Plant Physiology, May 1999, Vol. 120, pp. 257–274, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Two SNF1-Related Protein Kinases from Spinach Leaf Phosphorylate and Inactivate 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase, Nitrate Reductase, and Sucrose Phosphate Synthase in Vitro[1]

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We resolved from spinach (*Spinacia oleracea*) leaf extracts four Ca^{2+}-independent protein kinase activities that phosphorylate the AMARAASAAALARRR (AMARA) and HMRSAMSGLHLVKRR (SAMS) peptides, originally designed as specific substrates for mammalian AMP-activated protein kinase and its yeast homolog, SNF1. The two major activities, HRK-A and HRK-C (3-hydroxy-3-methylglutaryl-coenzyme A reductase kinase A and C) were extensively purified and shown to be members of the plant SnRK1 (SNF1-related protein kinase 1) family using the following criteria: (a) They contain 58-kD polypeptides that cross-react with an antibody against a peptide sequence characteristic of the SnRK1 family; (b) they have similar native molecular masses and specificity for peptide substrates to mammalian AMP-activated protein kinase and the cauliflower homolog; (c) they are inactivated by homogeneous protein phosphatases and can be reactivated using the mammalian upstream kinase; and (d) they phosphorylate 3-hydroxy-3-methylglutaryl-coenzyme A reductase from Arabidopsis at the inactivating site, serine (Ser)-577. We propose that HRK-A and HRK-C represent either distinct SnRK1 isoforms or the same catalytic subunit complexed with different regulatory subunits. Both kinases also rapidly phosphorylate nitrate reductase purified from spinach, which is associated with inactivation of the enzyme that is observed only in the presence of 14-3-3 protein, a characteristic of phosphorylation at Ser-543. Both kinases also inactivate spinach sucrose phosphate synthase via phosphorylation at Ser-158. The SNF1-related kinases therefore potentially regulate several major biosynthetic pathways in plants: isoprenoid synthesis, sucrose synthesis, and nitrogen assimilation for the synthesis of amino acids and nucleotides.

Recent studies have defined a subfamily of plant protein kinases that are related to mammalian AMPK and the SNF1 protein kinase from the yeast *Saccharomyces cerevisiae* (for review, see Hardie and Carling, 1997; Halford and Hardie, 1998; Hardie et al., 1998). Mammalian AMPK switches off ATP-consuming anabolic pathways and switches on ATP-producing catabolic pathways by phosphorylating key regulatory enzymes such as HMG-CoA reductase (Corton et al., 1995). AMPK is activated by increased AMP and decreased ATP via a complex mechanism involving allosteric regulation (Corton et al., 1995), promotion of phosphorylation by an upstream protein kinase (AMPKK) (Hawley et al., 1995), and inhibition of dephosphorylation (Davies et al., 1995). Since AMP is elevated under conditions in which ATP is depleted because of the action of adenylate kinase, the kinase cascade is activated in a sensitive manner in response to cellular stresses that cause ATP depletion. We propose that AMPK acts as a "fuel gauge," protecting cells against the effects of environmental or nutritional stresses that deplete ATP (Hardie and Carling, 1997; Hardie et al., 1998).

A 1992 study (MacKintosh et al., 1992) reported that extracts of several plant species contained protein kinase(s) that phosphorylated the HMRSAMSGLHLVKRR (SAMS) peptide, a synthetic peptide designed as a specific substrate for mammalian AMPK (Davies et al., 1989). One of these protein kinases was purified from cauliflower (MacKintosh et al., 1992) and was shown to have properties very similar to those of mammalian AMPK in terms of both specificity for peptide substrates (Weekes et al., 1993; Dale et al., 1995b) and regulation by phosphorylation (MacKintosh et al., 1992). It also phosphorylated and inactivated HMG1, an HMG-CoA reductase from Arabidopsis, at a site (Ser-577) equivalent to that at which AMPK phosphorylated mam-

[1] This work was supported by a project grant from the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom, by research studentships from the BBSRC to P.G.D. and C.S., and by grant-aided support from the BBSRC to IACR (N.G.H.).

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Abbreviations: AMPK, AMP-activated protein kinase; AMPKK, AMPK kinase; HMG, 3-hydroxy-3-methylglutaryl; HRK, HMG-CoA reductase kinase; NR, nitrate reductase; PP2A, protein phosphatase-2A; PP2C, protein phosphatase-2C; SPS, Suc phosphate synthase; TFA, trifluoroacetic acid.

malian HMG-CoA reductase (Dale et al., 1995a). The one feature that was different between the plant and animal kinases was that the former was not activated by AMP, so that we could not adopt the name AMPK. Since HMG-CoA reductase was likely to be a physiological substrate, we tentatively named it HRK (HMG-CoA reductase kinase). It was subsequently termed HRK-A to distinguish it from a distinct form, HRK-B, that was purified from cauliflower and had a similar substrate specificity but a lower native molecular mass (Ball et al., 1994).

The *Saccharomyces cerevisiae SNF1* gene (also known as *CAT1* or *CCR1*) was identified via mutants that would not grow on carbon sources other than Glc, such as Suc or Gal. Genes required for growth on these carbon sources are repressed by Glc, and a functional *SNF1* gene is required for derepression (Gancedo, 1998). *SNF1* encodes a protein kinase (Snf1p) (Celenza and Carlson, 1986) that is closely related to the catalytic subunit of AMPK (Carling et al., 1994; Mitchelhill et al., 1994), and the two accessory subunits associated with these catalytic subunits in mammals and yeast are also closely related (Hardie et al., 1998). The yeast SNF1 kinase complex is rapidly and dramatically activated by phosphorylation in response to Glc deprivation, and this is associated with large increases in the cellular AMP:ATP ratio (Wilson et al., 1996). Therefore, there are obvious analogies between the roles of the AMPK and SNF1 systems, although, like cauliflower HRK-A, the SNF1 complex is not directly activated by AMP.

In 1991 a DNA encoding an Snf1 homolog was cloned from rye (Alderson et al., 1991), and homologs were subsequently cloned from barley (Halford et al., 1992; Hannappel et al., 1995), Arabidopsis (Le Guen et al., 1992), tobacco (Muranaka et al., 1994), and potato (Man et al., 1997). These higher plant kinases are now termed the SnRK1 (SNF1-related protein kinase 1) subfamily to distinguish them from other plant kinases somewhat more distantly related to Snf1 (Halford and Hardie, 1998). DNAs encoding rye (Alderson et al., 1991) or tobacco (Muranaka et al., 1994) SnRK1 complemented *snf1* mutations in *S. cerevisiae*. Plant SnRK1 DNAs predict protein products of approximately 58 kD, and it was shown that the catalytic subunit of cauliflower HRK-A was a 58-kD polypeptide that cross-reacted with antibodies raised against a sequence, the PFDDDNIPNLFKKIK (NIP) peptide, which is conserved in the SnRK1 family (Ball et al., 1994, 1995). Evidence was also obtained that the barley *SnRK1* gene (*BKIN12*) encoded a protein kinase with a similar specificity to cauliflower HRK-A (Barker et al., 1996). Recently, expression of potato *SnRK1* DNA in the antisense orientation has been shown to dramatically decrease expression of Suc synthase mRNA in tubers and to abolish the induction of Suc synthase mRNA by Suc in leaves (Purcell et al., 1998). Since Suc synthase catalyzes a primary step in Suc catabolism in these tissues, this reinforces the idea that one function of SnRK1 kinases is to promote catabolic pathways, in this case via changes in gene expression.

Our biochemical characterization of the SnRK1 kinases in plants was originally performed using cauliflower inflorescences (MacKintosh et al., 1992; Ball et al., 1994, 1995; Dale et al., 1995a, 1995b), an abundant source for kinase purification. We have now switched our attention to spinach (*Spinacia oleracea*) leaves, partly because this system is more amenable to physiological studies, but also because there have been more extensive biochemical and metabolic studies using this system. We were particularly interested in the possibility that Ca^{2+}-independent protein kinases resolved from spinach leaf extracts by anion-exchange chromatography, which regulate SPS (McMichael et al., 1995a) or NR (Douglas et al., 1997), might be members of the SnRK1 family. This seemed possible because the sequences around the regulatory sites on both SPS (McMichael et al., 1993) and NR (Douglas et al., 1995; Bachmann et al., 1996b; Su et al., 1996) matched the recognition motif that we had previously established for cauliflower HRK-A (Dale et al., 1995b).

McMichael et al. (1995a) resolved three kinase activities, of which the third (peak III) was Ca^{2+} independent. Peak III inactivated SPS and had a native molecular mass (150 kD) similar to HRK-A but was not purified beyond the initial column and remained poorly characterized. Douglas et al. (1997) also resolved three kinase peaks of which the third (PK_{III}) was Ca^{2+} independent. PK_{III} appeared similar to HRK-A in that it phosphorylated the SAMS peptide and Arabidopsis HMG-CoA reductase, was inactivated by protein phosphatases, had a similar specificity with synthetic peptides, and had a similar native molecular mass (140 kD). Fractions containing PK_{III} also contained an activity that inactivated NR in an ATP- and 14-3-3 protein-dependent manner and a 58-kD polypeptide that cross-reacted with an antibody raised against a fusion between maltose-binding protein and a rye SnRK1 (MBP-RKIN1). However, the latter result was not conclusive proof that PK_{III} was an snRK1 kinase, because the 58-kD polypeptide did not comigrate exactly with the peptide kinase activity.

Although these results suggested that PK_{III} (Douglas et al., 1997) and/or peak III (McMichael et al., 1995a) might be members of the SnRK1 family, several important questions remained: Was peak III (McMichael et al., 1995a) the same entity as PK_{III} (Douglas et al., 1997)? Although there were certain similarities, peak III was reported not to regulate NR, whereas PK_{III} had not been tested on SPS. Were peak III and/or PK_{III} members of the SnRK1 kinase family? Were the NR kinase and the peptide kinase in the PK_{III} fractions the same molecular entity?

In this paper we have re-examined these questions. We report that spinach leaf contains at least four Ca^{2+}-independent protein kinases that phosphorylate synthetic peptides designed as substrates for AMPK/SNF1-related protein kinases, that at least two of these (HRK-A and -C) are indeed members of the SnRK1 family, and that HRK-A and HRK-C can phosphorylate and inactivate HMG-CoA reductase, NR, and SPS in vitro. HRK-C may correspond to peak III (McMichael et al., 1995a) and PK_{III} (Douglas et al., 1997), but it has now been more extensively purified and characterized. HRK-B, HRK-C, and HRK-D appear to represent previously undescribed entities.

MATERIALS AND METHODS

Materials

Spinach (*Spinacia oleracea* var Medina) was grown under greenhouse conditions at IACR-Long Ashton. The leaves were harvested after 30 d, frozen in dry ice, and stored at −20°C. AMP-agarose (catalog no. A3019), benzamidine hydrochloride, Brij-35, DTT, Glc-6-P, Fru-6-P, PMSF, PVPP, staurosporine, Tween 20, and UDP-Glc were from Sigma. Glc-6-P was also obtained from Boehringer Mannheim. [γ-^{32}P]ATP, Hyperfilm-MP, and Hyperfilm-ECL were from Amersham. Okadaic acid and Miracloth were from Calbiochem. ATP and trypsin (sequencing grade) were from Boehringer Mannheim. Chromatography columns and packings were from Pharmacia Biotech. ATP-γ-Sepharose (Haystead et al., 1993) was a generous gift from Dr. Tim Haystead (University of Virginia, Charlottesville). Vivaspin concentrators were from Vivascience (Binbrook, UK). Other reagents were from Merck-BDH (Poole, UK) and were of analytical grade.

We purified commercial samples of Glc-6-P (supplied as disodium salts) by dissolving them in water and passing them through a column containing Chelex 100 (2.5 mL, Na^+ form) layered on top of the ion-exchange resin AG50 (1 mL, H^+ form). Fractions were collected in preweighed tubes and freeze-dried. The weighed Glc-6-P (now in the free acid form) was dissolved in water and neutralized with NaOH to make a 500 mM stock solution.

Peptides, Proteins, and Antibodies

The AMARAASAAALARRR (AMARA), HMRSAMSGLHLVKRR (SAMS), and SP (KGRJRRISSVEJ, J = norleucine) peptides and variants of AMARA were synthesized as described previously (Dale et al., 1995b; Douglas et al., 1997). The GRMRRISSVEMMDNWANTFK (GRM) peptide corresponding to residues 151 to 170 in spinach SPS was synthesized by the Peptide Synthesis Facility (Bristol University, UK). The catalytic domain of Arabidopsis HMG-CoA reductase (HMG1cd) was expressed and purified from *Escherichia coli* (Dale et al., 1995a). 14-3-3 protein was the *BMH2* gene product from *Saccharomyces cerevisiae* expressed in *E. coli* (Moorhead et al., 1996). Cauliflower HRK-A was purified as described previously (Ball et al., 1994). PP2C (protein phosphatase 2Cα from human) and PP2A (protein phosphatase 2A from bovine heart, catalytic subunit) were purified as described previously (Davies et al., 1995). AMPKK was purified from rat liver (Hawley et al., 1996). The anti-NIP antibody was described previously (Ball et al., 1995). A rabbit antibody recognizing spinach SPS that is not phosphorylated at Ser-158 was a gift from Hendrik Weiner (Heidelberg University, Germany). This antibody was raised against the peptide CGRMRRISSVEMMDN, corresponding to residues 151 to 164 in spinach SPS (Weiner, 1995). The second antibody used was affinity-purified goat anti-rabbit IgG (conjugated with horseradish peroxidase) from Bio-Rad. Markers for SDS-PAGE were Prosieve protein markers (FMC BioProducts, Rockland, ME).

Enzyme Assays

Peptide kinase assays were conducted as described previously (Davies et al., 1989) except that AMP was omitted and, unless stated otherwise, 200 μM AMARA peptide was used in place of SAMS. One unit of peptide kinase is the amount that incorporates 1 nmol of phosphate into the peptide in 1 min at 30°C. AMPKK (Hawley et al., 1996) and PP2A and PP2C (Cohen et al., 1988; McGowan and Cohen, 1988) were assayed as described previously. NR was assayed as described by Mackintosh et al. (1995) except that 7.5 μM phenazine methosulfate was included in the zinc acetate stopping solution. One unit of NR is the amount that produces 1 μmol of nitrite in 1 min at 30°C. SPS was assayed as the Fru-6-P and UDP-Glc-dependent production of Suc and Suc-P (Huber and Huber, 1991). It was routinely assayed under "nonlimiting" conditions (10 mM Fru-6-P and 40 mM Glc-6-P), but to study the effects of phosphorylation it was also assayed under "limiting" conditions (3 mM Fru-6-P, 12 mM Glc-6-P, and 10 mM Pi). One unit of SPS produces 1 μmol of Suc-P in 1 min at 30°C under nonlimiting conditions. To study the effects of phosphorylation, the activity was expressed as an activity ratio: the activity under limiting conditions divided by the activity under nonlimiting conditions. Results are expressed as means ± SE of this ratio from triplicate assays.

Purification of Peptide Kinases from Spinach Leaf

The petioles were trimmed off and the spinach leaves (600 g) were homogenized in a kitchen blender with 900 mL of ice-cold homogenization buffer (0.25 M mannitol, 100 mM Tris-HCl, pH 8.2, at 4°C, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, and 0.1 mM PMSF). All remaining steps were performed at 4°C. The homogenate was centrifuged at 10,000*g* for 10 min and the supernatant was passed through two layers of cheesecloth. The extract was made 11% (w/v) with respect to PEG 6000 and was stirred gently for 20 min. Precipitated protein was collected by centrifugation at 22,000*g* for 25 min, and the pellet was resuspended in a minimal volume of buffer A (50 mM Tris-HCl, pH 7.8, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 0.02% [v/v] Brij-35, and 10% [v/v] glycerol). The volume was adjusted to 150 mL with buffer A, and the suspension was clarified by centrifugation at 15,000*g* for 15 min.

The supernatant was stirred with an equal volume of DEAE-Sepharose slurry equilibrated in buffer A, and the slurry was packed into a column. The column was washed with buffer A until the A_{280} of the eluate was <0.15, and kinases were eluted using 300 mL of buffer A plus 0.25 M NaCl. The eluate was made 12% (w/v) with respect to PEG 6000 and stirred for 20 min, and the precipitated protein was collected by centrifugation at 22,000*g* for 20 min. The pellet was resuspended in 30 mL of buffer B (buffer A at pH 7.5) and clarified by centrifugation at 10,000*g* for 5 min. The supernatant was loaded at 3 mL/min onto a 20-mL Q-Sepharose HiLoad 16/10 column (equilibrated in buffer B) at 3 mL/min. The column was washed with buffer B until the A_{280} had returned to baseline, and then kinases

were eluted using a 180-mL gradient from 0 to 0.4 M NaCl in buffer B.

Two peaks of AMARA kinase activity were resolved: These were pooled and mixed together, and protein was precipitated by adding $(NH_4)_2SO_4$ to 50% saturation, stirring for 20 min, and centrifuging at 22,000*g* for 20 min. The pellet was resuspended in 5 mL of buffer C (50 mM Na Hepes, pH 8.0, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 0.02% [w/v] Brij-35, and 10% [v/v] glycerol) and was dialyzed overnight against three changes of buffer C. The volume was made up to 20 mL, and the suspension was clarified by centrifugation at 10,000*g* for 5 min. The supernatant was applied to a 1-mL Mono-Q HR 5/5 column equilibrated in buffer C at 1 mL/min. The column was eluted using a 33-mL gradient from 0 to 0.4 M NaCl in buffer C (Fig. 1).

Three peaks of AMARA kinase activity (I, II, and III) were pooled separately, concentrated to approximately 500 μL in a Vivaspin-30 (Vivascience, Lincoln, UK), frozen in liquid N_2, and stored at −80°C. For further purification, peaks I, II, and III were diluted to 1 mL with buffer D (buffer C at pH 7.4 and without NaF) and applied individually to 1-mL Mono-Q HR 5/5 columns equilibrated in buffer D. The columns were eluted with gradients from 0 to 100 mM (peaks I and II) or 0 to 200 mM (peak III) $MgCl_2$ in buffer D (Fig. 2). Peaks of AMARA kinase activity (HRK-A, HRK-B, HRK-C, and HRK-D) were concentrated in a Vivaspin-30, frozen in liquid N_2, and stored at −80°C.

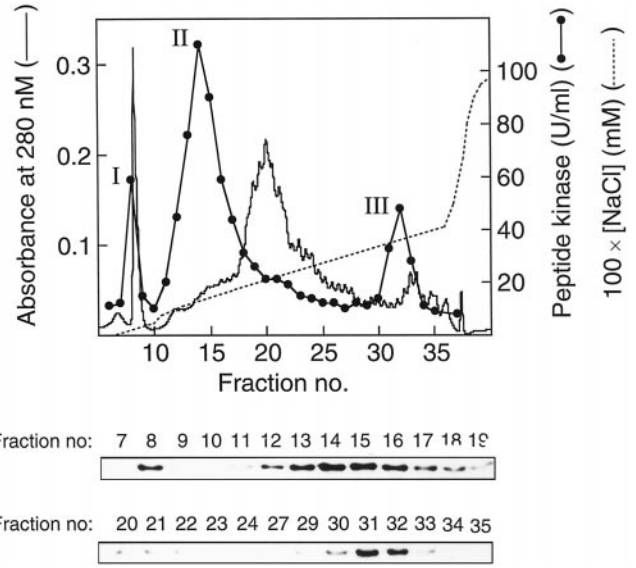

Figure 1. Separation of AMARA peptide kinases and immunoreactive 58-kD polypeptide(s) from spinach leaf by Mono-Q chromatography. The column was eluted with a gradient of increasing NaCl. Upper panel, Fractions were analyzed for peptide kinase activity (●). Peaks I, II, and III are indicated. Protein content in the eluate (continuous line) was monitored by A_{280}, and the NaCl concentration (dashed line) was monitered by conductivity. U, Units. Lower panels, Individual numbered fractions from the column analyzed by western blotting.

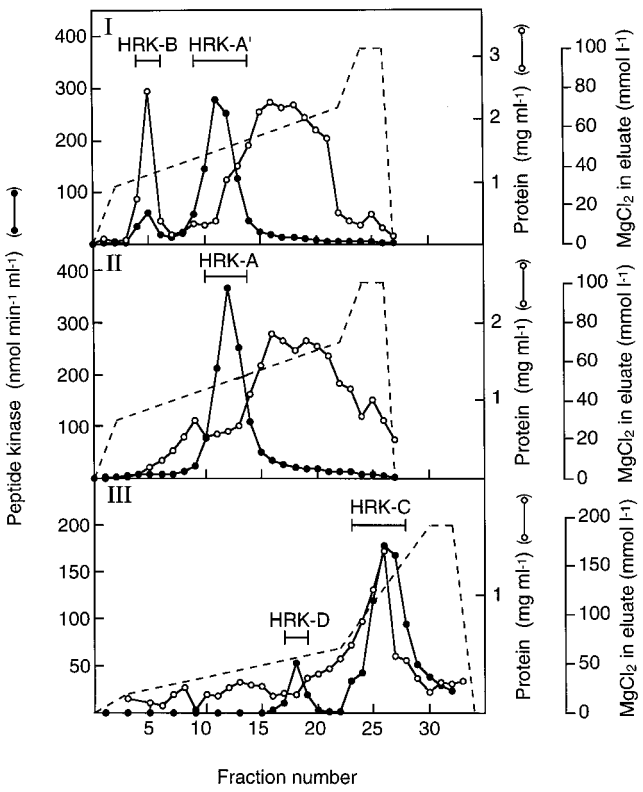

Figure 2. Rechromatography of fractions I, II, and III on Mono-Q, with elution using a gradient of increasing $MgCl_2$ (dashed line). Other symbols are as for Figure 1.

For some studies HRK-A and HRK-C were purified by a modification of the above method, referred to in the text as "protocol 2." Homogenization was identical except that the leaves (600 g) were homogenized in 1.2 L of homogenization buffer containing 8 g of PVPP. The homogenate was centrifuged at 10,500*g* for 15 min, and the supernatant was squeezed through two layers of cheesecloth and then one layer of Miracloth. Solid $(NH_4)_2SO_4$ was added with stirring to give 50% saturation. The suspension was centrifuged at 28,000*g* for 20 min, and the pellet was resuspended in buffer A and dialyzed against two changes of buffer A. The dialyzed sample was applied to DEAE-Sepharose equilibrated in 160 mL of buffer A, the column was washed extensively with buffer A, and the kinases were eluted with a 500-mL linear gradient from 0 to 0.5 M NaCl in buffer A. Fractions containing AMARA peptide kinase activity were pooled and $(NH_4)_2SO_4$ was added to 50% saturation.

The suspension was centrifuged (24,000*g*; 20 min), and the pellet was resuspended in buffer E (buffer C at pH 7.0), dialyzed against two changes of buffer E, and applied at 1 mL/min to a reactive Blue-Sepharose 30-mL column. After the sample was washed extensively with buffer E, the kinases were eluted with buffer E containing 0.5 M NaCl. Fractions containing AMARA peptide kinase activity were pooled and dialyzed against two changes of buffer C. The

suspension was centrifuged at 24,000*g* for 10 min and applied to a Mono-Q column equilibrated with buffer C at 1 mL/min. After the sample was washed extensively with buffer C, the kinases were eluted with a gradient from 0 to 50 mM NaCl (1 mL), 50 to 400 mM NaCl (20 mL), and then 0.4 to 1.0 M NaCl (1 mL), all in buffer C.

Four peaks of AMARA peptide kinase activity were obtained, eluting at approximately 130, 200, 250, and 350 mM NaCl. The profile (not shown) was similar to that in Figure 1, except that HRK-D appeared as a discrete peak eluting between HRK-A and HRK-C. The four peaks were pooled separately, desalted by repeated dilution with buffer D and concentration in a Vivaspin-30, and applied to a Mono-Q column equilibrated in buffer D. After the sample was washed with buffer D, HRK-A, HRK-B, and HRK-D were eluted using gradients in buffer D from 0 to 30 mM $MgCl_2$ (2 mL), 30 to 70 mM $MgCl_2$ (13 mL), and then 70 to 100 mM $MgCl_2$ (5 mL) or 0 to 30 mM $MgCl_2$ (2 mL); then HRK-C using a gradient from 30 to 100 mM $MgCl_2$ (23 mL). HRK-A, HRK-B, HRK-C, and HRK-D eluted at approximately 50, 35, 90, and 70 mM $MgCl_2$ respectively. This step removed substantial contamination of HRK-B and HRK-D by HRK-A and a small contamination of HRK-C by HRK-D. Kinase peaks were pooled, concentrated in a Vivaspin-30, made 50% (v/v) with respect to glycerol, and stored at −20°C.

Further Purification of Peptide Kinases on ATP-γ-Sepharose

A 0.4-mL ATP-γ-Sepharose column (Davies et al., 1994) was equilibrated in buffer F (buffer D with 50 mM NaCl and 50 mM $MgCl_2$). Samples of HRK-A or HRK-C were diluted to 4 mL with buffer F and applied to the column at 0.5 mL/min. The column was washed with 20 mL of buffer F, and kinase was eluted using 20 mL of buffer F containing 5 mM ATP. The eluted AMARA kinase peaks were concentrated in a Vivaspin-30, frozen in liquid N_2, and stored at −80°C.

Native Molecular Mass Determination

Samples of peaks I, II, and III from the first Mono-Q column (Fig. 1) were diluted to 1 mL with buffer G (50 mM Na-Hepes, pH 7.4, 100 mM NaF, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 0.02% [w/v] Brij-35, and 10% [v/v] glycerol) with or without 0.2 M NaCl and applied to a Superdex 200 column (60 × 1.6 cm) equilibrated in the same buffer. The column was run at 1 mL/min and 1-mL fractions were assayed for AMARA kinase activity. The column was calibrated using 445-kD horse spleen apoferritin, 200-kD sweet potato β-amylase, 66-kD BSA, and 29-kD carbonic anhydrase.

Phosphorylation of HMG-CoA Reductase and Analysis of Phosphorylation Site

Purified recombinant HMG1cd (0.15–0.2 mg/mL) was phosphorylated at 30°C using spinach HRK-A or HRK-C or cauliflower HRK-A, at 5 units/mL in 20 mM Tris-HCl, 30 mM Na-Hepes, pH 7.0, 12 mM NaCl, 40 μM EDTA, 2.6 mM DTT, 20% (v/v) glycerol, 5 mM $MgCl_2$, and 0.2 mM [γ-^{32}P]ATP (300–600 cpm/pmol by Cerenkov counting). Incubations were terminated by adding SDS-PAGE sample buffer, and proteins were separated by SDS-PAGE in 12% gels. Analysis of the site of phosphorylation on HMG-CoA reductase was as described previously (Dale et al., 1995a).

Partial Purification of NR

All purification steps were carried out a 4°C. Frozen spinach leaves (100 g with petioles removed) were homogenized in a kitchen blender with 250 mL of buffer H (50 mM Na-Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 10 μM FAD, and 5% [v/v] glycerol). The homogenate was centrifuged at 28,000*g* for 10 min and the supernatant was passed through two layers of cheesecloth and one layer of Miracloth. The clarified supernatant (approximately 200 mL) was added to 40 mL of packed Reactive Blue II Sepharose that had been equilibrated in buffer H, and the mixture was stirred and allowed to settle for 20 min. After that time, the mixture was restirred and allowed to settle again for 20 min, and the column was washed with buffer H until the A_{280} decreased below 0.02. NR was eluted with 100 μM NADH in buffer H, and active fractions were pooled and concentrated to 200 μL using a Vivaspin-30. The sample was diluted 25-fold in buffer I (buffer H plus 50 mM $MgCl_2$) and loaded onto an ATP-γ-Sepharose column (0.5 mL) that was washed with 20 mL of buffer I. The flow-through and wash fractions (which contained the NR activity) were pooled and concentrated to approximately 200 μL in a Vivaspin-30. Glycerol was added to 50% (v/v) final volume, and the protein was stored at −20°C.

Partial Purification of Spinach SPS

All purification steps were carried out at 4°C. Frozen spinach leaves (400 g with petioles removed) were homogenized in a kitchen blender with 5 g of PVPP and 800 mL of buffer J (50 mM Na-Mops, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, and 0.1% [v/v] Triton X-100). The homogenate was squeezed through one layer of cheesecloth and one layer of Miracloth. After the sample was centrifuged at 30,100*g* for 15 min to remove insoluble material, the supernatant was precipitated by the addition of 40% (w/v) PEG 8000 and brought to a final concentration of 5% (w/v). After 5 min of stirring, the suspension was centrifuged at 30,100*g* for 15 min, and the supernatant was a 12% final concentration of PEG 8000. After the sample was centrifuged at 30,100*g* for 15 min, the pellet was resuspended in 150 mL of buffer K (50 mM Na-Mops, pH 7.5, and 10 mM $MgCl_2$) using a ground-glass homogenizer, clarified by centrifugation at 12,100*g* for 5 min, and applied to a 15-mL Reactive Blue II Sepharose column connected in series to a 30-mL Q-Sepharose anion-exchange column, both equilibrated in buffer K.

After the sample was loaded, the Blue-Sepharose column was washed with 30 mL of buffer K to wash any remaining unbound proteins onto the Q-Sepharose and then discon-

nected. The Q-Sepharose column was washed extensively with buffer K containing 0.2 M NaCl until the A_{280} fell below 0.15, and then the protein was eluted with buffer K containing 0.5 M NaCl. SPS activity was measured in the 4-mL fractions collected, and active fractions were pooled and concentrated to 2.5 mL in a Vivaspin-30 before they were applied to a PD10 desalting column. The desalted sample was further concentrated to approximately 1 mL, diluted 3-fold with buffer K, and added to 4 mL of AMP-agarose resin. The suspension was stirred and allowed to settle for 15 min in a column, and this procedure was repeated twice. The column was washed with 20 mL of buffer K and then eluted with the same volume of buffer K containing 0.5 M NaCl. Fractions (1.4 mL) were collected throughout the wash and elution steps and were assayed for SPS and AMARA peptide kinase activity. Fractions containing SPS activity in the initial wash (SPS1) and in 0.5 M NaCl eluate (SPS2) were pooled separately, and both were concentrated to approximately 0.3 mL in a Vivaspin-30. Glycerol was added to a final concentration of 20% (v/v), and the samples were snap-frozen in small aliquots and stored at −80°C.

Pretreatment of SPS with Protein Phosphatase

Purified SPS samples were treated with the catalytic subunit of PP2A prior to phosphorylation studies. SPS (0.6 unit/mL) in buffer L (50 mM Na-Mops, pH 7.5) was treated with PP2A (3.5 millliunits/mL) at 30°C for 30 min. The phosphatase was then inhibited by the addition of okadaic acid to a final concentration of 200 nM.

Phosphorylation and Inactivation of SPS

To study the effects of phosphorylation on SPS activity, 0.5 unit/mL SPS was incubated at 30°C in the presence or absence of 200 μM ATP and kinase (as specified in the text), in buffer L containing 2 mM $MgCl_2$, 200 nM okadaic acid, 2.5 mM DTT, 0.1 mM PMSF, 1 mM benzamidine, and 1 μg/mL soybean trypsin inhibitor. After the sample was incubated for 30 min, triplicate 10-μL aliquots were removed and SPS activity was determined under limiting and nonlimiting conditions.

To study phosphorylation of SPS, incubations were identical except that [γ-^{32}P]ATP (500–1000 cpm/pmol) was used. The reaction was terminated by the addition of SDS sample buffer, and the sample was boiled and subjected to SDS-PAGE in 8% gels. After Coomassie Blue staining the gel was dried and subjected to autoradiography to determine the incorporation of ^{32}P into the 130-kD SPS polypeptide. To estimate the stoichiometry of phosphorylation, the amount of the 130-kD polypeptide was estimated by densitometry of stained gels by comparison with BSA standards run on the same gel. The 130-kD polypeptide was then excised from the gel and its radioactivity was determined by Cerenkov counting.

Peptide Analysis and Sequencing

GRM peptide (56 μM) was incubated for 10 min at 30°C with 1.5 units/mL HRK-A or HRK-C in buffer M (50 mM Na-Hepes, pH 7.0) with 5 mM $MgCl_2$ and 200 μM [γ-^{32}P]ATP (200 cpm/pmol). The reaction was stopped by injecting the mixture onto a Sep-Pak Plus C_{18} cartridge (Waters) equilibrated with 0.1% (v/v) TFA. The cartridge was washed with 0.1% TFA until all unbound radioactivity was removed and then with 0.1% TFA in 50% (v/v) acetonitrile to elute the labeled peptides. The peptides were taken to near dryness on a centrifugal vacuum evaporator, adjusted to pH 8.0 by addition of Tris base, and digested with trypsin (1:10, w/w) for 20 h at 37°C.

PP2A-treated SPS1 was phosphorylated and analyzed by SDS-PAGE as in the previous section. The gel was dried, and the ^{32}P-labeled SPS polypeptide was detected by autoradiography and excised from the gel. The gel slice was then rehydrated in water and redried on a centrifugal vacuum evaporator. It was then rehydrated in buffer N (50 mM Tris-HCl, pH 8.0, and 0.05% [v/v] Zwittergent 3-16) at 37°C for 60 min and then digested with trypsin (1:10, w/w) at 37°C for 60 min. The supernatant was removed and the gel slice was washed twice with buffer N. The combined supernatants were taken to near dryness on a centrifugal vacuum evaporator and diluted in 0.1% (v/v) TFA.

^{32}P-labeled peptides were analyzed on a C_{18} protein and peptide HPLC column (0.25 × 45 cm; Vydac, Hesperia, CA) on an HPLC system (Gilson, Middleton, WI) run in 0.1% (v/v) TFA. Peptides were eluted in the gradient from water to acetonitrile, as indicated in Figure 12, and detected by Cerenkov counting using an analytical on-line monitor (Reeve Analytical, Glasgow, UK). To determine which residue was phosphorylated, peptides were subjected to solid-phase sequencing using a procedure based on that of Bodwell et al. (1991).

PAGE, Western Blotting, and Other Analytical Procedures

SDS-PAGE in 10% gels was performed according to the method of Laemmli (1970). Proteins were transferred electrophoretically to nitrocellulose membranes (Hybond N, Amersham) using a trans-blot apparatus (Mini Protean II, Bio-Rad). Membranes were blocked by incubating in TBS (25 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 0.1% (w/v) Tween 20 and 5% (w/v) nonfat milk powder. Membranes were placed on laboratory film, antibody (1.5 mL diluted in TBS-Tween) was added, and the membranes were incubated without shaking for 2 h. The blots were washed five times for 5 min each with TBS-Tween and then for 1 h with a second antibody in TBS-Tween-5% milk powder. Detection of second antibody utilized the ECL system (Amersham). Determination of protein concentration was as described by Bradford (1976). K_m values were determined by direct fitting of initial velocity data to the Michaelis-Menten equation using the Kaleidagraph program (Synergy Software, Reading, PA). Values are ±SE.

RESULTS

Resolution of AMARA Kinases from Spinach Leaf Extracts by Anion-Exchange Chromatography

Cauliflower HRK-A was originally assayed (MacKintosh et al., 1992; Ball et al., 1994) using the SAMS peptide, which had been designed as a specific substrate for AMPK (Davies et al., 1989) and which is also a reasonably specific substrate for the yeast SNF1 complex (Wilson et al., 1996). However the AMARA peptide is a better substrate for cauliflower HRK-A than SAMS (Dale et al., 1995b). AMARA has the key residues required for recognition, but apart from the three Arg residues at the C terminus, which allow binding to phosphocellulose paper, other residues have been replaced by Ala. We used AMARA for routine assays in this study.

Protein kinases in spinach leaf extracts were extracted in buffers containing 50 mM NaF to inhibit protein phosphatases. They were initially purified by PEG precipitation and chromatography on DEAE-Sepharose, the latter using a step (rather than gradient) elution. No significant resolution of multiple forms of peptide kinase occurred in this step. Chromatography on Q-Sepharose with elution using an NaCl gradient resulted in two broad peaks of AMARA kinase activity (not shown). These were recombined and then separated on a Mono-Q column eluted with an NaCl gradient (Fig. 1), which resulted in the resolution of three peaks of peptide kinase activity designated I, II, and III. Peaks I and II eluted from Mono-Q at NaCl concentrations similar to those of HRK-B and HRK-A previously purified from cauliflower (Ball et al., 1994). Fraction II indeed appears to be very similar to cauliflower HRK-A by several criteria (see below) and will henceforth be referred to as HRK-A. However, fraction I turned out to be a mixture of two kinases. The kinase(s) corresponding to fraction III was not observed in the previous study of cauliflower (Ball et al., 1994).

If the fractions from the Mono-Q column were assayed with the SAMS peptide (used to purify the cauliflower kinases by Ball et al. [1994]) or the SP peptide (based on the phosphorylation site on SPS; see "Materials and Methods"), identical profiles were obtained, but the specific activities were approximately one-half those obtained with the AMARA peptide (not shown). None of the three AMARA kinase peaks was inhibited by EGTA or stimulated by Ca^{2+}; 1 mM Ca^{2+} caused a slight inhibition of all activities (not shown).

If fraction I was rechromatographed using the same gradient, only 56% of the recovered activity eluted in the fraction I position, whereas 44% eluted in the same position as fraction II/HRK-A (not shown). Fraction I from the first column therefore contained at least two protein kinase activities. Fractions I, II, and III from the first column were further purified by chromatography on Mono-Q with elution using a gradient of increasing $MgCl_2$ rather than NaCl (Fig. 2). This resulted in the resolution of the peptide kinase activities from a considerable amount of contaminating protein, particularly for fractions I and II. When fraction I was analyzed in this way, approximately 80% of the activity eluted in exactly the same position as fraction II/HRK-A (although we suspect it to be identical to HRK-A, this fraction will be designated HRK-A′), with 20% eluting as a discrete earlier peak designated HRK-B.

When fraction III was analyzed using the $MgCl_2$ gradient, approximately 90% of the activity (designated HRK-C) eluted right at the end of the gradient, with about 10% (designated HRK-D) eluting at an intermediate position between HRK-A and HRK-C. At this stage the specific activities of HRK-A, HRK-B, HRK-C, and HRK-D were increased 95-, 46-, 120-, and 100-fold, respectively, compared with the initial extract (2.5 units/mg). However, these figures are underestimates of the purification achieved, because the activity of the initial extract represents the sum of the activity of all four kinases.

Analysis of AMARA Kinases by Gel Filtration

Fractions I, II, and III from the first Mono-Q column were also analyzed by gel filtration on Superdex 200 (Fig. 3). Fraction II (HRK-A) eluted as a single symmetrical peak just ahead of the 150-kD marker. Fraction I resolved into two peaks, a major peak (HRK-A′, approximately 80% of total activity) that comigrated exactly with HRK-A and a minor peak (HRK-B, approximately 20%) migrating between the 66- and 29-kD markers with an apparent molecular mass of approximately 45 kD. Fraction III also mi-

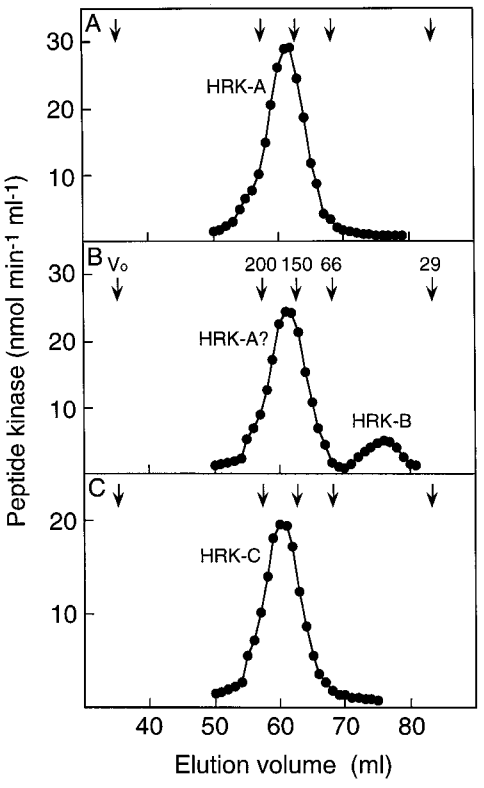

Figure 3. Analysis of fractions II (A), I (B), and III (C) by gel filtration on Superdex 200. Fractions were analyzed for peptide kinase activity (●). Elution volumes of marker proteins are indicated by arrows, and molecular masses in kilodaltons are shown in B.

grated as a single symmetrical peak (HRK-C) between the 200- and 150-kD markers. When the profiles for HRK-C and HRK-A were superimposed, HRK-C appeared to elute one fraction ahead, indicating a slightly higher molecular mass.

Further Purification of HRK-A on ATP-γ-Sepharose

HRK-A (Fig. 4) and HRK-C (not shown) could be separated on a small scale from the bulk of contaminating proteins by chromatography on ATP-γ-Sepharose (Haystead et al., 1993; Davies et al., 1994) with elution using MgATP. Unfortunately, the protein content in the ATP eluate was too low to allow accurate determination of specific activity, and it was also difficult to determine the recovery of activity because of the high ATP concentration, which diluted [γ-^{32}P]ATP in the assay, in the elution buffer. However SDS-PAGE analysis (Fig. 4) indicated that a very substantial purification had been achieved. When the purified HRK-A was analyzed by SDS-PAGE and silver staining, a triplet of polypeptides of approximately 56 to 60 kD were detected, along with a few others of higher and lower

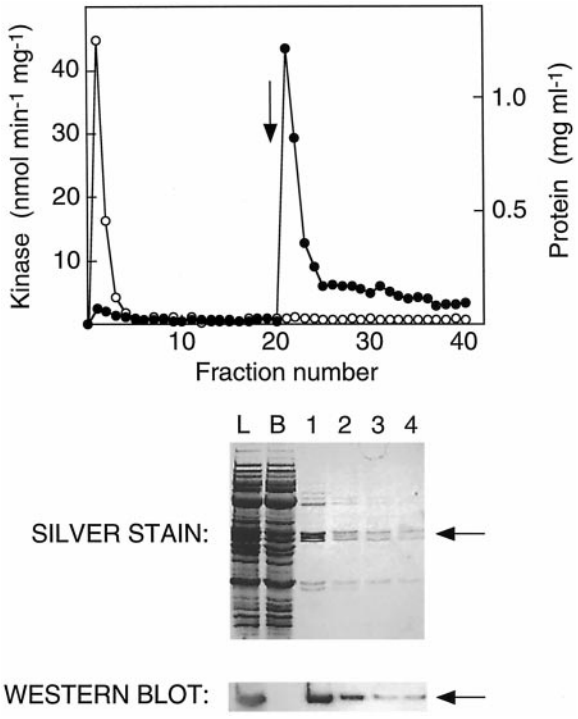

Figure 4. Purification of HRK-A (derived from a separation similar to Fig. 2) on ATP-γ-Sepharose. Top, Protein/activity profile. The bulk of proteins did not bind to the column, as shown by the protein peak (○) in fractions 1 to 3. After extensive washing, the column was eluted with buffer containing ATP. Fractions were analyzed for peptide kinase activity (●). Middle, SDS-PAGE analysis followed by silver staining. Lane L, Load; lane B, breakthrough (fractions 1-3); lane 1, fractions 21 to 25; lane 2, fractions 26 to 30; lane 3, fractions 31 to 35; and lane 4, fractions 36 to 40. Bottom, Western-blot analysis of fractions using anti-NIP antibody. Arrows at right indicate that the 58-kD polypeptide detected with the antibody aligns with the central band of the silver-stained triplet.

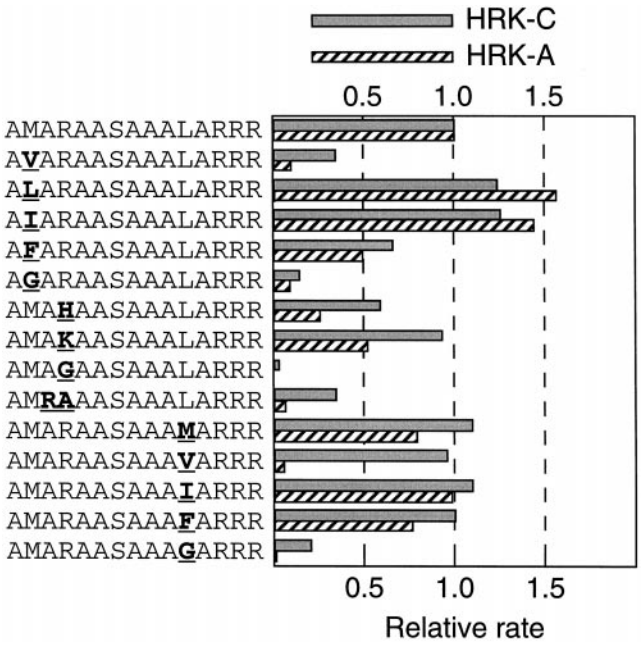

Figure 5. Rate of phosphorylation of variants of the AMARA peptide (40 μM) by HRK-A and HRK-C. The sequence of the peptide is shown at left, with the amino acids changed from the parent AMARA peptide highlighted in bold and underlined. Results are expressed relative to the initial velocity obtained with the parent AMARA peptide.

molecular mass. When HRK-C was purified on ATP-γ-Sepharose, a 58-kD polypeptide was also detectable by silver staining (not shown).

Analysis of Peptide Kinase Peaks by Western Blotting and Sequence Analysis

The various peptide kinase peaks were analyzed by western blotting using an antibody raised against the NIP peptide, which is highly conserved in all sequenced members of the higher-plant SnRK1 subfamily. Polypeptides of an apparent molecular mass of 58 kD, which were indistinguishable in size from each other, correlated with fractions I, II, and III across the first Mono-Q column (Fig. 1). Polypeptides of an apparent molecular mass of 58 kD also correlated with kinase activity when HRK-A (Fig. 4) or HRK-C (not shown) were fractionated on ATP-γ-Sepharose. The cross-reacting polypeptide in Figure 4 corresponded to the middle band of the triplet, migrating at 58 kD. The upper and lower bands but not the middle band of the triplet comigrated with major polypeptides that were also present in the breakthrough fraction (Fig. 4, lane B). The lower band was identified by amino acid sequencing as the large subunit of Rubisco (predicted mass of 53 kD).

Specificity of HRK-A and HRK-C with Synthetic Peptides

Further studies were conducted using HRK-A and HRK-C as purified on Mono-Q (Fig. 2, $MgCl_2$ gradient). Although HRK-A′ (the form of HRK-A that comigrated with HRK-B in the initial NaCl gradient) appeared to be-

have identically to HRK-A, it was not studied further. HRK-A and HRK-C had K_m values for the AMARA and SAMS peptides that were similar but not identical (HRK-A: AMARA, 6 ± 1 μM, and SAMS, 90 ± 6 μM; HRK-C: AMARA, 11 ± 2 μM, and SAMS, 38 ± 5 μM). The rates of phosphorylation of variants of the AMARA peptide at an arbitrary concentration of 40 μM are shown in Figure 5. These results are considered further in the "Discussion."

Phosphorylation of HMG-CoA Reductase

Both HRK-A and HRK-C readily phosphorylated the bacterially expressed catalytic domain of HMG-CoA reductase, HMG1cd (Dale et al., 1995a), from Arabidopsis. Radioactivity from [γ–^{32}P]ATP was incorporated exclusively into the 50-kD polypeptide of HMG1cd (not shown). Samples of HMG1cd were phosphorylated using spinach HRK-A or HRK-C or cauliflower HRK-A. The ^{32}P-labeled 50-kD polypeptide in each case was excised from the gel and digested with CNBr, and the resultant peptides were analyzed by reversed-phase HPLC. After phosphorylation by any of the three kinases, a single major comigrating radioactive peptide was obtained (not shown), which we have previously shown for cauliflower HRK-A to have the sequence KYNRSSRDISGATT, which corresponds to the C-terminal CNBr peptide of Arabidopsis HMG1 (Dale et al., 1995a). Solid-phase sequencing of these peptides (not shown) showed that in every case the radioactivity was released at cycle 5, corresponding to Ser-577 in the predicted sequence of HMG1.

Phosphorylation of NR

For studies of NR phosphorylation, HRK-A and HRK-C were purified by protocol 2. To examine whether the kinases phosphorylated NR, spinach NR was partially purified by specific elution from Reactive Blue II Sepharose using NADH (Douglas et al., 1995). Preliminary experiments indicated that this preparation was still contaminated with endogenous protein kinases, but these could be almost completely removed by passage through ATP-γ-Sepharose (Davies et al., 1994) to which NR does not bind (not shown). Coomassie Blue staining after SDS-PAGE revealed that this preparation contained a doublet migrating just behind the 100-kD marker (Fig. 6). The autoradiogram (Fig. 6) also shows that HRK-A or HRK-C phosphorylated both of these polypeptides in the doublet, as well as a third slightly larger polypeptide that was barely visible in the stained gel. Full-length NR has a predicted mass of 104 kD, but it has been shown that it is susceptible to proteolysis at the N terminus during purification from spinach leaf (Douglas et al., 1995), and we suspect that all three polypeptides may be derived from NR. Figure 7 shows that incubation of NR with HRK-A or HRK-C resulted in almost complete inactivation but only if the incubation included MgATP and the 14-3-3 protein.

Phosphorylation of SPS

For studies of SPS phosphorylation, the kinases were purified by protocol 2. In preliminary studies we noted that SPS purified from spinach leaf by PEG precipitation and Blue- and Q-Sepharose chromatography was still contaminated with protein kinase(s) that would phosphorylate the AMARA and the SP peptides. The SP peptide is based on the sequence around Ser-158 on spinach SPS but with norleucine (J) in place of Met. The SPS in these preparations was inactivated in a time-dependent manner in the presence of MgATP even when no exogenous kinase was added (not shown). The peptide kinase and SPS-inactivating activities were likely to be a function of the same enzyme, because high concentrations of the SP or AMARA peptides inhibited the ATP-dependent inactivation (not shown). The endogenous peptide kinase and SPS-inactivating activities could be largely but not completely removed by passing the SPS preparation through an AMP-agarose column. This resulted in two pools of SPS activity (Fig. 8A): SPS1, which passed straight through the column and was relatively free of peptide kinase and SPS-inactivating activity, and SPS2, which bound to the column, was eluted with 0.5 M NaCl, and was still heavily contaminated with peptide kinase(s) and SPS-inactivating activity (not shown).

The SPS specific activity of the SPS1 fraction was 0.4 μmol min^{-1} mg^{-1}. When analyzed by SDS-PAGE, the preparation contained a number of polypeptides, but there was a prominent one with an apparent molecular mass of 130 kD. Although this is slightly higher than the mass predicted from the DNA sequence of spinach SPS (122 kD), this polypeptide cross-reacted with anti-SPS antibody (Fig. 8B). When the amount of protein in the 130-kD polypeptide was estimated by densitometry of Coomassie Blue-stained gels using BSA as a standard, specific activities of 150 ± 50

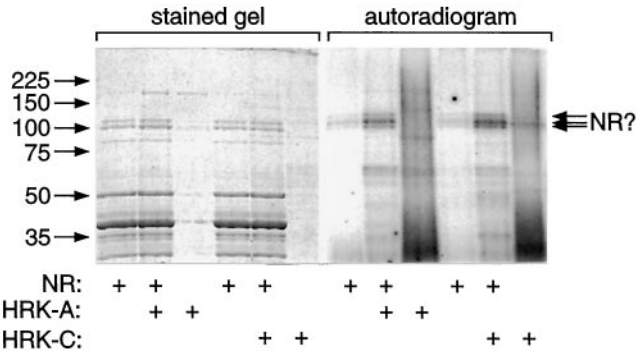

Figure 6. Coomassie Blue-stained gel (left) and corresponding autoradiogram (right) after incubation of partially purified NR with [γ-^{32}P]ATP and HRK-A or HRK-C (both 1 unit/mL, 25 min). The presence or absence of NR or kinases in individual lanes is indicated at the bottom. Migrations of marker proteins (in kilodaltons) are indicated on the left. Identification of NR polypeptides (arrows at right) is based on their molecular masses and previous studies using a similar purification protocol (Douglas et al., 1995). NR (0.2 unit/mL) was incubated with [γ-^{32}P]ATP (200 μM, approximately 1000 cpm/pmol) in a total volume of 20 μL of 50 mM Hepes-NaOH, pH 7.4, 10 mM $MgCl_2$, 10 μM FAD, and 1 mM DTT in the presence or absence of HRK-A or HRK-C (1 unit/mL). After 10 min at 30°C the reactions were terminated by the addition of SDS-PAGE sample buffer, and the samples were boiled and loaded onto an 8% gel. After Coomassie Blue staining, the dried gels were subjected to autoradiography using Hyperfilm-MP.

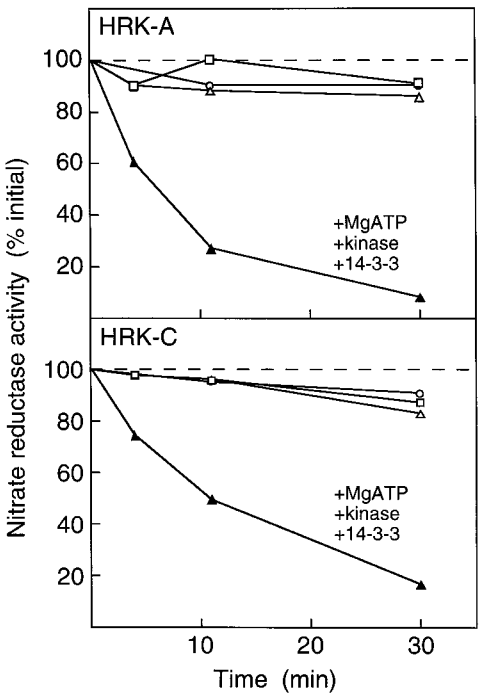

Figure 7. Effect of incubation with MgATP with or without HRK-A (top) or HRK-C (bottom) with or without 14-3-3 protein, on the activity of partially purified NR. NR was incubated with MgATP alone (○), MgATP plus kinase (□), MgATP plus 14-3-3 protein (△), or MgATP plus kinase plus 14-3-3 protein (▲). NR (4 milliunits/mL) was incubated at 30°C in a total volume of 850 μL of 50 mM Na-Hepes, pH 7.4, 10 mM $MgCl_2$, 10 μM FAD, 1 mM DTT, and 1 mg/mL BSA. When added, MgATP was ATP (200 μM) plus $MgCl_2$ (5 mM), HRK-A or HRK-C were at 1 unit/mL, and 14-3-3 protein (yeast Bmh2 protein) was at 0.02 mg/mL. Aliquots (200 μL) were removed at various times and assayed for NR activity in the presence of 5 nM staurosporine, which inhibits HRK-A and HRK-C and blocks further phosphorylation. Results are expressed as the percentages of initial activity, which was 4 milliunits/mL in both cases.

μmol min^{-1} mg^{-1} (means ± SE) were calculated for three different SPS1 preparations. This is identical to the value quoted by Huber and Huber (1996) for the specific activity of pure SPS. The SPS1 fraction was still contaminated with trace amounts of peptide kinase and SPS-inactivating activities. However, the residual peptide kinase activity could be inactivated in a time- and dose-dependent manner by treatment with the homogeneous catalytic subunit of PP2A; this treatment also almost completely abolished the ATP-dependent SPS inactivation (not shown). The PP2A-treated SPS1 fraction represents an essentially kinase-free SPS preparation that was used for further studies, PP2A being inhibited before subsequent studies by the addition of okadaic acid.

We assessed the effect of phosphorylation on SPS activity by measuring the ratio of activities under limiting conditions (3 mM Fru-6-P, 12 mM Glc-6-P, and 10 mM Pi) and nonlimiting conditions (10 mM Fru-6-P and 40 mM Glc-6-P), a protocol established by Huber et al. (1989). Incubation of PP2A-treated SPS1 with MgATP alone did not give a significant decrease in the activity ratio, but further addition of HRK-A, HRK-B, HRK-C, or HRK-D (equal amounts of peptide kinase activity) led to a decrease in activity ratio in every case from 0.4 to 0.20 to 0.25 (Fig. 9). This was associated with phosphorylation of the 130-kD SPS polypeptide (Fig. 9). At least some of this phosphorylation occurred at Ser-158, because after incubation with MgATP and added kinases, but not with MgATP alone, the signal obtained in western blots using an antibody that only recognizes the dephospho form of the Ser-158 sequence (Weiner, 1995) was significantly diminished (Fig. 9).

Because HRK-B and HRK-D are less well characterized than HRK-A and HRK-C and because HRK-C was available in larger amounts than HRK-A, more detailed studies of the inactivation of SPS were conducted with HRK-C. No inactivation of SPS by HRK-C was observed if MgATP was omitted, and inactivation was dependent on the dose of HRK-C added (not shown). Both the decrease in the activity ratio and the phosphorylation of the 130-kD polypeptide of SPS produced by HRK-C were time dependent (Fig. 10). With the particular SPS1 preparation used in Figure 10, there was some decrease in the activity ratio and an increase in the phosphorylation of SPS, even in the absence of added kinase. However, the addition of HRK-C greatly stimulated both effects. At later times the phosphorylation produced by HRK-C reached a stoichiometry of about 0.3 mol phosphate mol^{-1} 130-kD subunit. This represents an approximate estimate only, because the SPS protein content was estimated by densitometric comparison of the Coomassie Blue-stained 130-kD polypeptide using known amounts of BSA as standard. Inactivation of SPS could be at least partially reversed by subsequent incubation with homogeneous mammalian PP2A or PP2C (Fig. 11). This experiment does not distinguish whether the reactivation was due to reversal of phosphorylation by the endogenous kinase or by HRK-C. However, since both phosphorylate Ser-158 exclusively (see below), this does not affect the interpretation.

Since SPS is phosphorylated at multiple sites, not all of which are regulatory (Huber and Huber, 1996), it was important to examine the site(s) of phosphorylation. The experiment presented in Figure 9 using the dephospho-specific antibody suggested that HRK-A, HRK-B, HRK-C, and HRK-D all phosphorylated SPS on Ser-158 but did not reveal whether all of the phosphate incorporated into the 130-kD polypeptide was located at this site. To address this, we synthesized the GRM peptide, which corresponds to residues 151 to 170 on spinach SPS. This peptide was an excellent substrate for both HRK-A and HRK-C (K_m[HRK-A] = 6.4 ± 0.8 μM; K_m[HRK-C] = 10.9 ± 1.2 μM). Solid-phase sequence analysis (not shown) demonstrated that all of the phosphate was released at cycle 8, as would be expected if the Ser residue corresponding to Ser-158 in the full-length sequence was phosphorylated. The phosphorylated peptide was digested with trypsin (which would cleave after Arg-5, yielding the limit tryptic peptide ISS(p)VEMMDNWANTFK) and purified by reversed-phase HPLC (Fig. 12, A and B). The major radioactive peptide retained by the column eluted at 66 min for HRK-A and HRK-C. Solid-phase sequencing of these peptides indicated that almost all of the radioactivity was now released at cycle 3 as expected (not shown).

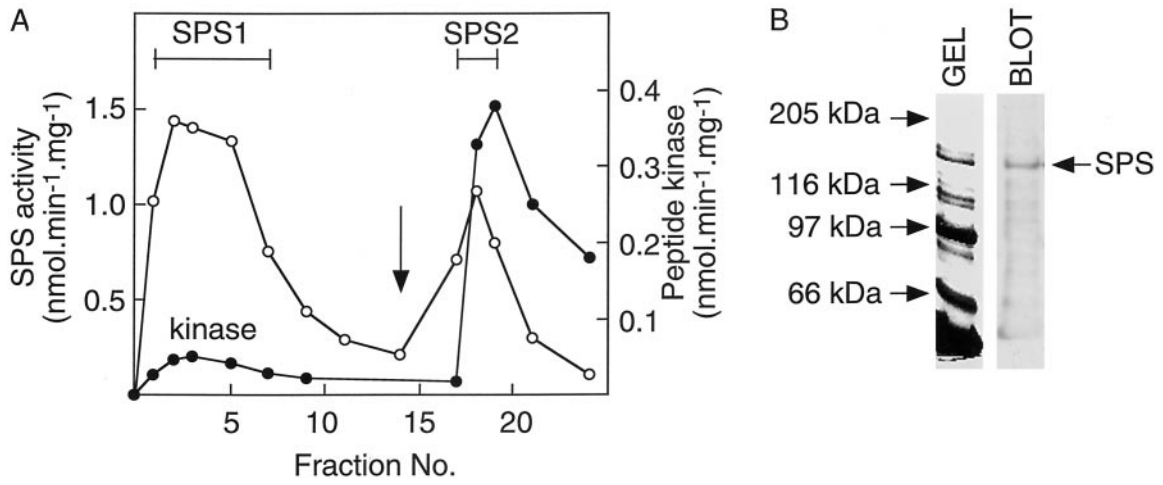

Figure 8. A, Partial resolution of SPS activity and AMARA peptide kinase activity on AMP-agarose. An SPS fraction purified to the Q-Sepharose step as described in "Materials and Methods" was applied to a column of AMP-agarose, which was washed with starting buffer. At the point shown by the arrow, bound proteins were eluted with buffer plus 0.5 M NaCl. Fractions were assayed for SPS (○) and AMARA peptide kinase activity (●). B, SPS1 fraction was analyzed by SDS-PAGE in 8% gels. Gels were either stained with Coomassie Blue (left) or the proteins were electrophoretically transferred to nitrocellulose and the membranes probed with an anti-SPS antibody (right). The migration of marker proteins are indicated on the left.

The PP2A-treated SPS1 preparation was phosphorylated using [γ-^{32}P]ATP and HRK-A or HRK-C, and proteins were separated by SDS-PAGE. A gel slice containing the 130-kD SPS polypeptide was dried and digested with trypsin, leading to release of 70% to 80% of the radioactivity into solution. On analysis by reversed-phase HPLC, the radioactivity retained by the column was recovered as a major peptide eluting at 66 min (exactly comigrating with the tryptic peptide obtained after phosphorylation of the GRM peptide), plus a minor peptide eluting just ahead of it at 64 min (Fig. 12, C and D). This minor form was also present in the analyses of the synthetic peptides (Fig. 12, A and B), although it was much less prominent. Solid-phase sequencing of the peptides eluting at 64 or 66 min revealed that the radioactivity was released at cycle 3 in every case (not shown). Although the peptide eluting at 64 min was not conclusively identified, we suspect that it is a form of the ISS(p)VEMMDNWANTFK peptide in which one or both of the Met residues had been oxidized during preparation.

Regulation of HRK-A and HRK-C by Phosphorylation

Both HRK-A and HRK-C could be inactivated by incubation with homogeneous mammalian protein phosphatases, i.e. the catalytic subunit of PP2A or PP2C. They differed in their susceptibilities to protein phosphatase treatment, since HRK-C was inactivated by PP2A more slowly than HRK-A, whereas inactivation by PP2C occurred at similar rates (Fig. 13). After inactivation by PP2A, both HRK-A and HRK-C could be reactivated 3- to 4-fold in a time-dependent manner by incubation with mammalian AMPKK in the presence of MgATP (not shown).

Lack of Effect of Glc-6-P on HRK-A and HRK-C

McMichael et al. (1995b) reported that a partially purified SPS kinase from spinach leaf (peak III) was inhibited 50% to 60% by 10 mM Glc-6-P. Since we suspected that peak III corresponded to HRK-C (see "Discussion"), we tested the effects of Glc-6-P on HRK-A and HRK-C (purified by protocol 2). When AMARA was used as a substrate, one commercial preparation of Glc-6-P inhibited both kinases at 10 mM, whereas another did not (not shown). This anomaly was eventually traced to contaminants in the Glc-6-P, probably Ba^{2+} ions that are used in the commercial preparation of hexose phosphates. After purification to remove these contaminants (see "Materials and Methods"), neither sample of commercial Glc-6-P caused any inhibition of HRK-A or HRK-C at 10 mM, although there was a slight inhibition at 100 mM (not shown).

DISCUSSION

The key findings of this paper are that two Ca^{2+}-independent protein kinases from spinach leaf, currently termed HRK-A and HRK-C, phosphorylate and inactivate not only HMG-CoA reductase but also SPS and NR. Our results also provide compelling evidence that these kinases represent members of the SNF1-related SnRK1 family. Our results pull together several previous studies of the regulation of HMG-CoA reductase, SPS, and NR via phosphorylation by Ca^{2+}-independent protein kinases (Dale et al., 1995a; McMichael et al., 1995a; Douglas et al., 1997). However, in these previous studies the protein kinases were poorly characterized in molecular terms, and it was not clear that single protein kinases could regulate all three metabolic enzymes. Although further work is required to prove that these enzymes are targets for the SnRK1 kinases in vivo, this work shows that the SnRK1 kinases could potentially regulate both isoprenoid and Suc synthesis and nitrogen assimilation for amino acid and nucleotide biosynthesis. These are three major biosynthetic pathways in the plant, and it is interesting to note that the mammalian SNF1 homolog, AMPK, also regulates multiple biosyn-

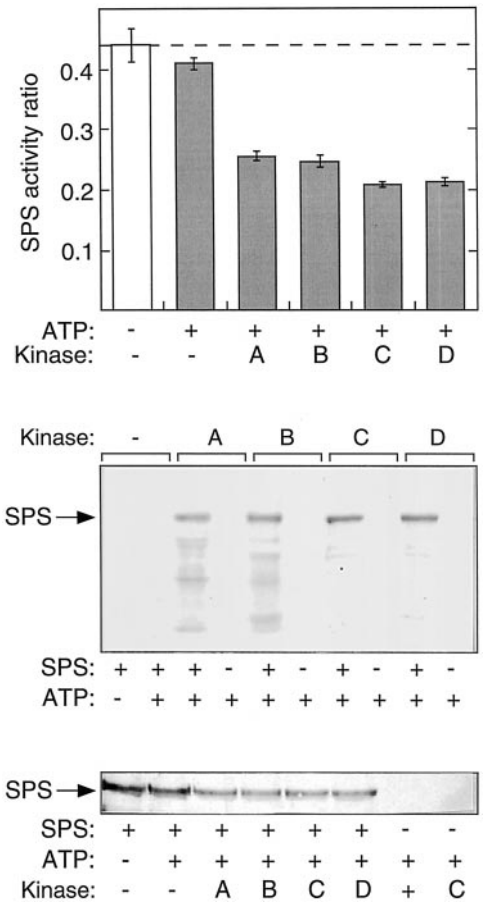

Figure 9. Inactivation and phosphorylation of SPS by HRK-A, HRK-B, HRK-C, and HRK-D. SPS1 was pretreated with PP2A to inactivate the endogenous kinase. Incubations (30 min at 30°C) contained SPS, MgATP, and HRK-A, HRK-B, HRK-C, or HRK-D (0.34 unit/mL) as indicated. Top, Aliquots were assayed for SPS activity. Results (means ± SE for triplicate assays) are expressed as the ratio of activities under limiting conditions (3 mM Fru-6-P, 12 mM Glc-6-P, and 10 mM Pi) and nonlimiting conditions (10 mM Fru-6-P and 40 mM Glc-6-P). Middle, Incubations utilized [γ-^{32}P]ATP and were analyzed by SDS-PAGE and autoradiography. The 130-kD SPS polypeptide is indicated by the arrow on the left. Bottom, Incubations were analyzed by western blotting using an anti-peptide antibody that only recognizes SPS when it is not phosphorylated at Ser-158.

thetic pathways in animals (Hardie and Carling, 1997; Hardie et al., 1998).

Using peptides designed as substrates for the AMPK/SNF1 protein kinase subfamily, this study also shows that the situation in spinach leaves is more complex than that in cauliflower inflorescences (Ball et al., 1994). In the latter case only two Ca^{2+}-independent peptide kinases were defined, HRK-A and HRK-B. HRK-A had a catalytic subunit of 58 kD, which cross-reacted with an antibody raised against a peptide conserved in the plant SnRK1 subfamily (Ball et al., 1995) and had a native molecular mass of 160 to 200 kD (Ball et al., 1994). In these and several other respects, such as elution positions on Mono-Q in NaCl and $MgCl_2$ gradients, peptide specificity, inactivation by protein phosphatases, and reactivation by mammalian kinase kinase (MacKintosh et al., 1992; Ball et al., 1994; Dale et al., 1995b), cauliflower and spinach HRK-A are very similar. Spinach HRK-B did not cross-react with the plant SnRK1 antibody and had a native molecular mass of 45 kD: In this respect it appears to be similar to the previously defined cauliflower HRK-B (Ball et al., 1994).

The analysis of spinach HRK-A and HRK-B was complicated by the finding that after the initial Mono-Q column fraction I (containing HRK-B) appeared to be contaminated with a large amount of an activity (HRK-A′) that seemed indistinguishable from HRK-A. Although the HRK-A′ that was recovered by these procedures appeared to be identical to the HRK-A in fraction II from the first column, it was not studied further. Kinase(s) eluting in fraction III had not been observed in the study with cauliflower inflorescences (Ball et al., 1994). Fraction III was heterogeneous in terms of kinase activity and could be resolved into two peaks, HRK-C and HRK-D, using a Mono-Q column eluted with a $MgCl_2$ gradient (Fig. 2). However, HRK-D was a rather minor activity and was not studied further.

Subsequent studies concentrated on the two major peptide kinase activities, HRK-A and HRK-C. We propose that the HRK-A and HRK-C represent different forms of the AMPK/SNF1-related SnRK1 kinase subfamily. The evidence for this can be summarized as follows:

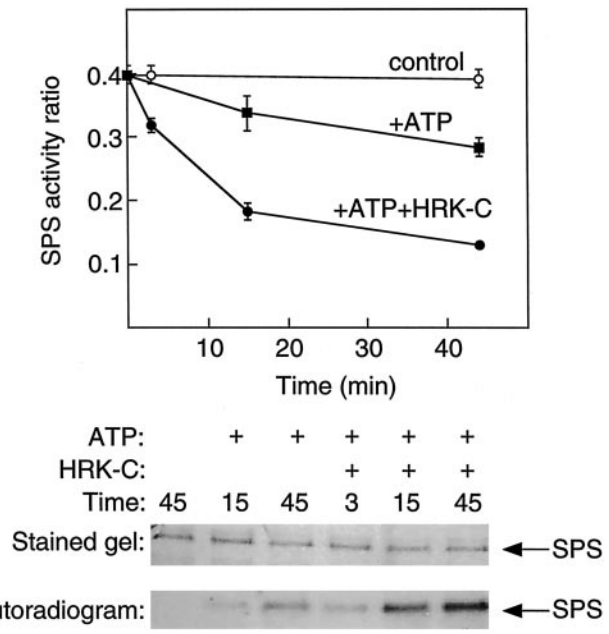

Figure 10. Time course of inactivation of SPS by HRK-C. The conditions were the same as in Figure 9, except that a different SPS1 preparation was used and HRK-C was at 0.4 unit/mL. Top, Aliquots were taken at various times for SPS assay. The results (means ± SE for triplicate assays) are expressed as the ratios of activities under limiting and nonlimiting conditions. Bottom, Parallel experiment performed using [γ-^{32}P]ATP. At various times aliquots were analyzed by SDS-PAGE. Results of Coomassie Blue staining (top) and autoradiography (bottom) of the 130-kD SPS polypeptide are shown. There were other prominent polypeptides on the stained gel but, as in Figure 9, the 130-kD polypeptide was the only one that was significantly labeled with ^{32}P.

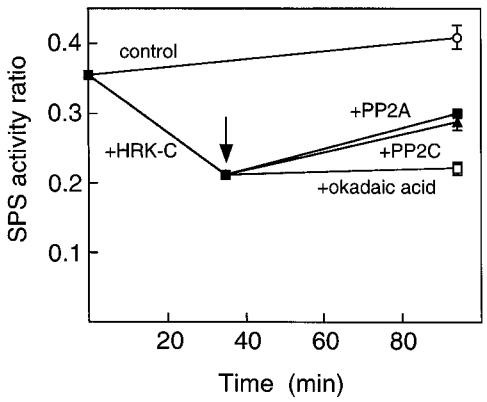

Figure 11. Inactivation of SPS by phosphorylation can be partially reversed by dephosphorylation. Inactivation was as for Figure 5 except that the SPS1 was not pretreated with PP2A (this explains the slightly lower activity ratio in the control, since the sample contained higher endogenous kinase activity). The SPS was inactivated for 35 min at 30°C with HRK-C and 1 mM ATP. At the point shown by the arrow, 10 nM staurosporine was added to inhibit the kinase, followed by 4 milliunits/mL PP2A, 4 milliunits/mL PP2C, or 200 nM okadaic acid. Separate experiments showed that staurosporine inhibited HRK-C with an IC_{50} of 1 nM. Staurosporine (10 nM) did not affect SPS activity, and was also added to the SPS assays.

First, HRK-A and HRK-C phosphorylate the AMARA and SAMS peptides with K_m values in the low micromolar range. Both peptides were originally designed as specific substrates for mammalian AMPK (Davies et al., 1989; Dale et al., 1995b) and are also rather specific substrates for the SNF1 protein kinase in *S. cerevisiae* (Wilson et al., 1996).

Second, using variants of the AMARA peptide, both HRK-A and HRK-C appear to have similar minimal recognition motifs to rat AMPK, yeast SNF1, and cauliflower HRK-A (Hyd-X-Basic-X-X-Ser-X-X-X-Hyd, where Hyd is an amino acid with a bulky hydrophobic side chain). Their peptide specificities are discussed in more detail below.

Third, across the eluate from at least two different columns, the activities of HRK-A and HRK-C correlated with the content of 58-kD polypeptides detectable by western blotting using an antibody raised against the NIP peptide. Although the NIP sequence is derived from the sequence of a rye SnRK1 (RKIN1; Alderson et al., 1991), 13 of the 15 residues are also conserved in the barley, Arabidopsis, tobacco, and potato homologs. The DNA sequences of all of the SnRK1 family currently cloned from higher plants predict protein products of 57 to 59 kD.

Fourth, the native forms of HRK-A and HRK-C have apparent molecular masses by gel filtration very similar to those of mammalian AMPK (Davies et al., 1994) and cauliflower HRK-A (Ball et al., 1994).

Fifth, both HRK-A and HRK-C are inactivated by protein phosphatases and can be reactivated by the upstream protein kinase in the mammalian system, i.e. AMPKK. We have identified Thr-172 as the major site phosphorylated on AMPK by AMPKK (Hawley et al., 1996). This residue is conserved in all currently sequenced members of the plant SnRK1 subfamily, and the sequence surrounding it is also highly conserved among animals, yeast, and plants.

Although they appear to comigrate on SDS-PAGE, the 58-kD polypeptides in HRK-A and HRK-C may represent distinct isoforms of the catalytic subunit of spinach SnRK1 kinase, analogous to the α1 and α2 isoforms of mammalian AMPK (Stapleton et al., 1996). The HRK-A and HRK-C holoenzymes elute from Mono-Q columns at different times using two different elution protocols (NaCl and $MgCl_2$ gradients; Figs. 1 and 2), appear to be slightly different in native molecular mass (Fig. 3), differ in their relative susceptibilities to dephosphorylation by PP2A and PP2C (Fig. 13), and have subtle differences in specificity for peptide substrates. At least two DNA sequences homologous to Snf1 have been cloned from barley (Halford et al., 1992) and Arabidopsis (P. Lessard and M. Thomas, personal communication). Another possibility is that HRK-A and HRK-C may represent the same catalytic subunit complexed with different regulatory subunits or the same gene product(s) subject to different posttranslational modifications. Specific molecular probes are required to distinguish

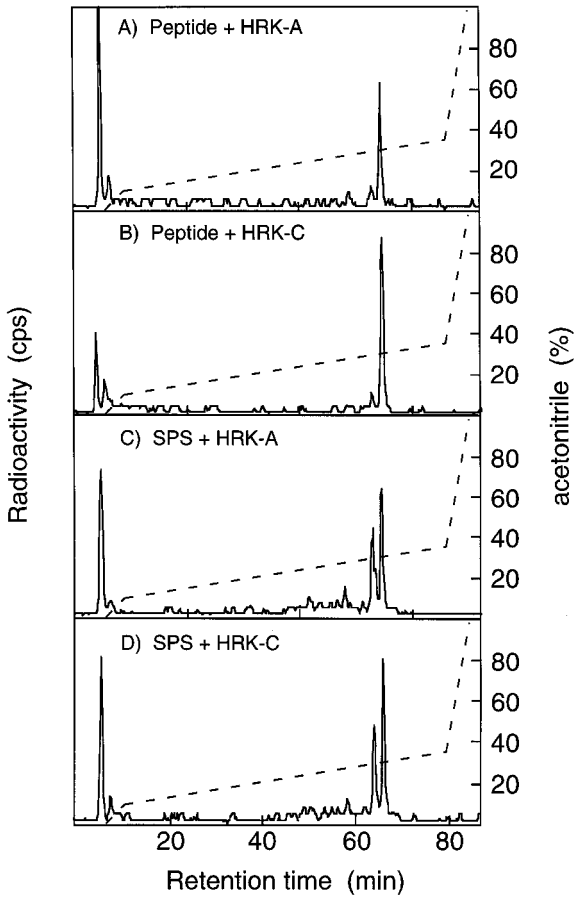

Figure 12. Reversed-phase HPLC analysis of ^{32}P-labeled tryptic peptides derived from synthetic *GRM* peptide phosphorylated by HRK-A (A); synthetic *GRM* peptide phosphorylated by HRK-C (B); purified SPS phosphorylated by HRK-A (C); and purified SPS phosphorylated by HRK-C (D). See "Materials and Methods" for experimental details. The continuous line shows the radioactivity determined by Cerenkov counting; the dashed line shows the percentage of acetonitrile in the eluant.

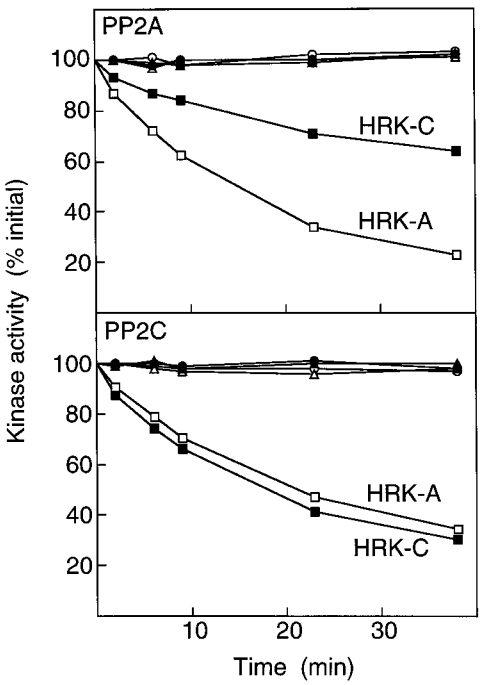

Figure 13. Inactivation of HRK-A (open symbols) and HRK-C (closed symbols) by PP2A (top) or PP2C (bottom). Incubations were performed with kinase alone (circles), kinase plus PP2A, or PP2C plus Mg^{2+} (squares), and kinase plus PP2A and okadaic acid, or PP2C minus Mg^{2+} (triangles). HRK-A and -C were purified as far as the Mono-Q column (Mg^{2+} gradient) but omitting NaF from the final purification buffer. The kinases were incubated at 30°C in 50 mM Na-Hepes, pH 7.0, 0.02% Brij-35 with PP2A. When added, PP2A or PP2C were at 10 units/mL, $MgCl_2$ at 10 mM, and okadaic acid at 100 nM. Samples were removed at various times for assay of peptide kinase activity. Results are expressed as the percentage of the initial activity.

among these various possibilities, which are not necessarily mutually exclusive.

The results in Figure 5 show that spinach HRK-A and HRK-C recognize primary sequence motifs on their substrates that are similar although not identical to those previously defined for mammalian AMPK, yeast SNF1, and cauliflower HRK-A (Dale et al., 1995b). Both require a bulky hydrophobic side chain at the P−5 position (i.e. five residues N-terminal to the phosphorylated Ser), since the peptide AGARAASAAALARRR was a poor substrate. The order of preference for particular hydrophobic residues at the P−5 position was Leu ≈ Ile > Met > Phe > Val for both kinases. Both kinases also preferred a bulky hydrophobic side chain at the P+4 position (i.e. four residues C-terminal to the phosphorylated Ser), since the peptide AMARAASAAAGARRR was a poor substrate, although substitution with Gly was much more deleterious for HRK-A than it was for HRK-C. Both kinases worked well with Leu, Ile, Met, or Phe at this position, but Val worked very poorly for HRK-A, although it was tolerated by HRK-C. Both kinases also required a basic residue at the P−3 position, since the peptide AMAGAASAAALARRR was a very poor substrate. The order of preference for both kinases was Arg > Lys > His. Another apparent difference between the two kinases was that HRK-C but not HRK-A would also phosphorylate the peptide AMRAAASAAALARRR, which has an Arg at the P−4 rather than the P−3 position. In this respect HRK-C is similar to mammalian AMPK and cauliflower HRK-A, although all AMPK/Snf1 kinases prefer the basic residue to be at the P−3 position.

Our studies show that the two Snf1-related protein kinases (HRK-A and HRK-C) from spinach also phosphorylated Arabidopsis HMG-CoA reductase, a key regulatory enzyme of isoprenoid biosynthesis. Phosphorylation occurs at a site (Ser-577) where phosphorylation was previously shown to cause complete inactivation (Dale et al., 1995a). HRK-A and HRK-C also phosphorylated and inactivated spinach NR, a key enzyme of nitrogen assimilation. Although the site phosphorylated on NR was not directly determined, the fact that phosphorylation caused inactivation, but only in the presence of the 14-3-3 protein, strongly suggests that it occurred at Ser-543 (Douglas et al., 1995; Bachmann et al., 1996a, 1996b; Moorhead et al., 1996; Su et al., 1996).

The results in this paper also show that HRK-A and HRK-C phosphorylate and inactivate SPS, a key regulatory enzyme of Suc synthesis. SPS is regulated by the allosteric activator Glc-6-P and the allosteric inhibitor Pi, as well as by phosphorylation (for review, see Huber and Huber, 1996). Phosphorylation of SPS by protein kinases, none of which have been well characterized, results in a decrease in activity under limiting conditions, i.e. low concentrations of the substrate Fru-6-P and a low ratio of Glc-6-P to Pi (Huber et al., 1989; Siegl and Stitt, 1990). Phosphorylation does not produce a change in activity under nonlimiting conditions (high Fru-6-P, high Glc-6-P, and no Pi). The inactivation under limiting conditions is produced by phosphorylation at a Ser residue corresponding to Ser-158 in spinach SPS (McMichael et al., 1993). The evidence that HRK-A and HRK-C regulate SPS by phosphorylation at Ser-158 may be summarized as follows: (a) Both kinases, which were extensively purified through five steps of purification, phosphorylated the 130-kD polypeptide of SPS, and this was associated with inactivation of the enzyme when the activity was measured in limiting but not nonlimiting conditions. (b) HRK-A and HRK-C phosphorylated spinach SPS at Ser-158, which was shown previously (McMichael et al., 1993) to be responsible for inactivation. (c) Both kinases were purified using the substrate peptide AMARAASAAALARRR, which contains the Hyd-X-Basic-X-X-Ser-X-X-X-Hyd motif that we have previously established as the minimal recognition motif (Dale et al., 1995b) for the cauliflower AMPK/SNF1 homolog (Ball et al., 1995) of SNF1. In all plant species in which this has been determined, the sequences around the phosphorylation site in SPS conform to this motif (Fig. 14).

SPS was also phosphorylated and inactivated by HRK-B and HRK-D, but these minor activities remain poorly characterized at present. As previously reported (Huber and Huber, 1990), partially purified SPS from spinach leaves contains an associated activity that inactivates the enzyme in an ATP-dependent manner. We found that our SPS preparations also contained activities that phosphorylated both the AMARA peptide and the 130-kD polypeptide. The

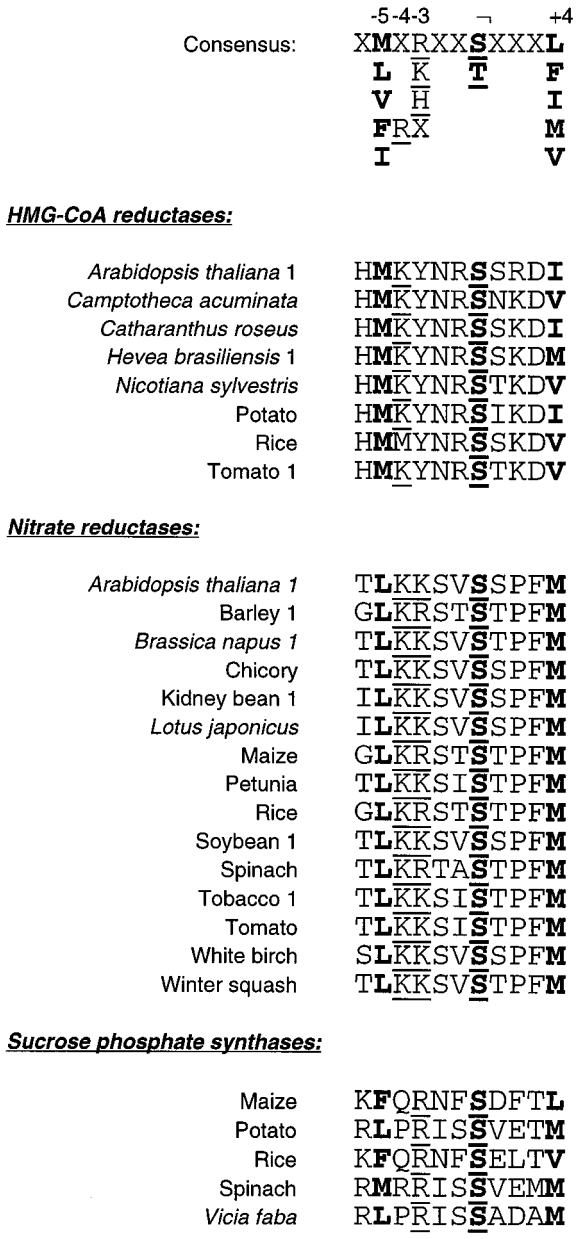

Figure 14. Consensus recognition motif for SnRK1 kinases (top), aligned with sequences around the regulatory phosphorylation sites on higher plant HMG-CoA reductases, NR, and SPS. Sequences were obtained from the SWISS-PROT and EMBL databases. Phosphorylated residues are in bold type and underlined; basic residues at P−3/P−4 are underlined, hydrophobic residues at P−5 and P+4 are in bold type. When a species has multiple isoforms only one is shown, although the other isoforms also conform to the motif.

SPS-inactivating activity was inhibited by the AMARA peptide, indicating that the peptide kinase and ATP-dependent SPS-inactivating activities may be functions of the same protein. All of these activities copurified with SPS through several conventional purification steps. Passing the sample through AMP-agarose was partially effective in removing the peptide/protein kinase and the SPS-inactivating activities (Fig. 8), but the key to production of an SPS preparation that was essentially free of these activities was the finding that the endogenous kinase(s) was inactivated by treatment with PP-2A. The identity of the kinase(s) contaminating the SPS preparations is not yet certain but may correspond to either or both HRK-A and HRK-C, since they also phosphorylate the AMARA and SP2 peptides and are inactivated by dephosphorylation (Fig. 13).

In Figure 14 we compare the consensus recognition motif for HRK-A and HRK-C with the sequences around Ser-577 on HMG-CoA reductase, Ser-543 on NR, and Ser-158 on SPS from several plant species. It is evident that the recognition motif is conserved in all higher-plant HMG-CoA reductase, NR, and SPS sequences currently in the sequence databases. This lends further support to the idea that these anabolic enzymes may be targets for the SnRK1 kinases in vivo.

It is important to compare our results with those of previous workers who have partially purified NR or SPS kinases from spinach leaf. Using a different extraction procedure, which omitted the protein phosphatase inhibitors EDTA and EGTA, McMichael et al. (1995a) resolved three NR/SPS kinases (peaks I, II, and III) by chromatography on Resource-Q. Peaks I and II were Ca^{2+} dependent and therefore unrelated to the kinases described here. Peak III was Ca^{2+} independent, inactivated SPS with little or no effect on NR, and had an apparent molecular mass of 150 kD on gel filtration. It therefore seems likely that peak III corresponded to either HRK-A or HRK-C, although based on its elution position on Resource-Q it is more similar to HRK-C. One thing that remains unclear is why peak III was reported to have such a low NR-inactivating activity (McMichael et al., 1995a).

A later study by Douglas et al. (1997) facilitates the comparison between our work and that of McMichael et al. (1995a). They extracted spinach leaves under conditions resembling those of McMichael et al. and under conditions more closely resembling ours (i.e. the presence of 50 mM NaF, EDTA, and EGTA). Although the preliminary purification steps were somewhat different from ours, they separated the protein kinases on a Mono-Q column using similar buffers and gradients and resolved three peaks of protein kinase activity (PK_I–PK_{III}) using as a substrate either the SP or the SAMS peptide. When prepared in the absence of NaF, PK_I and PK_{II} were Ca^{2+} dependent and therefore appear to correspond to peaks I and II of McMichael et al. (1995a). PK_{III} was Ca^{2+} independent under all conditions and appears to correspond to our HRK-C and to peak III of McMichael et al.

The similarities between PK_{III} and HRK-C may be summarized as follows: (a) They phosphorylate both AMARA and SAMS, with the former being the better substrate; (b) they phosphorylate Arabidopsis HMG-CoA reductase at Ser-577; (c) they elute from Mono-Q at the same salt concentration; (d) they have apparent molecular masses of approximately 150 kD on Superdex 200 chromatography; and (e) they both require a hydrophobic residue at the P+4 position. We suspect that we did not observe either of the Ca^{2+}-dependent protein kinases because AMARA and SAMS are relatively poor substrates (Douglas et al., 1997).

Douglas et al. (1997) also detected a 58-kD polypeptide in western blots of fractions containing PK_{III} using an antibody raised against a fusion protein between rye SnRK1 and maltose-binding protein. However, the PK_{III} activity did not exactly comigrate with the 58-kD polypeptide, whereas in our study it did (using the NIP peptide antibody). In our experience the RKIN1-MBP antibody was much less specific than the anti-NIP peptide antibody used here, and we propose that the 58-kD polypeptide detected using the former antibody (Douglas et al., 1997) may have been unrelated to the protein kinase activity detected. HRK-C was purified more extensively in this study than was PK_{III} and in our opinion the evidence in this paper that it is a member of the SnRK1 family is stronger than that presented by Douglas et al. (1997).

We therefore propose that the peak III SPS kinase (McMichael et al., 1995a) and the PK_{III} NR kinase (Douglas et al., 1997) both correspond to HRK-C, which is now firmly identified by our work as a member of the SnRK1 subfamily. In addition, there is a second member of the subfamily in spinach leaf (HRK-A), which appears to have been missed in these previous studies. HRK-A was probably not observed by McMichael et al. (1995a) because it is particularly susceptible to dephosphorylation by PP2A (Fig. 13), and because they did not include protein phosphatase inhibitors during purification. PK_{II} of Douglas et al. (1997), which was not studied in detail, was only partially Ca^{2+} dependent and may represent a mixture of HRK-A and a Ca^{2+}-dependent kinase, probably a CDPK, as suggested by Bachmann et al. (1996b).

Using synthetic peptide substrates, Huber and coworkers (McMichael et al., 1995b; unpublished data cited by Huber and Huber [1996]) proposed for peak III the recognition motif Basic-Hyd-X-Basic-X-X-Ser: basic residues at P−6 and P−3 (i.e. six and three residues upstream of the phosphorylated Ser) and a hydrophobic residue at P−5. This is similar but not identical to the motif (Hyd-X-Basic-X-X-Ser-X-X-X-Hyd) we have defined for cauliflower HRK-A and spinach HRK-A/HRK-C, i.e. hydrophobic residues at P−5 and P+4, and a basic residue at P−3 (Dale et al., 1995b and this study). This apparent discrepancy does not necessarily indicate that peak III and HRK-C are different. We have not specifically addressed the requirement for a basic residue at the P−6 position. Although the AMARA peptide has an Ala at this position, which might appear to indicate that a basic residue was not necessary, this is the N terminus of the peptide and it is possible that the free α-amino group fulfils the normal requirement for a basic residue. It is interesting that the SAMS peptide originally used to assay the plant AMPK-related kinases (MacKintosh et al., 1992; Ball et al., 1994) has a His at P−6, whereas most of the natural substrates for mammalian AMPK also have a basic residue at this position (Weekes et al., 1993). Toroser and Huber (1998), using variants of the SP2 peptide, recently reported that a Ca^{2+}-independent kinase (peak III) from cauliflower florets that is probably identical to HRK-A preferred a basic residue at the P−6 position. The apparent discrepancy in the requirement for a hydrophobic residue at the P+4 position may also be more apparent than real. Although both HRK-A and HRK-C preferred a hydrophobic residue at this position, it was only essential for HRK-A (Fig. 5). McMichael et al. (1995b) specifically address the requirement for a hydrophobic residue at this position. In any case, the sequence around Ser-158 predicted by all known SPS DNAs (Fig. 14) conforms both to our motif and to that established by Huber and coworkers.

McMichael et al. (1995b) reported that the peptide kinase activity of their peak III kinase was 54% inhibited by 10 mM Glc-6-P, implying that the latter might be a physiological regulator of SnRK1 kinases as well as an activator of SPS. Some commercial preparations of Glc-6-P did yield a similar inhibition of HRK-A and HRK-C, but others did not. This effect was eventually traced to contaminants in the Glc-6-P, probably Ba^{2+} ions. Although we cannot be certain that the preparation used by McMichael et al. (1995b) was similarly contaminated, this shows that caution is necessary when utilizing commercial sources of Glc-6-P that are not further purified.

Another theme that emerges from our study is that none of the peptide or protein substrates currently available are specific for a single protein kinase, and therefore molecular probes (e.g. immunoprecipitating antibodies) are required to develop specific assays for individual kinases. Detailed studies of the physiological roles of these kinases are probably premature until such specific assays are developed. Unfortunately, biochemical approaches (i.e. purification from plants) remain necessary to study the biochemical functions of members of the SnRK1 family, because production of catalytically active kinases from SnRK1 DNAs has not yet proved successful. Based on experience with the mammalian AMPK system (Dyck et al., 1996; Woods et al., 1996), this is probably because coexpression of the accessory β- and γ-subunits (not yet cloned from plants) is necessary to observe kinase activity when the catalytic (α) subunit DNA is expressed.

ACKNOWLEDGMENTS

We thank Simon Hawley for purified AMPKK, Albert Ferrer for provision of Arabidopsis HMG-CoA reductase, Hendrik Weiner for provision of SPS antibodies, Carol MacKintosh, Pauline Douglas, Greg Moorhead, and Sarah Meek (Dundee University) for providing yeast 14-3-3 protein and for helpful advice concerning NR, Tim Haystead for ATP-γ-Sepharose, Nick Helps for PP-2C, Barry Caudwell for solid-phase sequencing, and Valerie Pacquit for assistance with kinase preparations.

Received October 16, 1998; accepted January 28, 1999.

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