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Production and Characterization of an Antibody Specific for a Novel Protein Serine/Threonine Kinase, MPK38, Highly Expressed in Hematopoietic Cells

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Abstract

We report an antibody that selectively recognizes MPK38, a new protein serine/threonine kinase closely related to the SNF1 serine/threonine kinase family. This antibody recognized a region of the N-terminal kinase catalytic domain and part of the remaining C-terminal portion and was sensitive enough to detect a 72-kDa recombinant MPK38 in insect cells by Western blotting. Immunoblot analysis showed that the recombinant MPK38 was expressed in a time-dependent manner and reached a maximum after 48 h postinfection. In addition, the immune complex kinase assay revealed that the recombinant and endogenous MPK38 protein autophosphorylated in vitro. Phosphoamino acid analysis of autophosphorylated MPK38 protein showed that the phosphorylation was exclusively on serine and threonine residues, suggesting that MPK38 is a protein serine/threonine kinase. Thus, this antibody could be helpful for elucidating the biological functions of MPK38 in the MPK38-expressing cells.

Index Entries: Anti-MPK38 antibody; serine/threonine kinase; autophosphorylation; signal transduction; recombinant protein.

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Introduction

Protein phosphorylation plays a cardinal role in many signal transduction pathways. In higher eukaryotes, the regulation of protein phosphorylation is important in cellular events such as proliferation, oncogenesis, differentiation, and development *(1)*. The importance of protein kinases in regulating phosphorylation is underscored by the large number of protein kinase genes present in eukaryotes *(2)*. Recently nearly 2000 conventional protein kinases have been estimated. All protein kinases have been classified into two broad subfamilies based on substrate specificity: serine/threonine specific and tyrosine specific *(3)*.

We previously reported the cloning of MPK38 gene, a novel protein serine/threonine kinase, from a cDNA library constructed from the murine teratocarcinoma PCC4 cell line *(4)*. MPK38 was also recently cloned and given the name Melk for maternal embryonic leucine zipper kinase by Heyer et al. *(5)*. MPK38 is a putative member of the serine/threonine kinase family and shows extensive amino acid sequence homology with the SNF1 serine/threonine kinase family. In budding yeast, the derepression of the glucose-repressible genes is required for the function of a complex signaling when the yeast is deprived of glucose in the environment. One of these, SNF1, encodes a protein serine/threonine kinase and plays a major role in regulating glucose-repressed genes in response to glucose limitation *(6)*. Recently, several SNF1-related kinases have been identified from both mammals and plants and have been shown to complement functionally the sucrose nonfermenting 1 (snf1) mutants that are unable to utilize sucrose, raffinose, galactose, maltose, and other sugars as a carbon source *(7–11)*. Su et al. *(12)* recently reported that a novel serine/threonine kinase, called XEEK1, expressed in *Xenopus* was most similar to the SNF1 kinase with about 65% similarity within the catalytic domain, and that it could not functionally complement on snf1 mutation in yeast, suggesting that XEEK1 is not functionally related to the yeast SNF1 kinase or its related kinases. Therefore, it has been unclear whether or not the MPK38 kinase exhibiting approx 60% protein sequence identity can complement the yeast snf1 mutation.

In the present study, to provide a system for functional analysis of MPK38, we have expressed a truncated version encoding the catalytic kinase domain of MPK38 as a T7·Tag-tagged recombinant protein in bacteria and generated a specific antibody against the N-terminal catalytic domain of MPK38. In addition, we describe the biochemical characterizations of the MPK38 kinase.

Materials and Methods

Cell Culture and Reagents

Murine hematopoietic lineage cell lines (R1.1 and EL4, T lymphomas; A20, B lymphoma) were maintained in RPMI-1640 medium supplemented

with 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD) and 2 m*M* glutamine by standard procedures *(13)*. The plasmid vector pET-24a for expression in bacteria was purchased from Novagen (Madison, WI), and pGEX4T-1 was from Pharmacia (Uppsala, Sweden). Isopropyl-β-D-thiogalactopyranoside (IPTG), dithiothreitol (DTT), aprotinin, and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma (St. Louis, MO). [γ-^{32}P] ATP was obtained from Amersham International (Amersham, UK). Polyvinylidene difluoride (PVDF) membrane and peroxidase-conjugated donkey antirabbit IgG antibody were purchased from Millipore (Bedford, MA) and Pierce (Rockford, IL), respectively. Oligonucleotides were synthesized from Bioneer (Cheongwon, Chungbuk, Korea).

Plasmid Construction and Expression of Recombinant MPK38

One set of oligonucleotide primers corresponding to the full-length cDNA of the MPK38 gene was synthesized as follows: forward primer, 5'-CGCGGATCCATGAAAGATTATGAC-3'; reverse primer, 5'-CCGCTC GAGTCACATCTTGCAGCC-3'. The full-length cDNA of the MPK38 was directly used as the template for amplification. Polymerase chain reaction was performed on a DNA thermal cycler (Ericomp, San Diego, CA) as previously described *(13)*. The cycling parameters were 30 s at 94°C, 2 min at 54°C, and 1 min at 72°C. The amplified cDNA encoding the MPK38 kinase was subcloned into the *Bam*HI and *Xho*I sites of pGEX4T-1 and, after digestion with *Bam*HI and *Eco*RI, subcloned into the pET-24a. Cloned DNA (pET-MPK38) was subjected to DNA sequencing analysis on both strands by the dideoxynucleotide chain termination method with the T7 sequencing™ kit (Pharmacia). Similarly, pBac-MPK38, a plasmid vector for expression in insect cells, was constructed by ligating a *Bam*HI/*Xho*I cDNA fragment encoding amino acid 1 through the stop codon of MPK38 into the *Bam*HI/*Xho*I sites of pBacPAK9 (Clontech, Palo Alto, CA). The pET-MPK38 plasmid was introduced into *Escherichia coli* (BL21), and a single colony of the transformed cells was inoculated into Luria broth (LB) medium containing 10% tryptophan, 10% NaCl, and 5% yeast extract and grown overnight at 37°C. The seed culture was inoculated into a 100-fold volume of fresh LB medium containing 20 µg/mL of ampicillin and cultured for 3 h at 37°C. To this culture a final concentration of 1 m*M* IPTG was added and cultured for an additional 3 h at 37°C. The induced cell lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining.

Preparation of Antiserum

The anti-MPK38 polyclonal rabbit antiserum was raised against pET-MPK38 bacterial protein (see previous section). To purify the recombinant MPK38 protein, the transformed cells were induced in a shake flask containing in total 200 mL of culture and 1 m*M* IPTG at 37°C for 6 h, sonicated, and centrifuged at 14,328*g* for 20 min. The inclusion body was solubilized

with 8 *M* urea, and after electrophoresis, the recombinant protein band was excised. The gel fragment was then washed with phosphate-buffered saline (PBS) (137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* $Na_2HPO_4 \cdot H_2O$, 1.4 m*M* KH_2PO_4) and homogenized in the same volume of PBS. The resulting protein (100 µg) was emulsified in Freund's complete adjuvant and injected intramuscularly at several sites. The animals were boosted three times every 2 wk with the emulsified proteins in Freund's incomplete adjuvant and bled from the ear vein 10 d after the last injection. Titer of the antiserum was measured by Western blotting at each booster. Antiserum was removed by centrifugation (896*g*, 15 min) after incubation at 37°C for 3 h.

Western Blotting

After SDS-PAGE, proteins were transferred to a PVDF membrane. Blocking was performed overnight in PBS containing 5% (w/v) skim milk (Difco, Detroit, MI). After incubation with anti-MPK38-specific polyclonal antibody (diluted 1:1000) for 2 h, blots were subsequently washed three times with PBST (0.05% Tween-20 and 3% bovine serum albumin in PBS), then incubated with peroxidase-conjugated donkey antirabbit IgG antibody (diluted 1:5000), washed again with PBST, and developed using an ECL detection system (Amersham).

Preparation and Characterization of Recombinant MPK38 in Insect Cells

Sf21 cells were maintained at 27°C in supplemented Grace's insect medium containing 10% FBS. Recombinant baculovirus was produced by cotransfecting Sf21 cells with pBac-MPK38 recombinant vector and Bsu36 I-digested BacPAK6 viral DNA (Clontech) using the Lipofectin (Gibco BRL) method. To obtain recombinant virus, after incubation at 27°C for 72 h, the supernatant was collected by centrifugation. To amplify the recombinant virus, Sf21 cells (5×10^5/well) were seeded in six-well flat-bottomed microplates (Costar, Cambridge, MA) and cultured at 27°C for 2 h. After removal of the medium, the cells were infected with 100 µL of the supernatant containing the recombinant virus and incubated for 1 h. Then 2 mL of Grace's insect medium containing 10% FBS was added and incubated at 28°C for 3 to 4 d until the cells looked well infected. To determine the production of recombinant MPK38, the cells (4×10^6) were seeded on 75-cm^2 flasks and maintained for 24 h, and the medium was removed from the cells. Cells were infected with the recombinant virus and incubated at room temperature for 1 h, and 10 mL of Grace's insect medium containing 10% FBS was added. Cells were harvested at the indicated times and analyzed by immunoblotting.

Immune Complex Kinase Assay

Cells were washed three times with cold PBS and resuspended in lysis buffer (1% Nonidet P-40; 150 m*M* NaCl; 50 m*M* Tris, pH 8.0; 100 µ*M* sodium

orthovanadate; 5 µg/mL aprotinin; and 10 µg/mL PMSF), and incubated on ice for 30 min and treated with anti-MPK38 immune and preimmune serum. Immune complexes were precipitated with protein A–Sepharose CL-4B (Pharmacia). After washing the precipitate three times with lysis buffer, then twice with kinase buffer (25 m*M* HEPES, pH 7.4; 1 m*M* DTT; and 10 m*M* $MgCl_2$), the immune complex was incubated with 5 µCi of [γ-^{32}P]-ATP at 37°C for 10 min, separated by 8% SDS-PAGE, and transferred to PVDF membranes and detected by autoradiography.

Phosphoamino Acid Analysis

Autophosphorylated proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, visualized by autoradiography, and excised. The samples were hydrolyzed in 6 *M* HCl at 110°C for 1 h. The supernatant fluid was dried with a Speedvac evaporator (Labconco, Kansas City, MO), resuspended in 10 µL of buffer (0.58 *M* formic acid and 1.36 *M* glacial acetic acid, pH 1.9) containing 1 µL of nonradioactive phosphoamino acid standards mixture [Ser(P), Thr(P), and Tyr(P), 1 mg/mL each], and subjected to two-dimensional electrophoresis on cellulose acetate thin-layer plates. The first dimension was run at pH 1.9 and 1.5 kV for 20 min, and the second dimension was run at pH 3.5 and 1.3 kV for 16 min. Then the plate was removed and dried in an oven at 80°C for 20 min. To visualize the phosphoamino acid standards, the plate was stained with 0.25% ninhydrin in acetone and reheated in the oven for 10 min. Autoradiogram was prepared with intensifying screens at –70°C. Following autoradiography, alignment was performed to identify radioactive phosphoamino acids.

Results and Discussion

To express the recombinant MPK38 protein in *E. coli*, the *E. coli* strain BL21(DE3), which contains the T7 RNA polymerase gene under the control of the lacUV5 promoter, was transformed with either the pET-24a or pET-MPK38 plasmid, and, after the induction of the transformed cells with IPTG, the expression levels were compared by SDS-PAGE analysis of the cell lysates. The results showed that the expected recombinant MPK38 protein corresponding to ~60 kDa was successfully induced and purified to near homogeneity (Fig. 1). Antiserum was raised in rabbits against the purified recombinant MPK38 protein excised from SDS-PAGE as described under Materials and Methods.

To explore the enzymatic characteristics of MPK38 protein and characterization of the generated antibody, we expressed the recombinant MPK38 from the plasmid harboring the full-length cDNA using recombinant baculovirus, in which expression is driven by the strong viral polyhedrin promoter. To determine the time course of expression, infected Sf21 cells were harvested every 24 h postinfection and lysed, and the lysates were subjected to SDS-PAGE and immunoblot analysis. Expression of recombinant MPK38, with a predicted molecular weight of 72 kDa, was

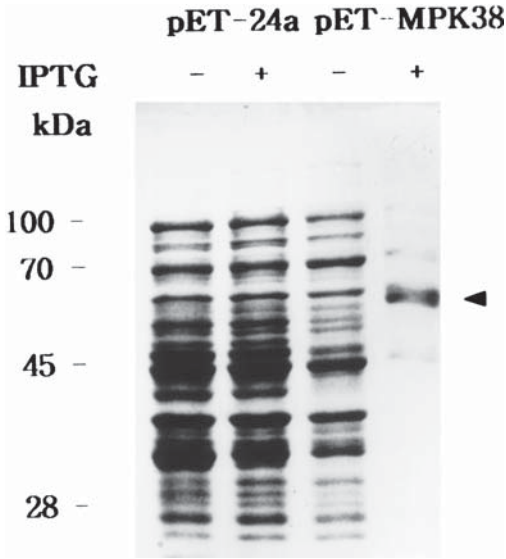

Fig. 1. Expression of recombinant MPK38 in *E. coli*. A bacterial expression vector (pET-MPK38) for recombinant MPK38 protein was constructed as described in Materials and Methods. The bacterial expression construct of MPK38 (pET-MPK38) or the expression vector (pET-24a) alone was subsequently transformed into bacterial strain BL21(DE3) and either induced by the addition of 1 m*M* IPTG (+) or noninduced (–) to produce the recombinant MPK38 protein. The cell lysate was then resolved by 10% SDS-PAGE and stained with Coomassie blue.

already detectable at 24 h postinfection (Fig. 2). Thereafter, expression levels increased in a time-dependent manner, and maximum expression of the recombinant MPK38 was observed approx 48 h after postinfection.

To support further the identification of the recombinant MPK38 expressed in insect cells as a protein kinase, immune complex kinase assay was performed to determine whether anti-MPK38 antibody could immunoprecipitate protein kinase activity from cells. Nonidet P-40 extracts of insect cells infected with recombinant baculovirus were incubated with anti-MPK38 antibody. The proteins specifically adsorbed were incubated with [γ-^{32}P] ATP to allow the autophosphorylation or phosphorylation of associated proteins. Phosphoproteins present were separated by SDS-PAGE, transferred to a PVDF membrane, and detected by autoradiography. As shown in Fig. 3, immunoprecipitates from the extracts of insect cells infected with recombinant baculovirus yielded an approx 72-kDa phosphoprotein, but no similarly sized phosphoproteins were detected using control cells that were not infected with recombinant baculovirus (Fig. 3, top). Moreover, the anti-MPK antibody specifically recognized a protein that migrated at the same apparent molecular weight as the 72-kDa phosphoprotein from the same membrane by immunoblotting (Fig. 3, bottom), suggesting that the authentic MPK38 gene product also migrates at 72 kDa. In addition, autophosphorylation significantly increased when Mg^{2+} was

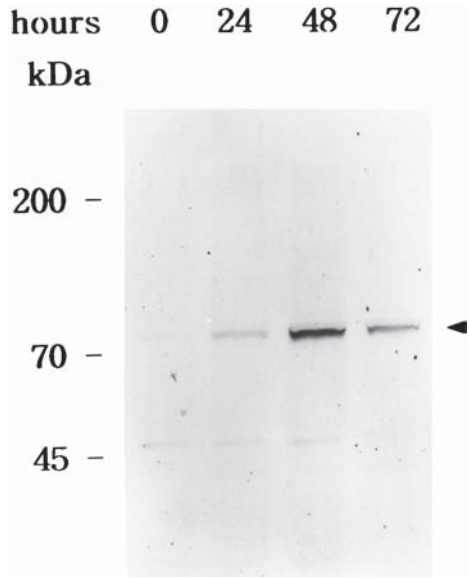

Fig. 2. Immunoblot analysis of extracts from Sf21 cells infected with recombinant MPK38 baculovirus. The recombinant baculovirus containing the full-length cDNA of MPK38 was obtained by cotransfecting Sf21 cells with pBac-MPK38 plasmid and Bsu36 I-digested BacPAK6 viral DNA. The infected cells were harvested every 24 h postinfection and lysed, and the lysates were subjected to SDS-PAGE, immunoblotted with anti-MPK38 serum, and visualized by ECL.

present in a kinase buffer compared to Mn^{2+} (data not shown). The preference of Mg^{2+} over Mn^{2+} for MPK38 kinase activity is not similar to that described for *Xenopus* XEEK1, a serine/threonine kinase, sharing ~65% similarity with yeast SNF1 kinase within the kinase domain *(12)*.

To characterize the endogenous MPK38 in MPK38-expressing cells, we also investigated the autophosphorylation of MPK38 in an in vitro kinase assay with immunoprecipitates. As shown in Fig. 4A, MPK38 was capable of autophosphorylation in cells of hematopoietic lineage, R1.1 and EL4 T lymphomas, and A20 B lymphoma. However, phosphorylation of this band was not seen when preimmune serum was used. This is consistent with the previous data on the pattern of MPK38 expression, which was determined by Northern blot analysis *(4)*. Phosphoamino acid analysis of autophosphorylated MPK38 shows that the phosphorylation was exclusively on serine and threonine residues (Fig. 4B). Together, these data demonstrate that MPK38 gene product apparently has a protein serine/threonine kinase activity in MPK38-expressing cells. In the MPK38 amino acid sequence, a well-conserved threonine residue (T^{167}) within kinase subdomain VIII, a potential site of autophosphorylation, found in many protein serine/threonine kinases was present *(14)*. Other studies have provided evidence that sites of autophosphorylation lie within 20 residues upstream of the A^{176}-P^{177}-E^{178} consensus triplet, which has been implicated

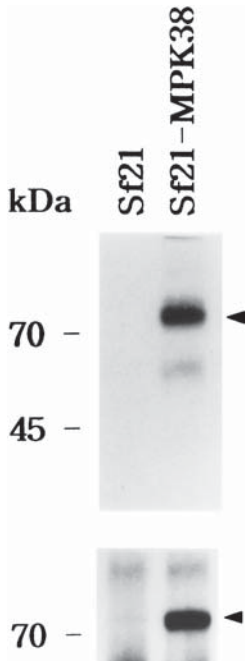

Fig. 3. Autophosphorylation of recombinant MPK38 protein in anti-MPK38 immune complex. Proteins were immunoprecipitated from 1% Nonidet P-40 extracts of control Sf21 cells (Sf21) or Sf21 cells infected with recombinant MPK38 baculovirus containing pBac-MPK38 plasmid (Sf21-MPK38) using anti-MPK38 serum. Immunoprecipitates were incubated with [γ-^{32}P]-ATP, separated by SDS-PAGE, and transferred to a PVDF membrane. Phosphoproteins present on membrane were detected by autoradiography (**left panel**). The presence of recombinant MPK38 protein in the immune complexes was verified by immunoblotting with anti-MPK38 antibody (**right panel**). Arrows indicate the migration position of the recombinant MPK38 protein.

in catalysis *(4,14,15)*. The role of this autophosphorylation site is not entirely known, but for several protein kinases there is evidence that phosphorylation of this site leads to increased catalytic activity *(16–18)*.

Reports on the relationship between kinase activity and autophosphorylation suggest that autophosphorylation may result in a conformational change that allows better access of substrates to the active site. It remains to be conclusively shown which residues are autophosphorylated on MPK38. However, the increased phosphorylation of a protein of proteins in the 72-kDa range following immunoprecipitation by anti-MPK38 antibody could represent autophosphorylation or phosphorylation by a second protein kinase. In an effort to identify an in vitro substrate of MPK38, two protein kinase substrates available in our laboratory were tested, including myelin basic protein and enolase, but no phosphorylation was observed under our assay conditions (data not shown). These results raise the possibility that MPK38 may have a narrow substrate specificity.

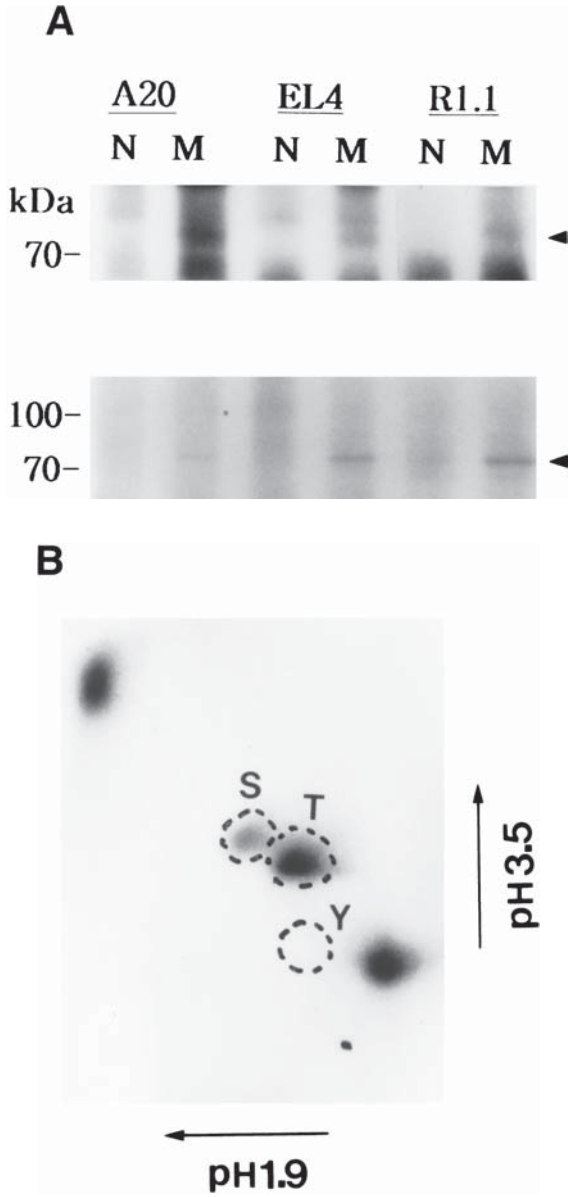

Fig. 4. Identification of protein serine/threonine kinase activity in immune complexes. **(A)** Autophosphorylation of endogenous MPK38. 1% Nonidet P-40 extracts of cells were immunoprecipitated with preimmune serum (N) or anti-MPK38 antibody (M). Immunoprecipitates were purified by coupling to protein A-Sepharose beads, resuspended in kinase buffer containing 10 m*M* $MgCl_2$, and incubated with [γ-^{32}P] ATP. The samples were separated by 8% SDS-PAGE, transferred to a PVDF membrane, and detected by autoradiography (**top panel**). The presence of endogenous MPK38 protein in the immune complexes was verified by immunoblotting with anti-MPK38 antibody (**bottom panel**). Arrows indicate the migration position of the endogenous MPK38 protein. **(B)** Phosphoamino acid analysis of MPK38. Phosphoamino acid analysis of phosphorylated MPK38 was performed by excision of the appropriate radiolabeled protein band, and phosphoamino acids were separated by two-dimensional electrophoresis as described in Materials and Methods. The positions of authentic phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y), determined by ninhydrin staining, are indicated.

Further understanding of the physiological functions of MPK38 in hematopoietic cells will result from the identification of its cellular substrates and examination of the regulation of MPK38 expression by drugs that affect signal transduction pathways. In this regard, our anti-MPK38 antibody against the N-terminal region of MPK38 could provide valuable information about cellular events in which MPK38 kinase or associated proteins play a cardinal role.

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References

1. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991), *Cell* **64,** 281–302.
2. Hunter, T. (1995), *Cell* **80,** 225–236.
3. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988), *Science* **241,** 42–52.
4. Gil, M., Yang, Y., Lee, Y., Choi, I., and Ha, H. (1997), *Gene* **195,** 295–301.
5. Heyer, B. S., Warsowe, J., Solter, D., Knowles, B. B., and Ackerman, S. L. (1997), *Mol. Reprod. Develop.* **47,** 148–156.
6. Celenza, J. L. and Carlson, M. (1986), *Science* **233,** 1175–1180.
7. Alderson, A., Sabelli, P. A., Dickinson, J. R., Cole, D., Richarson, M., Kreis, M., Shewry, P. R., and Halford, N. G. (1991), *Proc. Natl. Acad. Sci. USA* **88,** 8602–8605.
8. 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,** 11,442–11,448.
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. Muranaka, T., Banno, H., and Machida, Y. (1994), *Mol. Cell. Biol.* **14,** 2958–2965.
11. Woods, A., Munday, M. R., Scott, J., Yang, X., Carlson, M., and Carling, D. (1994), *J. Biol. Chem.* **269,** 19,509–19,515.
12. Su, J. Y., Erikson, E., and Maller, J. L. (1996), *J. Biol. Chem.* **271,** 14,430–14,437.
13. Yang, Y., Yoo, H. M., Choi, I., Pyun, K. H., Byun, S. M., and Ha, H. (1996), *J. Invest. Dermatol.* **107,** 367–372.
14. Hunter, T. and Cooper, J. A. (1986), in *The Enzymes*, vol. 17, Boyer, P. D. and Krebs, E. G., eds., Academic, Orlando, FL, pp. 191–246.
15. Shoji, S., Ericsson, L. H., Walsh, K. A., Fischer, E. H., and Titani, K. (1983), *Biochemistry* **22,** 3702–3709.
16. Weinmaster, G., Zoller, M. J., Smith, M., Hinze, E., and Pawson, T. (1984), *Cell* **37,** 559–568.
17. Weinmaster, G. and Pawson, T. (1986), *J. Biol. Chem.* **261,** 328–333.
18. Kmiecik, T. E. and Shalloway, D. (1987), *Cell* **49,** 65–73.