Immunological evidence that HMG-CoA reductase kinase-A is the cauliflower homologue of the RKIN1 subfamily of plant protein kinases

Kathryn L. Ball[a], Jacqueline Barker[b], Nigel G. Halford[b], D. Grahame Hardie[a,*]

[a]*Department of Biochemistry, The University, Dundee DD1 4HN, Scotland, UK*
[b]*IACR, Long Ashton Research Station, Department of Agricultural Sciences, Bristol University, Bristol BS18 9AF, UK*

Received 6 November 1995

Abstract Three different antibodies against the *RKIN1* and *BKIN12* gene products from rye and barley recognized the 58 kDa subunit of HMG-CoA reductase kinase-A (HRK-A) from *Brassica oleracea* on Western blots. HRK-A was also detected by an antipeptide antibody in enzyme-linked immunoassays, and this was competed by the peptide antigen. HRK-A was not recognized by antibodies against plant, mammalian and *Saccharomyces cerevisiae* relatives of RKIN1, i.e. wheat PKABA1, rat AMP-activated protein kinase and *S. cerevisiae* Snf1p. RKIN1/HMG-CoA reductase kinase-A are now among the first protein kinases in plants to be well characterized at both the molecular and biochemical levels.

Key words: HMG-CoA reductase kinase; AMP-activated protein kinase; SNF1; RKIN1; BKIN12; AKIN10

1. Introduction

We have recently purified and characterized a protein kinase, termed HMG-CoA reductase kinase-A (HRK-A), from the developing inflorescence of the cauliflower (*Brassica oleracea*) [1,2]. By biochemical criteria, the protein kinase was very similar to mammalian AMP-activated protein kinase (AMPK) in that it had a very similar substrate specificity against proteins and peptides [1,3,4], and in that it was inactivated by mammalian protein phosphatase-2A and reactivated by the mammalian protein kinase which lies upstream of AMPK, AMP-activated protein kinase kinase. Mammalian AMPK phosphorylates and inactivates a number of metabolic enzymes, including the key regulatory enzyme of sterol/isoprenoid synthesis, HMG-CoA reductase [5,6]. It is thought to exert a protective role when the cellular energy status is compromised during environmental stress, by inhibiting biosynthetic pathways [7]. The physiological substrates of HRK-A remain unknown, although we have recently shown that it inactivates HMG-CoA reductase (*HMG1* isoform) from *Arabidopsis thaliana* by phosphorylating serine-577, exactly equivalent to the site at which AMPK phosphorylates mammalian HMG-CoA reductase [8].

It is now clear that AMPK is the mammalian homologue of the *Saccharomyces cerevisiae* SNF1 protein kinase, which comprises a minimal complex of the *SNF1* and *SNF4* gene products, and is involved in yeast in the response to nutritional stress (starvation for glucose) [9–12]. A number of DNA sequences encoding proteins related to Snf1p have been cloned from higher plants, including *RKIN1* from rye [13], *BKIN12* and *BKIN2* from barley [14,15], *AKIN10* from *A. thaliana* [16], and *NPK5* from tobacco [17]. These appear to be true homologues of *SNF1*, since *RKIN1* or *NPK5* DNAs rescue *snf1* disrupted mutants of *S. cerevisiae* in that they allow growth on non-fermentable carbon sources [13,17]. However apart from this relationship with Snf1p, nothing was known about the biochemical properties or function of the RKIN1 subfamily of plant protein kinases. We recently identified the catalytic subunit of HRK-A as a polypeptide of 58 kDa [2]. Since the DNAs encoding the plant Snf1p homologues predicted protein products of this size, and given the biochemical similarities between AMPK and HRK-A, it seemed likely that the latter was the cauliflower homologue of the *RKIN1* gene product and other plant SNF1-related kinases. In this paper this has been tested using antibodies raised against the *RKIN1* and *BKIN12* gene products.

2. Materials and methods

2.1. Materials and antibodies

The 'NIP' antipeptide antibody was raised in rabbits using the peptide PFDDDNIPNLFKKIK, corresponding to the predicted amino acid sequence from residues 214 to 228 of the rye *RKIN1* gene product. The peptide was coupled to bovine serum albumin. Antibodies were also raised in rabbits against two bacterially expressed fusion proteins. MBP-RKIN1 contained the maltose binding protein MalE fused to the entire open reading frame of the rye *RKIN1* gene product. MBP-BKIN12 contained MalE fused to the C-terminal 40% of the barley BKIN12 gene product, commencing at residue 304 of the latter. Antibodies against wheat PKABA1, *S. cerevisiae* Snf1p and rat AMPK were gifts (see Acknowledgements). The horseradish peroxidase-conjugated swine anti-rabbit secondary antibody was from Dako. Other materials were as described [2].

2.2. Purification and assay of HRK-A

HRK-A was purified and assayed as described [2] as far as step 4 (Mono Q). It was further purified by chromatography on ATP-γ-Sepharose as described for AMPK [18], using ATP rather than AMP for elution. This yielded kinase with a similar specific activity to that obtained with the six step procedure used previously [2]. By Coomassie Blue staining the 58 kDa polypeptide was a major component, but other polypeptides were also evident (not shown).

2.3. Western blotting

Proteins were separated by SDS-PAGE in 10% gels [19] and transferred electrophoretically to nitrocellulose. Filters were blocked in 5% non-fat dried milk in phosphate-buffered saline [20] containing 0.2% Tween 20 (PBST) for 1 h, washed in PBST and incubated with antisera diluted (as described in the figure legends) with 5% non-fat dried milk-PBST for 1 h. Filters were washed with PBST, and incubated for a

*Corresponding author. Fax: (44) (382) 20-1063.
E-mail: d.g.hardie@dundee.ac.uk

Abbreviations: AMPK, AMP-activated protein kinase; HMG-, 3-hydroxy-3-methylglutaryl-; HRK-A, HMG-CoA reductase kinase-A; HRK-B, HMG-CoA reductase kinase-B; SNF1, sucrose non-fermenting-1; ELISA, enzyme-linked immunoassay; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing Tween 20.

SSDI 0014-5793(95)01343-1

further 1 h with horseradish peroxidase-conjugated swine anti-rabbit antibody diluted 1:1000 in 5% milk-PBST. After removing excess secondary antibody by washing with PBST, bound protein was detected by enhanced chemiluminescence (ECL) reaction using Hyperfilm-MP (Amersham, UK).

2.4. Enzyme-linked immunoassays

Purified HRK-A was coated onto ELISA plates (10–100 ng/well) in 50 mM bicarbonate buffer and incubated overnight at 4°C, then washed with 3 × phosphate-buffered saline (PBS; [20]). The plates were blocked with 5% non-fat dried milk-PBS for 2 h at room temperature. Primary antibody ('NIP' peptide antisera diluted 1:500 in 3% milk-PBS with or with out 'NIP' peptide) was added to the plates for 3 h at room temperature, the plates were washed with 3 × PBS and the antibody was detected with a secondary horseradish peroxidase-conjugated swine anti-rabbit antibody diluted 1:1000 in 3% non-fat dried milk-PBS. Binding was quantitated using *N,N,N',N'*-tetramethyl benzidine with H_2O_2 [20]. After neutralisation with H_2SO_4, the plates were read using a Dynatech ELISA plate reader.

3. Results

3.1. An antipeptide antibody against RKIN1 recognizes the 58 kDa polypeptide of HRK-A

An antibody was raised against the peptide PFDDDNIPNLFKKIK, corresponding to the predicted amino acid sequence from residues 214 to 228 of the rye *RKIN1* gene product. In the sequences of other SNF1-related protein kinases from barley, *A. thaliana*, tobacco and potato (N.G.H., unpublished) at least 13 of these 15 residues are conserved. The antibody was used to screen pooled fractions obtained at each step during the purification of HRK-A from *B. oleracea*. Fig. 1 (left panel) shows that after DEAE-Sepharose chromatography the antibody recognized a polypeptide migrating with an apparent molecular mass of ≈58 kDa, which became a larger proportion of total protein as the purification progressed through the Blue-Sepharose, Mono Q and ATP-γ-Sepharose steps. At some stages of purification the 58 kDa polypeptide appears to migrate as a doublet. Using the ATP-γ-Sepharose purified HRK-A, detection of the polypeptide was completely abolished by competition with the 'NIP' peptide used to raise the antibody (Fig. 1, right panel). We have previously identified the 58 kDa polypeptide as the catalytic subunit of HRK-A both by autophosphorylation, and by labelling with [^{14}C]fluorosulphonylbenzoyl adenosine, a reactive ATP analogue which inactivated HRK-A [2]. The reason why it often migrates as a doublet remains unclear: there is no shift in mobility when HRK-A is inactivated by treatment with protein phosphatase-2C (C. Sugden and D.G.H., unpublished).

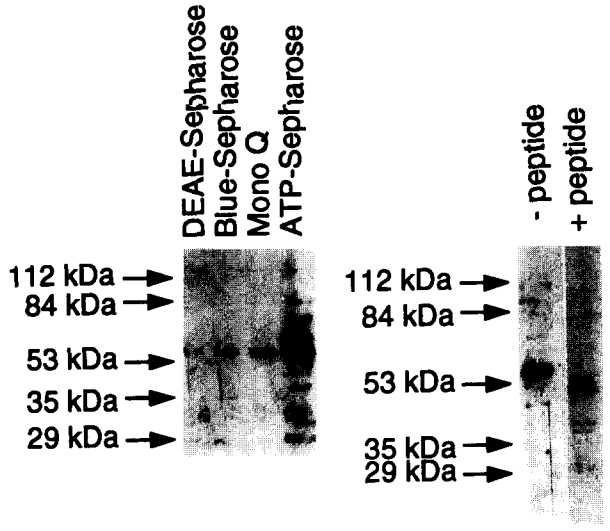

Fig. 1. (Left panel) Western blotting of various fractions using RKIN1 antipeptide antiserum. Samples of HRK-A after purification through DEAE-Sepharose (25 μg), Blue Sepharose (25 μg), Mono Q (25 μg) and ATP-Sepharose (0.5 μg) were analysed by SDS-PAGE, transferred to nitrocellulose, and probed using antipeptide antiserum at 1:500 final dilution. (Right panel) ATP-Sepharose purified HRK-A was analysed as above except that the probing with antibody was in the presence or absence of 1 mg/ml of the antigen peptide. Mobilities of pre-stained molecular weight markers (Sigma) are shown.

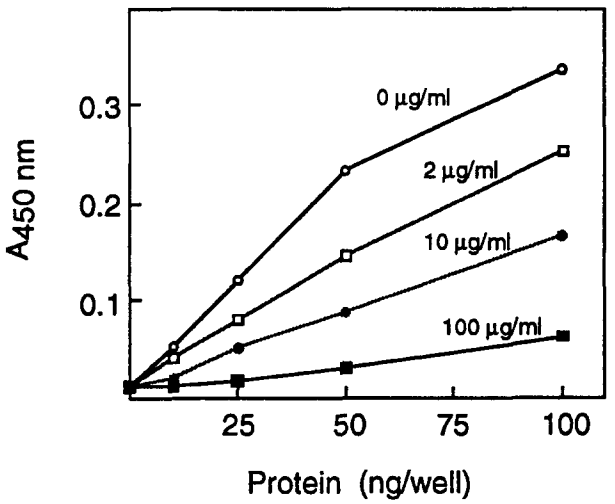

Fig. 2. Enzyme-linked immunoassay using increasing amounts of HRK-A protein (0–100 ng/well, ATP-Sepharose step) bound to the ELISA plate, showing the effect of competition with increasing amounts (0–100 μg/ml) of antigen peptide.

We also analysed the reaction between ATP-γ-Sepharose purified HRK-A and the antiserum using enzyme-linked immunoassay (ELISA). Fig. 2 shows that the antiserum bound to HRK-A which was coated onto the wells of an ELISA plate, but that increasing concentrations of the 'NIP' peptide antigen progressively prevented this binding and eventually reduced it to background levels.

3.2. The 'NIP' antipeptide antibody recognizes HRK-A but not HRK-B

During the purification of HRK-A from *B. oleracea* we had detected a second protein kinase activity termed HMG-CoA reductase kinase-B (HRK-B). HRK-B had a very similar specificity to HRK-A and was also inactivated by dephosphorylation, but differed in having a smaller subunit molecular mass (40–45 kDa vs. 58 kDa for HRK-A) and a smaller native molecular mass (≈45 kDa vs. ≈200 kDa) [2]. Fig. 3 shows Western blotting across the first Mono Q column (step 3), which is the first step in which HRK-A and -B are fully resolved. This revealed that the 58 kDa doublet detected by the antibody exactly comigrated with HRK-A activity, but that the antibody did not recognize HRK-B.

3.3. Detection of HRK-A using other antibodies to SNF1-related protein kinases

While this work was in progress, two additional antibodies

against the plant RKIN1/BKIN12 protein kinases became available. They were raised against two bacterially expressed fusions with maltose binding protein (MalE), i.e. MBP-RKIN1, which contained MalE plus the entire coding region of rye RKIN1, and MBP-BKIN12, which contained MalE plus the C-terminal 40% of BKIN12, i.e. excluding the kinase domain. Fig. 4 shows that both of these antisera recognized the 58/56 kDa doublet of HRK-A purified to the ATP-γ-Sepharose step, although the 'NIP' antipeptide antiserum gave a stronger signal. At the same dilution the various preimmune sera did not react with any polypeptides in these preparations (not shown).

In contrast to the results with the anti-RKIN1 and anti-BKIN12 sera, the 58 kDa subunit was not recognized (not shown) by antipeptide antibodies raised against sequences from rat AMPK, *S. cerevisiae* Snf1p, or wheat PKABA1 [21]. The latter is a representative of a subfamily of plant protein kinases which are also related to Snf1p, albeit somewhat more distantly than RKIN1/BKIN12 [22].

4. Discussion

These results provide strong evidence that HRK-A from *B. oleracea* represents the homologue of the plant SNF1-related protein kinases RKIN1, BKIN2, BKIN12, AKIN10 and NPK5. The evidence may be summarized as follows.

1) The 58 kDa polypeptide previously identified as the catalytic subunit of HRK-A [2] was recognized by antibodies raised against a peptide whose sequence is highly conserved in all of the known members of the plant SNF1-related protein kinase subfamily. In both Western blots and enzyme-linked immunoassays, binding of the antibody to HRK-A was completely abolished by increasing concentrations of the synthetic peptide antigen. The 58 kDa polypeptide was also recognized by antibodies raised against expressed recombinant *RKIN1* from rye and *BKIN12* from barley.

2) The predicted molecular masses of the rye *(RKIN1)*, barley *(BKIN12)*, *A. thaliana (AKIN10)* and tobacco *(NPK5)* gene products all lie within the range 57,700–58,700 Da [13,14,16,17], consistent with the estimated molecular mass of the catalytic subunit of HRK-A by SDS-PAGE [2].

3) HRK-A has a specificity for synthetic peptides almost identical with those of rat AMPK and *S. cerevisiae* SNF1 [4]. RKIN1 clearly represents the rye homologue of Snf1p, since DNA encoding RKIN1 rescues yeast *snf1* mutants, while

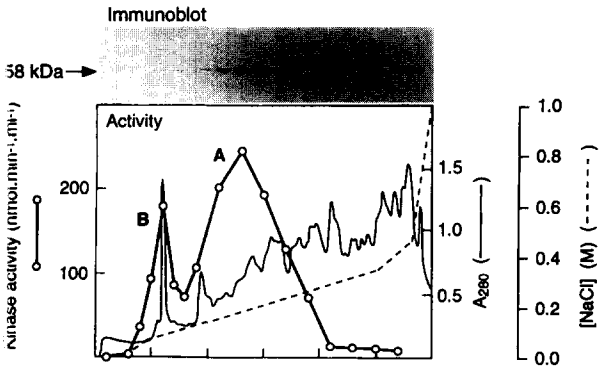

Fig. 3. Separation of HRK-A and HRK-B during Mono Q chromatography showing (upper panel) Western blotting of fractions across the column with RKIN1 antipeptide antiserum.

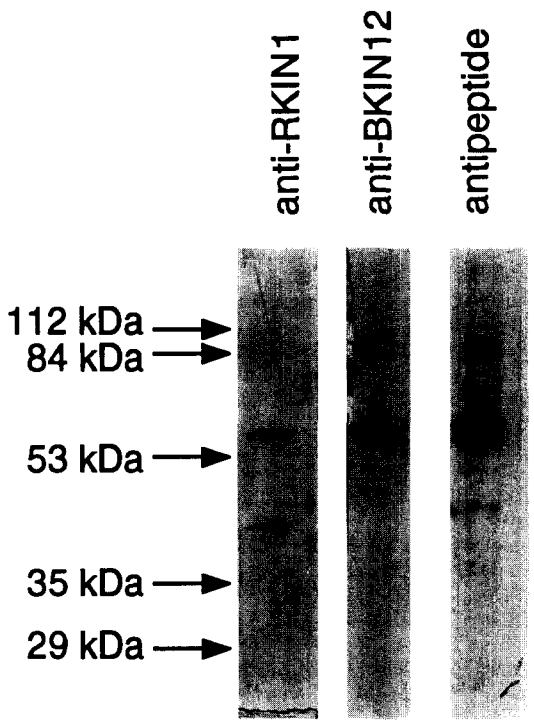

Fig. 4. Western blotting of ATP-Sepharose purified HRK-A (0.5 μg) using antisera raised against a full-length expressed RKIN1 fusion protein at a dilution of 1:250 (anti-RKIN1), against a BKIN12 fusion protein at 1:250 (anti-BKIN12) and against the 'NIP' peptide at 1:500 (antipeptide).

RKIN1 and Snf1p are 60% identical in sequence (within the kinase domain) at the amino acid level [13].

We regard these findings as important because, although at least 40 putative protein kinases have now been cloned from higher plants [23], the biochemical properties or physiological targets were not known for any of them, other than by analogy with animal or yeast homologues. By contrast, other plant protein kinases such as those acting on pyruvate dehydrogenase, phosphoenolpyruvate carboxylase, sucrose phosphate synthase and nitrate reductase are characterized at the biochemical level, but not at the molecular level. The HRK-A/RKIN1 group now represent the first plant protein kinase subfamily which are not only well defined at the molecular level, but for which the biochemical properties (including the motif for recognition of targets [4]) are also well characterized. At least one potential physiological target (HMG-CoA reductase) for HRK-A has already been identified [8], although there are likely to be others. The exact physiological function of the HRK-A/RKIN1 subfamily remains unclear, but the established roles of the animal and yeast homologues in the response to environmental and nutritional stress provide clues for framing testable hypotheses. With the molecular probes and biochemical assays now available to us, it should prove possible to define the physiological roles of the plant SNF1-related protein kinases.

Acknowledgements: This work was supported by a Project Grant to D.G.H. from the BOMRIP inititative of the UK Biotechnology and Biological Sciences Research Council (BBSRC). IACR receives grant-aided support from the BBSRC. N.G.H. was also supported by the

European Communities BIOTECH programme, as part of the Project of Technological Priority 1993-6. We thank Peter Shewry for helpful discussions, and the following for donation of additional antisera: David Carling (AMPK), Marian Carlson (SNF1) and Kay Walker-Simmons (PKABA1).

References

[1] MacKintosh, R.W., Davies, S.P., Clarke, P.R., Weekes, J., Gillespie, J.G., Gibb, B.J. and Hardie, D.G. (1992) Eur. J. Biochem. 209, 923–931.
[2] Ball, K.L., Dale, S., Weekes, J. and Hardie, D.G. (1994) Eur. J. Biochem. 219, 743–750.
[3] Weekes, J., Ball, K.L., Caudwell, F.B. and Hardie, D.G. (1993) FEBS Lett. 334, 335–339.
[4] Dale, S., Wilson, W.A., Edelman, A.M. and Hardie, D.G. (1994) FEBS Lett. 361, 191–195.
[5] Clarke, P.R. and Hardie, D.G. (1990) EMBO J. 9, 2439–2446.
[6] Gillespie, J.G. and Hardie, D.G. (1992) FEBS Lett. 306, 59–62.
[7] Corton, J.M., Gillespie, J.G. and Hardie, D.G. (1994) Curr. Biol. 4, 315–324.
[8] Dale, S., Arró, M., Becerra, B., Morrice, N.G., Boronat, A., Hardie, D.G. and Ferrer, A. (1995) Eur. J. Biochem. (in press).
[9] Mitchelhill, K.I., Stapleton, D., Gao, G., House, C., Michell, B., Katsis, F., Witters, L.A. and Kemp, B.E. (1994) J. Biol. Chem. 269, 2361–2364.
[10] Stapleton, D., Gao, G., Michell, B.J., Widmer, J., Mitchelhill, K., Teh, T., House, C.M., Witters, L.A. and Kemp, B.E. (1994) J. Biol. Chem. 269, 29343–29346.
[11] Woods, A., Munday, M.R., Scott, J., Yang, X., Carlson, M. and Carling, D. (1994) J. Biol. Chem. 269, 19509–19515.
[12] Carling, D., Aguan, K., Woods, A., Verhoeven, A.J.M., Beri, R.K., Brennan, C.H., Sidebottom, C., Davison, M.D. and Scott, J. (1994) J. Biol. Chem. 269, 11442–11448.
[13] Alderson, A., Sabelli, P.A., Dickinson, J.R., Cole, D., Richardson, M., Kreis, M., Shewry, P.R. and Halford, N.G. (1991) Proc. Natl. Acad. Sci. USA 88, 8602–8605.
[14] Halford, N.G., Vicente-Carbajosa, J., Sabelli, P.A., Shewry, P.R., Hannapel, U. and Kreis, M. (1992) Plant J. 2, 791–797.
[15] Hannappel, U., Vicentecarbajosa, J., Barker, J.H.A., Shewry, P.R. and Halford, N.G. (1995) Plant Mol. Biol. 27, 1235–1240.
[16] LeGuen, L., Thomas, M., Bianchi, M., Halford, N.G. and Kreis, M. (1992) Gene 120, 249–254.
[17] Muranaka, T., Banno, H. and Machida, Y. (1994) Mol. Cell. Biol. 14, 2958–2965.
[18] Davies, S.P., Hawley, S.A., Woods, A., Carling, D., Haystead, T.A.J. and Hardie, D.G. (1994) Eur. J. Biochem. 223, 351–357.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Harlow, E. and Lane, D. (1988) Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 726 pp.
[21] Anderberg, R.J. and Walker-Simmons, M.K. (1992) Proc. Natl. Acad. Sci. USA 89, 10183–10187.
[22] Hardie, D.G., Carling, D. and Halford, N.G. (1994) Semin. Cell Biol. 5, 409–416.
[23] Hanks, S.K. and Hunter, T. (1995) in: (Hardie, D.G. and Hanks, S.K., eds.) vol. 1, pp. 7–47, Academic Press, London.