A novel Jun N-terminal kinase (JNK)-binding protein that enhances the activation of JNK by MEK kinase 1 and TGF-β-activated kinase 1

Satoru Koyano[a,b], Michihiko Ito[a], Nobuhiko Takamatsu[a], Tadayoshi Shiba[a], Ken-ichi Yamamoto[b], Katsuji Yoshioka[b,*]

[a] *Department of Biosciences, School of Science, Kitasato University, 1-15-1 Kitasato, Kanagawa 228-8555, Japan*
[b] *Department of Molecular Pathology, Cancer Research Institute, Kanazawa University, 13-1 Takaramachi, Kanazawa 920-0934, Japan*

Received 9 July 1999; received in revised form 4 August 1999

Abstract We have identified a novel Jun N-terminal kinase (JNK)-binding protein, termed JNKBP1, and examined its binding affinity for JNK1, JNK2, JNK3, and extracellular signal-regulated kinase 2 (ERK2) in COS-7 cells. JNKBP1 preferentially interacted with the JNKs, but not with ERK2. Furthermore, we investigated the effect of overexpressing JNKBP1 on the JNK and ERK signaling pathways in COS-7 cells. JNKBP1 alone had only a marginal effect on JNK activity. However, the activation of JNK by MEK kinase 1 and TGF-β-activated kinase 1 was significantly enhanced in the presence of JNKBP1. In contrast, JNKBP1 had no or very little effect on the ERK signaling pathway. These results suggest that JNKBP1 functions to facilitate the specific and efficient activation of the JNK signaling pathways.

Key words: Signal transduction; Mitogen-activated protein kinase; Jun N-terminal kinase; Stress-activated protein kinase

1. Introduction

Mitogen-activated protein kinase (MAPK) cascades, which consist of MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK) as the major components, are conserved eukaryotic signaling pathways [1–4]. MAPK is activated by dual phosphorylation on threonine and tyrosine residues catalyzed by MAPKK, and MAPKK is activated by serine/threonine phosphorylation catalyzed by MAPKKK. The Jun N-terminal kinases (JNKs) represent a group of MAPKs that are strongly activated by proinflammatory cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα), and extracellular stresses, such as UV irradiation and osmotic shock [5–7]. In the JNK cascades MAPKs are JNK1, JNK2, and JNK3 (also known as SAPKγ, SAPKα, and SAPKβ, respectively) [8–12], and MAPKKs are SEK1 (also known as MKK4 and JNKK1) [13–15] and MKK7 (also known as JNKK2) [16–18]. A number of protein kinases, including apoptosis signal-regulating kinase 1 (ASK1) [19], MEK kinase 1 (MEKK1) [20–22], mixed lineage kinase 3 (MLK3) [23], and TGF-β-activated kinase 1 (TAK1) [24], have been identified as MAPKKKs in the JNK cascades. The extracellular signal-regulated kinases (ERKs) represent another group of MAPKs, which are mostly responsive to mitogenic and differentiation stimuli [25]. In the ERK cascades, ERK (ERK1 and ERK2), MEK (MEK1 and MEK2), and Raf (Raf-1, A-Raf, B-Raf) correspond to MAPK, MAPKK, and MAPKKK, respectively [7]. The identification of numerous components of the MAPK cascades suggests that there are a number of these distinct signaling pathways in cells. To date, however, the molecular mechanism by which the specificity and efficiency of the MAPK cascades is maintained remains unclear.

As a step toward clarifying this mechanism, we searched for proteins that interact with JNK. In the present study we molecularly cloned a novel JNK-binding protein, termed JNKBP1, and examined its binding affinity for JNK and ERK MAPKs. We further investigated the effect of overexpressing JNKBP1 on the JNK and ERK cascades.

2. Materials and methods

2.1. Two-hybrid screening and isolation of JNKBP1 cDNA

The entire coding sequence of mouse JNK3 was inserted into the pAS2 vector (Clontech). A mouse brain cDNA library in pGAD10 (Clontech) was transfected into the yeast strain CG1945 harboring pAS2-JNK3. The Clontech yeast two-hybrid system was used according to the manufacturer's instructions. A ZAPII mouse brain cDNA library (Stratagene) was screened using the partial JNKBP1 cDNA fragment, obtained from the yeast two-hybrid system, as a probe.

2.2. Northern blotting analysis

Northern blotting analysis was performed as described previously [26] using ^{32}P-labeled JNKBP1 and β-actin cDNA probes.

2.3. Plasmid construction

JNK1, JNK2, and JNK3 cDNAs were isolated from a ZAPII mouse brain cDNA library, and MEKK1 cDNA from a ZAPII mouse spleen cDNA library (Stratagene). TAK1 cDNA was a generous gift from K. Matsumoto. The full-length human Raf-1 cDNA was obtained from Health Science Research Resources Bank, Japan. The ORF of ERK2 was amplified by polymerase chain reaction from human lymphocyte cDNA. To prepare glutathione *S*-transferase (GST)-JNK3 fusion protein, the entire coding sequence of mouse JNK3 was inserted into the pGEX-2T vector (Pharmacia). For in vitro translation, the entire or portions of the coding sequence of JNKBP1 were inserted into a modified pcDNA3 vector (Invitrogen) encoding a histidine (His) tag and an S tag (Novagen). To construct mammalian expression vectors for His-S-MAPKs and His-S-MEKK1, the entire coding sequences were inserted into the modified pcDNA3-His-S vector. For the expression of Myc-JNKBP1 (residues 1–1508 and 1063–1331), the entire or portions of the coding sequence of JNKBP1 was inserted into a modified pcDNA3 vector encoding a Myc tag. The expression vectors of full-length TAK1 and truncated Raf-1 (ΔRaf; residues 316–648) were constructed using a modified pcDNA3 vector encoding a Flag tag.

*Corresponding author. Fax: (81) (76) 234-4517.
E-mail: katsuji@kenroku.kanazawa-u.ac.jp

Abbreviations: JNK, Jun N-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEKK1, MEK kinase 1; TAK1, TGF-β-activated kinase 1; GST, glutathione *S*-transferase

0014-5793/99/$20.00
PII: S0014-5793(99)01084-4

2.4. Analyses of protein-protein interactions in vitro and in vivo

GST and GST-JNK3 proteins were expressed in *Escherichia coli* and purified with glutathione-agarose according to the manufacturer's instructions. The entire and various portions of JNKBP1 ORF were translated in vitro in the presence of [^{35}S]methionine using the TNT T7 Quick Coupled Transcription/Translation System (Promega). The ^{35}S-labeled JNKBP1 proteins were mixed with either immobilized GST or GST-JNK3 in buffer A (20 mM HEPES pH 7.5, 50 mM NaCl, 0.5 mM EGTA, and 1% NP-40), rotated for 2 h at 4°C, spun, and washed three times with buffer A. The precipitates were separated by SDS-PAGE and subjected to autoradiography. For the analysis of protein-protein interactions in intact cells, the expression vectors were cotransfected into COS-7 cells with FuGENE6 (Boehringer Mannheim) according to the manufacturer's instructions. After 34 h, cells were lysed in buffer B (50 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM β-glycerophosphate, 2 mM Na_3VO_4, 1 mM PMSF, and 0.2 mM DTT), and precipitated by S-protein agarose. The recovered fractions were separated by SDS-PAGE, and transferred to Immobilon-P (Millipore). The membranes were probed with anti-Myc 9E10 monoclonal antibody (Boehringer Mannheim), and visualized with the ECL detection system (Amersham). Expression of His-S-MAPKs was examined by immunoblotting cell lysates with anti-His polyclonal antibody (Santa Cruz).

2.5. Reporter assay

5XGAL4-LUC reporter (Stratagene), GAL4-c-Jun or -Elk1 expression vector (Stratagene), and the expression vectors (see Fig. 4) were transiently transfected with FuGENE6 transfection reagent. After 34 h, the cells were harvested and their luciferase activity was measured. All transfections were standardized by cotransfecting a *Renilla* luciferase control vector (Promega).

3. Results and discussion

We used a yeast two-hybrid system to search for proteins that directly interact with JNK3. A positive clone encoding a protein, termed JNKBP1, was identified by screening approximately 2×10^6 transformants. To isolate overlapping cDNAs encompassing the entire open reading frame (ORF), we screened a mouse brain cDNA library with a partial JNKBP1 cDNA insert obtained from the yeast two-hybrid system. The full-length JNKBP1 cDNA was found to encode a protein of 1508 amino acids with a calculated relative molecular mass of 163 985 (Fig. 1A). Database searches suggested that JNKBP1 represents a mouse homologue of KIAA0596 (accession number AB011168), identified by the Kazusa cDNA project. No function has yet been ascribed to this human gene. JNKBP1 contains four WD40 repeats [27] (see Fig. 1A). We analyzed the tissue distributions of mouse JNKBP1 by Northern blotting analysis. The results indicate that the approximately 5.8-kb JNKBP1 mRNA is ubiquitously expressed at a very high level in brain (Fig. 1B).

To define the region of JNKBP1 that is responsible for its interaction with JNK, the entire and various portions of JNKBP1 ORF were translated in vitro in the presence of [^{35}S]methionine. The JNKBP1 proteins were mixed with glutathione-agarose bound to either GST or GST-JNK3, recovered, and analyzed by SDS-PAGE and autoradiography (Fig. 2). The results indicate that the JNK-binding region is located between residues 1063 and 1331 in JNKBP1. This region is almost completely overlapped with the region isolated using the yeast two-hybrid system (residues 1042–1331).

We next examined the binding affinity of JNKBP1 for JNK and ERK MAPKs using cotransfection experiments in COS-7 cells (Fig. 3). When full-length JNKBP1 was expressed in these cells, the expression level of the full-length protein was not high. We therefore expressed a Myc epitope-tagged portion of JNKBP1 (Myc-JNKBP1; residues 1063–1331), which contains the JNK-binding domain, and examined its ability to bind with His-S-tagged JNK1, JNK2, JNK3, or ERK2 (His-S-JNK1, -JNK2, -JNK3, and -ERK2, respectively) in COS-7 cells. The His-S-MAPK proteins were recovered from the cell lysates by affinity binding to S-protein agarose, and the precipitates were examined for the presence of the Myc-JNKBP1 protein by immunoblotting with an anti-Myc antibody. JNK1, JNK2, and JNK3 interacted with JNKBP1, while no (or very little) binding of ERK2 was observed under our assay conditions. JNK2 showed higher binding affinity to JNKBP1 than did JNK1 or JNK3.

We further examined the effect of overexpressing full-length JNKBP1 on the JNK cascades in COS-7 cells (Fig. 4A). The JNK activity in the transfected cells was detected by measuring the transcriptional activity of a GAL4-c-Jun (residues 1–223) fusion protein containing the c-Jun activation domain. Overexpression of full-length JNKBP1 had only a marginal

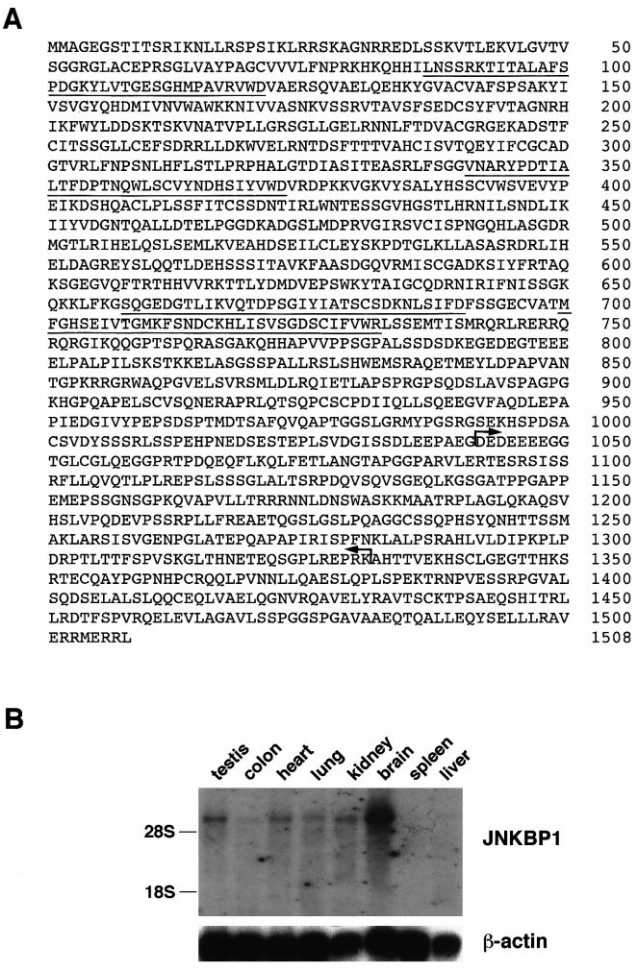

Fig. 1. A: Deduced amino acid sequence of JNKBP1. The region isolated using the yeast two-hybrid system (residues 1042–1331) is shown by angled arrows. The nucleotide sequence of mouse JNKBP1 has been submitted to DDBJ/EMBL/GenBank with accession number AB029482. The four copies of the WD40 repeats are underlined. B: Expression of JNKBP1 mRNA in mouse tissues. Poly(A)$^+$ RNA samples isolated from various mouse tissues were examined by Northern blotting analysis. The positions of 28S and 18S rRNAs are indicated on the left. β-Actin mRNA was included as a loading control.

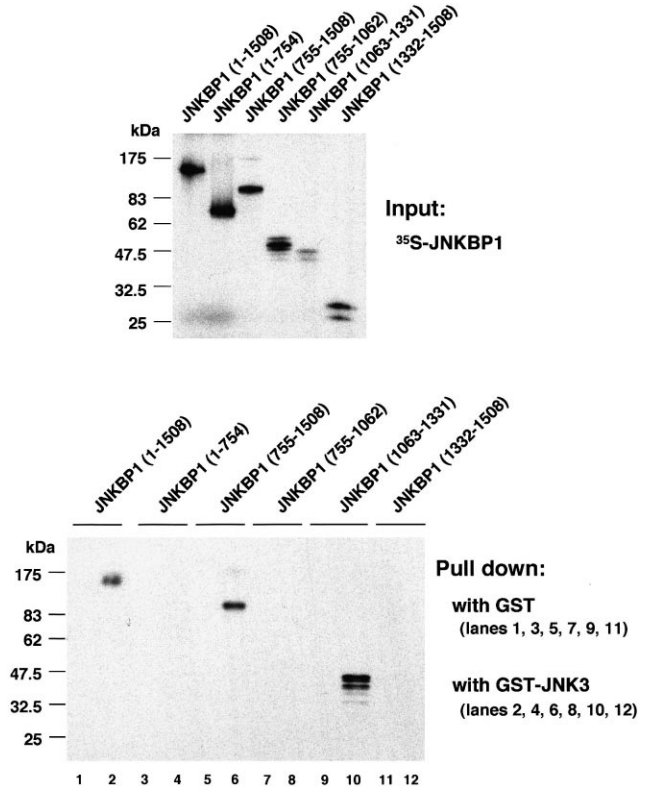

Fig. 2. Mapping of JNK-binding region in JNKBP1. The entire and various portions of JNKBP1 ORF were translated in vitro in the presence of [35S]methionine. The 35S-labeled JNKBP1 proteins were incubated with 1 μg of either immobilized GST (lanes 1, 3, 5, 7, and 9) or GST-JNK3 (lanes 2, 4, 6, 8, and 10). The protein complexes were extensively washed and the bound JNKBP1 proteins were detected by SDS-PAGE and autoradiography. One fourth of each 35S-labeled JNKBP1 protein used in the binding reaction was loaded as a control. The positions of molecular weight markers are indicated on the left.

effect on JNK activity. MEKK1 and TAK1, MAPKKKs in the JNK cascades, activated JNK by 3.0- and 3.2-fold, respectively, under our assay conditions. Interestingly, the activation of JNK by MEKK1 and TAK1 was further enhanced in the

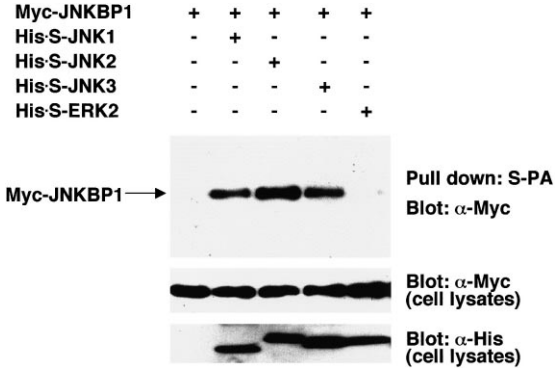

Fig. 3. Binding of JNKBP1 to JNK and ERK MAPKs. COS-7 cells were transiently transfected with 0.5 μg of pcDNA3-Myc-JNKBP1 with 1.5 μg of pcDNA3-His-S empty vector, pcDNA3-His-S-JNK1, -JNK2, -JNK3, or -ERK2, as indicated. Cell lysates were precipitated with S-protein-linked agarose (S-PA), and analyzed by immunoblotting with an antibody to Myc. Expression of Myc-JNKBP1 and His-S-MAPKs was examined by immunoblotting one tenth of the cell lysates used in the binding reactions.

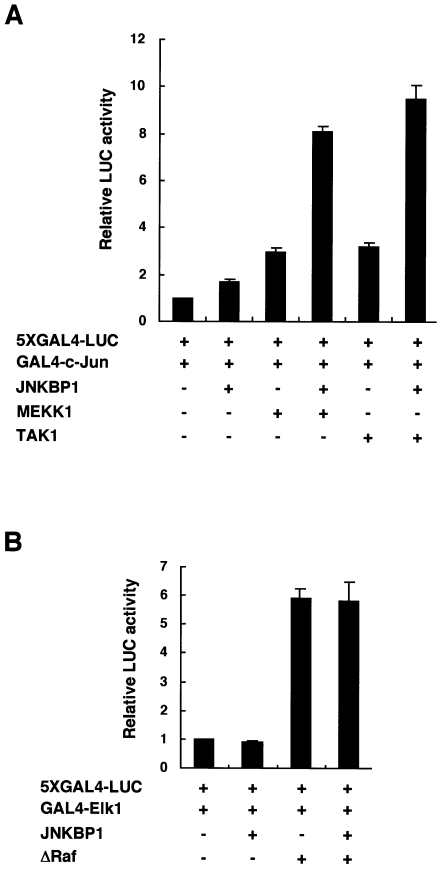

Fig. 4. Effect of overexpressed JNKBP1 on the JNK (A) and ERK (B) cascades. A: COS-7 cells were transiently cotransfected with 0.3 μg of 5XGAL4-LUC reporter, 0.03 μg of GAL4-c-Jun expression vector, and 0.03 μg of *Renilla* luciferase (RL) control vector in the absence or presence of 0.9 μg of pcDNA3-Myc-JNKBP1, 0.06 μg of pcDNA3-His-S-MEKK1, or 0.2 μg of pcDNA3-Flag-TAK1, as indicated. B: COS-7 cells were transiently cotransfected with 0.3 μg of 5XGAL4-LUC reporter, 0.03 μg of GAL4-Elk1 expression vector, and 0.03 μg of RL control vector in the absence or presence of 0.9 μg of pcDNA3-Myc-JNKBP1 or 0.2 μg of pcDNA3-Flag-ΔRaf, as indicated. Total DNA was kept at 1.5 μg per transfection with pcDNA3-Myc empty vector. 5XGAL4-driven luciferase activity was normalized with RL activity. Bars represent the mean ± S.E.M. ($n = 3$).

presence of JNKBP1 by 2.7- and 3.0-fold, respectively. We also examined the effect of overexpressing full-length JNKBP1 on the ERK cascade in transfected COS-7 cells (Fig. 4B). The ERK activity in the cells was detected by measuring the transcriptional activity of a GAL4-Elk1 (residues 307–427) fusion protein containing the Elk1 activation domain. Overexpression of full-length JNKBP1 had no or very little effect on ERK activity, nor did it affect the activation of ERK by truncated Raf-1 (ΔRaf; residues 316–648), a constitutively active Raf-1 [28].

As JNKBP1 seems not to possess a kinase domain, it is unlikely that JNKBP1 directly activates the component(s) of the JNK cascades by phosphorylation. Recently, Whitmarsh et al. [29] reported that the JIP-1 protein plays a scaffolding role, where it facilitates the specific and efficient activation of JNK cascades. JNKBP1 is likely to play a similar role. The JIP-1 scaffold protein binds multiple components of the JNK signaling pathway, including the JNK MAPK, the MKK7 MAPKK, and the MLK3 MAPKKK. Thus, it should be

interesting to examine whether JNKBP1 interacts with the SEK1 and MKK7 MAPKKs, and the MEKK1, TAK1, and MLK3 MAPKKKs in addition to the JNK MAPK.

Acknowledgements: We thank Dr. Kunihiro Matsumoto for providing TAK1 cDNA, Yuko Odama, Norihiro Tokita and Hinako Ishizaki for technical assistance, and Dr. Yoshiyuki Sakaki for encouragement. This work was supported in part by grants from the Kitasato Research Foundation (M.I.), the Waksman Foundation of Japan Inc. (T.S.), and the Kato Memorial Foundation (K.Y.).

References

[1] Davis, R.J. (1994) Trends Biochem. Sci. 19, 470–473.
[2] Cano, E. and Mahadevan, L.C. (1995) Trends Biochem. Sci. 20, 117–122.
[3] Herskowitz, I. (1995) Cell 80, 187–197.
[4] Waskiewicz, A.J. and Cooper, J.A. (1995) Curr. Opin. Cell Biol. 7, 798–805.
[5] Kyriakis, J.M. and Avruch, J. (1996) J. Biol. Chem. 271, 24313–24316.
[6] Minden, A. and Karin, M. (1997) Biochim. Biophys. Acta 1333, F85–F104.
[7] Robinson, M.J. and Cobb, M.H. (1997) Curr. Opin. Cell Biol. 9, 180–186.
[8] Dérijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M. and Davis, R.J. (1994) Cell 76, 1025–1037.
[9] Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J. and Woodgett, J.R. (1994) Nature 369, 156–160.
[10] Kallunki, T., Su, B., Tsigelny, I., Sluss, H.K., Dérijard, B., Moore, G., Davis, R.J. and Karin, M. (1994) Genes Dev. 8, 2996–3007.
[11] Gupta, S., Barrett, T., Whitmarsh, A.J., Cavanagh, J., Sluss, H.K., Dérijard, B. and Davis, R.J. (1996) EMBO J. 15, 2760–2770.
[12] Mohit, A.A., Martin, J.H. and Miller, C.A. (1995) Neuron 14, 67–78.
[13] Sánchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M. and Zon, L.I. (1994) Nature 372, 794–798.
[14] Lin, A., Minden, A., Martinetto, H., Claret, F.-X., Lange-Carter, C., Mercurio, F., Johnson, G.L. and Karin, M. (1995) Science 268, 286–290.
[15] Dérijard, B., Raingeaud, J., Barrett, T., Wu, I.-H., Han, J., Ulevitch, R.J. and Davis, R.J. (1995) Science 267, 682–685.
[16] Moriguchi, T., Toyoshima, F., Masuyama, N., Hanafusa, H., Gotoh, Y. and Nishida, E. (1997) EMBO J. 16, 7045–7053.
[17] Tournier, C., Whitmarsh, A.J., Cavanagh, J., Barrett, T. and Davis, R.J. (1997) Proc. Natl. Acad. Sci. USA 94, 7337–7342.
[18] Wu, Z., Wu, J., Jacinto, E. and Karin, M. (1997) Mol. Cell. Biol. 17, 7407–7416.
[19] Ichijo, H., Nishida, E., Irie, K., Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K. and Gotoh, Y. (1997) Science 275, 90–94.
[20] Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Dérijard, B., Davis, R.J., Johnson, G.L. and Karin, M. (1994) Science 266, 1719–1723.
[21] Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., Zon, L.I., Woodgett, J.R. and Templeton, D.J. (1994) Nature 372, 798–800.
[22] Xu, S., Robbins, D.J., Christerson, L.B., English, J.M., Vanderbilt, C.A. and Cobb, M.H. (1996) Proc. Natl. Acad. Sci. USA 93, 5291–5295.
[23] Tibbles, L.A., Ing, Y.L., Kiefer, F., Chan, J., Iscove, N., Woodgett, J.R. and Lassam, N.J. (1996) EMBO J. 15, 7026–7035.
[24] Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E. and Matsumoto, K. (1995) Science 270, 2008–2011.
[25] Cobb, M.H. and Goldsmith, E.J. (1995) J. Biol. Chem. 270, 14843–14846.
[26] Yoshioka, K., Deng, T., Cavigelli, M. and Karin, M. (1995) Proc. Natl. Acad. Sci. USA 92, 4972–4976.
[27] Neer, E.J., Schmidt, C.J., Nambudripad, R. and Smith, T.F. (1994) Nature 371, 297–300.
[28] Stanton Jr., V.P., Nichols, D.W., Laudano, A.P. and Cooper, G.M. (1989) Mol. Cell. Biol. 9, 639–647.
[29] Whitmarsh, A.J., Cavanagh, J., Tournier, C., Yasuda, J. and Davis, R.J. (1998) Science 281, 1671–1674.