was added, to the second portion the same amount of AtHSF1 was added, but ATP was omitted. Following the kinase reaction the beads were pelleted and the bound proteins were eluted with SDS sample buffer, fractionated on 12% SDS-polyacrylamide gel, and subjected to western blotting with anti-CDC2a antibody. One-half of the AtHSF1 samples were separated by electrophoresis on a 10% SDS-polyacrylamide gel and analyzed by western blotting with monoclonal anti-His_6 antibody. The other half of the AtHSF samples were tested for DNA binding. DNA-binding assays were performed in 15 μL of a binding buffer (25 mM Tris-HCl [pH 7.8], 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 10% glycerol) that contained 2 ng (22,000 cpm) of ^{32}P-labeled HSE oligonucleotide probe and 0.75 μg of polydI·dC as a nonspecific competitor DNA. Following 20 min of binding at room temperature, the reaction mixes were loaded onto a 3.5% native polyacrylamide gel, separated by electrophoresis in 0.5× Tris-borate-EDTA buffer at 250 V for 3 h, dried, and subjected to autoradiography as described (Foster et al., 1994). Retardation gels were analyzed using the Strom PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and HSF:HSE bands were quantitated with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Partial Purification of an HSF1 Kinase from Arabidopsis Cell Suspension

Expression of AtHSF1 was recently demonstrated to result in constitutive activation of *HSP70* gene expression in *Drosophila* spp. and human cells (Hübel et al., 1995). The fact that Arabidopsis HSF1 escaped deactivation by phosphorylation in animal cells raised the question whether protein kinases recognizing HSF1 as a substrate exist at all in plant cells. To search for protein kinases phosphorylating HSF1 in Arabidopsis, a biochemical approach was developed. Retaining its DNA-binding activity, the Arabidopsis HSF1 protein (AtHSF1) was fused to an N-terminal His tag using the Qiaexpress system. The His-tagged AtHSF1 protein was expressed in *Escherichia coli* and purified to homogeneity. Immunoblotting with anti-HSF1 antibodies indicated that the intact AtHSF1 protein (66 kD) co-purified on the Ni^{2+}-NTA matrix with a C-terminally truncated form of AtHSF1 (45 kD), representing a minor

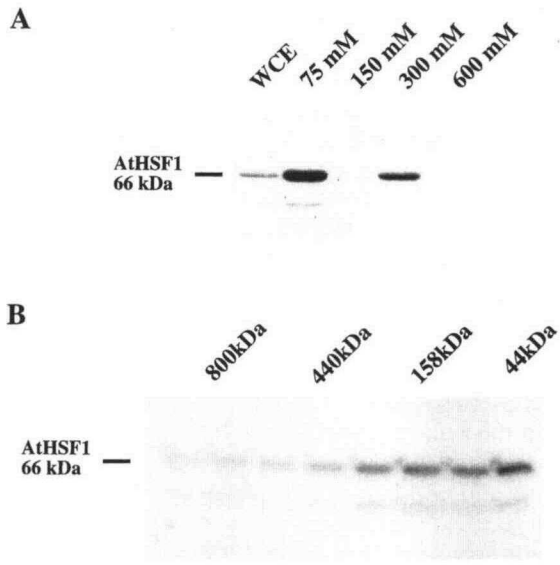

Figure 1. Chromatographic fractionation and detection of HSF1 kinase. A, Arabidopsis whole-cell extract (WCE) was fractionated on a DEAE anion-exchange column. Bound proteins were stepwise eluted with increasing concentrations of NaCl in buffer D. Aliquots of the column fractions were tested in protein kinase assays using the AtHSF1 protein as a substrate. Kinase reaction mixtures were resolved on a 10% SDS-polyacrylamide gel, and the phosphorylated AtHSF1 protein was detected by autoradiography. B, The active HSF1 kinase fraction, eluted by 75 mM NaCl from the DEAE anion-exchange column, was further purified on a Sephacryl S-300 gel-filtration column. Aliquots from every fourth fraction were tested in protein kinase assays, as described above. Elution positions of molecular mass marker proteins used to calibrate the column are shown at the top.

contaminant. This His-tagged AtHSF1 protein was used as the substrate in phosphorylation assays, with chromatographic fractions of proteins extracted from a quick-cycling suspension culture of dividing Arabidopsis cells.

Following preparation of a cleared cell lysate, the crude protein extract was first resolved on a DEAE-Sepharose anion-exchange column. Protein fractions eluted stepwise from the DEAE column using NaCl were assayed for HSF1 kinase activity. Following phosphorylation with [γ-^{32}P]ATP, the His-tagged AtHSF1 substrate was recovered from the reaction mixtures by adsorption onto Ni^{2+}-Sepharose beads, and resolved by electrophoresis on an SDS-polyacrylamide gel to monitor AtHSF1 phosphorylation by autoradiography (Fig. 1A) The bulk (90%) of HSF1 kinase activity was detected in the protein fraction eluted by 75 mM NaCl, but a minor amount (5%) of AtHSF1 kinase activity was also found in the fraction eluted by 300 mM NaCl. Proteins from the 75 mM NaCl fraction were concentrated by ammonium sulfate (60%), then subjected to size-separation by Sephacryl S-300 gel-filtration chromatography (see "Materials and Methods"). Every fourth fraction from the Sephacryl S-300 column was assayed for HSF1 kinase activity. High HSF1 kinase activities were detected in the Sephacryl S-300 chromatography fractions containing proteins between 150 and 50 kD. The peak of HSF1 kinase activity (Fig. 1B, lane 8) was found in a fraction corresponding to a mean molecular mass of 80 kD.

AtHSF1 Phosphorylation Occurs on Multiple Ser Residues

To identify amino acid residue(s) modified by the HSF1 kinase, the in vitro-phosphorylated AtHSF1 protein was hydrolyzed by HCl, and the released amino acids were resolved by thin-layer electrophoresis followed by autoradiography. Comparison of the mobility of ^{32}P-labeled amino acids to phosphoamino acid standards showed that AtHSF1 phosphorylation took place exclusively on Ser residues (Fig. 2A). To determine the number of Ser residues modified by the HSF1 kinase, a tryptic peptide mapping was performed. Two-dimensional electrophoretic separation of the products obtained by trypsin digestion of phosphorylated AtHSF1 revealed two phosphopeptides with a very similar migration property (Fig. 2B). The tryptic mapping data thus indicated that AtHSF1 is phosphorylated on at least two Ser residues that are separated by a trypsin cleavage site. Other fractions from the Sephacryl S-300 sizing column (fraction of 120 kD), neighboring the peak fraction of 80 kD of AtHSF1 kinase, resulted in similar phosphorylation of AtHSF1, yielding identical tryptic pep-

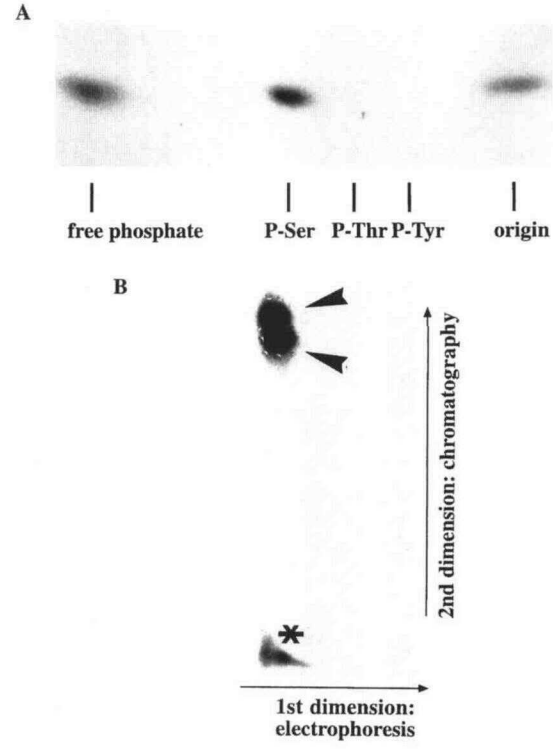

Figure 2. AtHSF1 is phosphorylated in vitro on multiple Ser residues. A, In vitro-phosphorylated AtHSF1 was hydrolyzed in 6 M HCl at 110°C. The released phosphoamino acids were electrophoretically resolved on cellulose-coated thin-layer plates and exposed to x-ray film. B, Phosphorylated AtHSF1 was digested with trypsin, and the resulting phosphopeptides were separated by thin-layer electrophoresis followed by TLC, and visualized by autoradiography. The asterisk indicates the origin of application of the probe, and the arrowheads indicate phosphopeptides.

tide maps (data not shown). The data, therefore, showed that the same AtHSF1-specific protein kinase is present in a broader range of chromatography fractions, suggesting some nonspecific, hydrophobic interaction between the gel-filtration matrix and kinase as shown in Figure 1B.

Identification of the HSF1 Kinase

Inspection of the amino acid sequence of AtHSF1 (Hübel and Schöffl, 1994) pointed to the presence of several potential phosphorylation sites for CKII (at AtHSF amino acid positions 14, 172, 329, 362, 381, 431, 450, and 479) and cyclin-dependent protein kinases (at amino acid positions 73, 321, and 403). To examine whether chromatography fractions of the HSF1 kinase contain the known Arabidopsis CKII, the kinase fractions were subjected to immunoprecipitation with an anti-peptide antibody recognizing the highly conserved N-terminal domain III within the catalytic subunit of CKII (Mizoguchi et al., 1993). Using the anti-CKII antibody no AtHSF1-phosphorylating activity could be detected in the immunoprecipitated AtHSF1 kinase fractions (Fig. 3A, lane 6). Instead, the total HSF1 kinase activity was retained in the supernatant (Fig. 3A, lane 5).

To investigate the possibility that the HSF1 kinase activity was due to a cyclin-dependent protein kinase, similar immunoprecipitation experiments were performed using antibodies recognizing the unique C-terminal domains of Arabidopsis CDC2a and CDC2b kinases. In vitro phosphorylation assays performed with the immunocomplexes revealed that the anti-CDC2a antibody precipitated a protein showing HSF1 kinase activity (Fig. 3A, lane 1), whereas the anti-CDC2b antibody tested in a similar immunoprecipitation reaction did not bind to the AtHSF1-phosphorylating protein kinase (data not shown). Due to the limiting amount of applicable anti-CDC2a antibody in these experiments, a considerable amount of kinase activity remained in the supernatant (Fig. 3A, lane 2). However, this remaining activity could be depleted from the supernatant by a second round of immunoprecipitation (Fig. 3A, lane 3). Together, with the fact that the preimmune serum of anti-CDC2a antibody did not precipitate any detectable AtHSF1 kinase activity (Fig. 3A, lane 4), the data indicated that a protein kinase cross-reacting with the anti-CDC2a antibody was responsible for specific phosphorylation of the AtHSF1 substrate in vitro.

To further characterize the HSF1 kinase, we took advantage of the observations showing that CDC2 kinases can specifically bind to the Suc1p protein (Bourne et al., 1996). The $p13^{suc1}$ protein was expressed in *E. coli*, purified to homogeneity, and coupled covalently to Sepharose beads. Aliquots from the HSF1 kinase peak fraction were incubated with the $p13^{suc1}$-Sepharose matrix, and then the matrix-bound proteins were assayed for HSF1 kinase activity. In fact, as expected for CDC2a kinase, the HSF1 kinase strongly bound to the $p13^{suc1}$-Sepharose matrix (Fig. 3A, lane 9). Only a very low but detectable amount of kinase was found to bind to mock-coupled (Fig. 3A, lane 7) or BSA-coupled (Fig. 3A, lane 8) control beads in the control experiments, demonstrating that the interaction of the

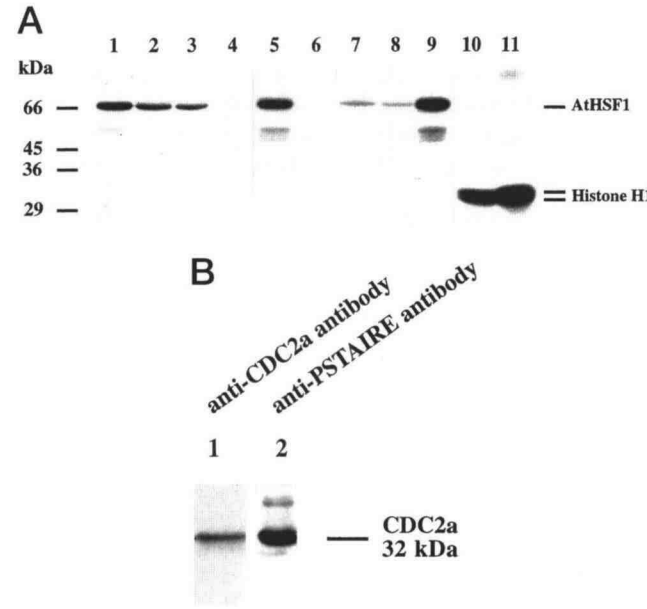

Figure 3. A, The HSF1 kinase was subjected to two successive rounds of immunoprecipitation with anti-CDC2a antibody (lanes 1 and 3) and with the preimmune serum of the same antibody (lane 4).The supernatant of the first immunoprecipitation reaction with anti-CDC2a antibody was analyzed in a kinase assay (lane 2). HSF kinase activity was also tested on immunocomplexes with anti-CKII antibody (lane 6) and in the supernatant of the anti-CKII immunoprecipitation reaction (lane 5). Kinase activity of proteins bound to $p13^{suc1}$-Sepharose beads were similarly assayed with AtHSF1 (lane 9) and compared with the kinase activities bound to control mock-coupled (lane 7) and BSA-coupled (lane 8) beads. The anti-CDC2a antibody precipitated and $p13^{suc1}$-Sepharose-bound kinases were tested in phosphorylation assays using histone H1 as the substrate (lanes 10 and 11, respectively). B, AtHSF1 protein was incubated with the HSF1 kinase fraction before binding to the Ni^{2+}-NTA beads. Proteins were eluted from the beads and immunoblotted with anti-CDC2a (lane 1) and anti-PSTAIRE (lane 2) antibodies.

kinase with $p13^{suc1}$-Sepharose is very specific. Both immunoprecipitated and $p13^{suc1}$-Sepharose-bound kinase fractions displayed histone H1 phosphorylation activity (Fig. 3A, lanes 10 and 11). These data lead to the conclusion that the HSF1 kinase is probably identical to the Arabidopsis CDC2a kinase. To confirm this conclusion, we tested whether the HSF1/CDC2a kinase could be precipitated with the His-tagged AtHSF1 substrate protein. Aliquots from the HSF1 kinase peak fraction were co-incubated with the His-tagged AtHSF1 substrate, which was subsequently bound to the Ni^{2+}-NTA beads. The beads were washed to remove the unbound proteins, and then the AtHSF1-associated proteins were eluted, fractionated on SDS-PAGE, and subjected to western blotting. The immunoblots were probed with an anti-PSTAIRE antibody, which recognized the conserved PSTAIRE motif of CDC2 kinases, as well as with an anti-CDC2a antibody, which detected a C-terminal peptide of Arabidopsis CDC2a kinase (Ferreira et al., 1991; Imajaku et al., 1992). Both anti-PSTAIRE and anti-CDC2a antibodies recognized a protein of 32 kD in agreement with the estimated molecular mass of Arabidop-

sis CDC2a kinase (Ferreira et al., 1991; Imajaku et al., 1992), indicating that AtHSF1 is capable of forming a relatively stable complex with the CDC2a kinase present in the HSF1 kinase fractions (Fig. 3B). Without AtHSF1 protein added to the kinase fraction, there was no detectable kinase on the Ni^{2+}-NTA beads (data not shown).

Phosphorylation Alters DNA-Binding Activity of AtHSF1

The effect of phosphorylation on AtHSF1 binding to cognate HSEs was analyzed by electrophoretic mobility shift assays using phosphorylated and nonphosphorylated AtHSF1 proteins. Increasing amounts of proteins from the Sephacryl S-300 peak kinase fraction of 80 kD were loaded onto $p13^{suc1}$ magnetic beads, and then 0.2 μg of AtHSF1 and ATP (100 μM) was added to one-half of the beads to initiate phosphorylation, whereas ATP was omitted from the control reactions performed with the other half of the beads. After the kinase reaction the beads were pelleted and washed, and one-half of the phosphorylated AtHSF1 protein samples was incubated with ^{32}P-labeled HSE oligonucleotides. The samples were subjected to native-PAGE, using 0.1 μg of untreated AtHSF1 protein incubated with wild-type HSE oligonucleotide (Fig. 4B, lane1) or with a mutant HSE (lane 2) as the controls. Comparison of the amounts of HSE-AtHSF1 complexes detected by the electrophoretic mobility shift assay showed that the kinase reaction in the presence of ATP decreased the DNA-binding activity of AtHSF1 in a kinase-dependent fashion: the more kinase was loaded onto the beads, the greater was the decrease in DNA binding (Fig. 4B, lanes 7–10).

Quantitation of the radioactivity in the retarded bands by a PhosphorImager revealed a maximum 3-fold reduction in HSE binding in comparison with binding activities observed with the same amounts of unphosphorylated AtHSF1 as the controls (Fig. 4C). AtHSF1 protein samples treated with the kinase without ATP showed no significant alteration of DNA-binding activity (Fig. 4B, lanes 3–6). To exclude the possibility that decreasing amounts of HSF1 proteins were present in the binding reactions, the second half of phosphorylated and unphosphorylated AtHSF1 samples was resolved on a polyacrylamide gel and immunoblotted with anti-His-6 antibodies (Fig. 4A, bottom). The result shows that the AtHSF1 protein was not degraded during the kinase reaction, and that comparable amounts of HSF1 were used in each DNA-binding reaction. Finally, the $p13^{suc1}$ beads were tested for the presence of bound-CDC2a kinase after eluting the bound proteins and subjecting them to immunoblotting with anti-CDC2a antibody following electrophoretic separation (Fig. 4A, top). As expected, the amount of CDC2a protein detected on the beads proportionally increased with the amount of fraction that was loaded.

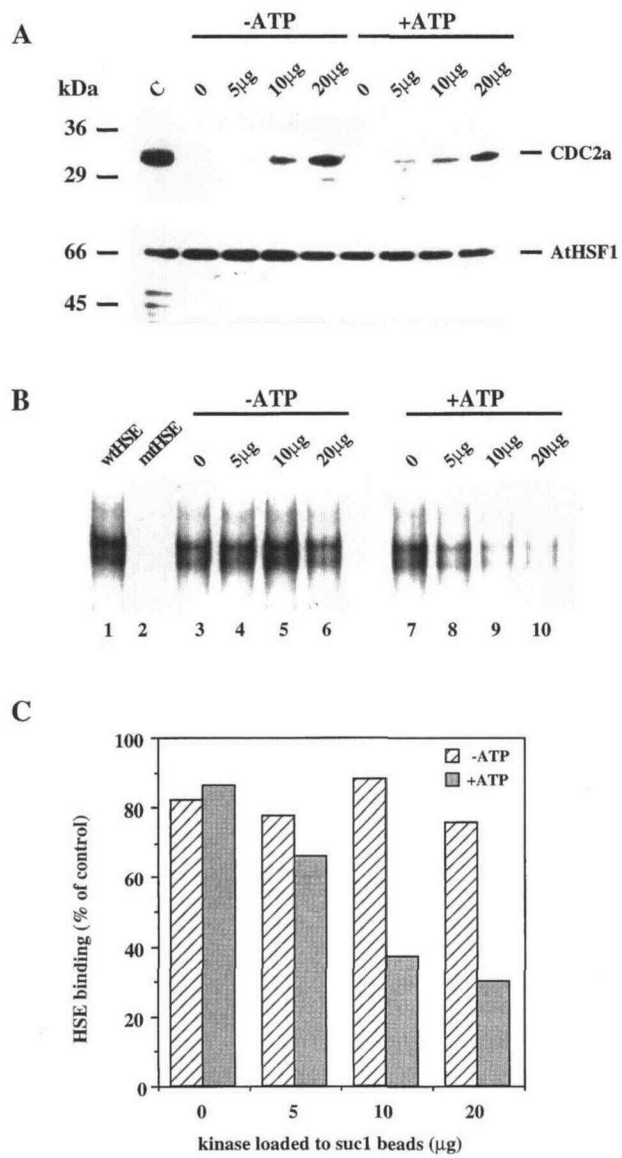

Figure 4. A, Increasing amounts from the Sephacryl S-300 peak kinase fraction of 80 kD were loaded to $p13^{suc1}$-magnetic beads. Proteins bound to the beads were electrophoresed on 12% SDS-polyacrylamide gel and immunoblotted with anti-CDC2a antibody (top). As a control, 25 μg of proteins from the peak fraction was electrophoresed on lane C. The amount of AtHSF1 present in the kinase reactions used for DNA-binding assays with or without ATP was monitored by loading one-half of the reaction mixtures on a 10% SDS polyacrylamide gel and immunoblotting the His-tagged AtHSF1 protein with anti-His-6 antibody (bottom). As a control, 0.1 μg of purified AtHSF1 was electrophoresed on lane C. B, Electrophoretic mobility-shift assays were performed with equivalent amounts of nonphosphorylated (lanes 3–6) or phosphorylated (lanes 7–10) AtHSF1 proteins using ^{32}P-labeled wild-type HSE oligonucleotide probe. As a control for the specificity of DNA binding, assays were done with the same amount of untreated AtHSF1 protein using wild-type HSE (lane 1) and mutant HSE (lane 2) oligonucleotides. The DNA-binding reactions were resolved on a native 3.5% polyacrylamide gel and evaluated by autoradiography. C, Radioactivity in the retardation bands was quantitated by PhosphorImager analysis. Activity in the retardation complexes obtained with the same amount of unphosphorylated AtHSF1 protein was taken as 100%.

DISCUSSION

Our data show that a protein kinase phosphorylating the heat-shock factor AtHSF1 is present in a quick-cycling suspension culture of dividing Arabidopsis cells. The HSF1 kinase was partially purified by ion-exchange and gel-

filtration chromatography, and observed to form a stable complex in pull-down kinase assays with a His-tagged AtHSF1 substrate protein immobilized on nickel-Sepharose. Phosphopeptide mapping indicates that the HSF1 kinase phosphorylates AtHSF1 on at least two different Ser residues. Antibodies raised against the catalytic subunits of Arabidopsis cyclin-dependent kinase CDC2b and CKII do not recognize the HSF1 kinase. In contrast, the HSF1 kinase binds specifically to an immobilized cell-cycle-control protein, Suc1p, and can be immunoprecipitated with an antibody recognizing the C-terminal domain of CDC2a kinase. The fact that immunocomplexes detected by the anti-CDC2 antibody bind and phosphorylate the AtHSF1 protein provides firm evidence that the HSF1 kinase is identical with the Arabidopsis cyclin-dependent protein kinase CDC2a of 32 kD. The activity peak of the HSF1 kinase elutes in gel-filtration chromatography with a peak molecular mass of 80 kD, which is in perfect agreement with the assumption that CDC2a occurs in association with a cyclin subunit of about 50 kD. Phosphorylation of AtHSF1 and/or molecular interactions with the HSF1 kinase result in a significant decrease of AtHSF1 DNA-binding activity to the cognate HSEs in vitro. Whatever the mechanism by which Cdc2a inhibits the DNA-binding activity of the Arabidopsis HSF1, deactivation of AtHSF1 by a cyclin-dependent protein kinase suggests an intriguing regulatory interaction between heat-shock response and cell-cycle control.

Except for DNA-PK, thus far no other protein kinase interacting with HSFs in vitro or in vivo is known in eukaryotes. Binding of HSF1 to DNA-PK in vitro and in the yeast two-hybrid system was observed and shown to be required for DNA-PK activation (Peterson et al., 1995). DNA-PKs are also found in association with other DNA-binding factors, including the Ku70 and Ku86 proteins (for review, see McConnel and Dynan, 1996). The Ku proteins appear to control DNA repair by targeting DNA-PK to DNA breaks induced by thermal radiation and other stress treatments (for review, see Jackson, 1996). Overexpression of Ku70 has been demonstrated to interfere with the *Hsp70* induction (Li, 1995), indicating that the Ku and HSF1 proteins compete for common HSE-binding sites within the *Hsp* promoters, as well as for limiting amounts of DNA-PK. Deficiency of DNA-PK in the *scid* mutant mice causes an extreme sensitivity to heat shock, inducing apoptosis (McConnel and Dynan, 1996). This correlates with the fact that HSF1 competes with Ku for targeting DNA-PK to *Hsp* promoters, where DNA-PK phosphorylates the C-terminal domain of RNA polymerase II, activating the *Hsp* gene expression (O'Brien et al., 1994; Li, 1995).

Data emerging from recent studies of check-point controls of the cell cycle in yeast and mammals demonstrate that stress-activated kinase pathways, including heat-shock signaling, control the activation of DNA-PKs (Cosulich and Clarke, 1996; Stewart and Enoch, 1996). Thus, maintaining the balance between homeostatic HSP synthesis and cell death appears to involve an intricate regulation by HSFs and DNA-PKs. Activation of DNA-PK in dividing cells results in S-phase arrest by inhibition of the activity of cell-cycle kinases in fission yeast and mammals (for review, see Lydall and Weinert, 1996; Stewart and Enoch, 1996). Based on the fact that HSF1 appears to be required for DNA-PK activation, the control of trimerization and nuclear transport of HSF1 probably plays a crucial role in cell-cycle control. Phosphorylation of HSF1 by a cell-cycle kinase in proliferating cells may ensure deactivation of HSF1, also when HSP70 levels are limiting, to avoid cell-cycle arrest. Therefore, the observed in vitro interaction between CDC2a and AtHSF1, as well as deactivation of AtHSF1 DNA-binding by CDC2a-mediated phosphorylation, may logically be inserted in the currently known regulatory pathways. The data described above thus open the way to use the phosphorylation-deficient forms of AtHSF1 to overcome a negative regulation by CDC2a and thereby test the significance of observed regulatory interaction between CDC2a and AtHSF1 in vivo.

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