FEBS Letters 353 (1994) 185–188

FEBS 14630

Isolation and characterization of mutants of human mitogen-activated protein kinase (ERK2)

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Received 22 August 1994

Abstract Site directed mutagenesis/charged-to-alanine scanning mutagenesis of the amino terminal portion of human ERK2 (from amino acids 1 to 150) purified as a glutathione-*S*-transferase fusion protein (GST-ERK2) from *E. coli* has been done to determine regions/amino acids important for activation by rabbit skeletal muscle MAP kinase kinase (rMEK) and kinase activity towards myelin basic protein (MBP). Five classes of mutants have been isolated. The first class of mutants comprises of G30A/G32A, A50D and R65A/R68A/E69A, that can be phosphorylated by rMEK and have no kinase activity towards MBP, the second class includes mutants D122A/H123A and N142A which have lower kinase activities but no change in their activation by rMEK; third class being Y34A, E58A/H59A, which have neutral effect towards either activity, the fourth class that includes completely inactive mutants D42A/K46A/R48A, the deletion mutant in the same region (−9aa[40–48]) and D104A/E107A/D109A and finally the fifth class that include K53A, E94A/K97A/D99A, K112A/K115A and R133A/K136A that are phosphorylated 140–240% but with kinase activity toward MBP ranging from 50–100% of the wild type.

Key words: Mitogen activated protein kinase; Charged-to-alanine scanning; Site directed mutagenesis; GST fusion protein

1. Introduction

A high percentage of human tumors, including tumors of the lung, pancreas, thyroid and colon, contain 'activating' mutations in the coding region of the guanine nucleotide binding protein *ras* [1,2]. The *ras* family of proto-oncogenes encode 21,000 Da molecular weight (p21) guanine nucleotide binding proteins that act as molecular switches, conveying signals from ligand activated tyrosine kinases (e.g. EGF, PDGF) to the serine/threonine kinase pathway (i.e. c-*raf*-1). A consequence of activating the *ras* pathway can be either proliferation [3] or cell cycle arrest and differentiation [4]. Recently it has been demonstrated, using both a genetic and biochemical approach, that the *ras* proto-oncogene binds and leads to the activation of the c-*raf*-1 Ser/Thr kinase, which in turn can phosphorylate and activate MAP kinase kinase (MEK) [5–10]. MEK has both threonine as well as tyrosine kinase activity; the only known substrate for MEK are the mitogen activated protein kinases, MAP kinases [11]. There are at least two members of this kinase family, also termed extracellular-signal regulated kinases, $p42^{MAPK}$ERK2 and $p44^{MAPK}$ERK1 [12–14]. Upon phosphorylation by MEK, the map kinases ERK1 and ERK2 translocate to the nucleus and phosphorylate transcription factors, such as c-Myc [15] or c-Jun [16] which leads to transcriptional activation of promoters that are responsive to these factors followed by either proliferation or developmental events.

We have sought to determine, by a combination of site-directed mutagenesis/charged-to-alanine scanning mutagenesis (replacing charged amino acids with alanine) [17] and in vitro biochemical analysis, which domain(s)/amino acids of ERK2 are important for its interaction with one of its substrates, myelin basic protein (MBP) and also its activation by MAP kinase kinase. The goal would ultimately be to construct peptides to inhibit ERK2 kinase activity/ERK2 activation by rMEK based on the data from mutagenesis studies. Specific inhibitors of the MAP kinase pathway (which will be equivalent to inhibiting the signal emanating from *Ras*) should be useful anti-proliferative agents. Our initial studies have focused on the amino-terminal portion of ERK2, from amino acid positions 1 through 150 (Fig. 1). We have discovered five classes of mutants. The first are ERK2 mutants that can be phosphorylated by rMEK in vitro but are unable to phosphorylate MBP, the second class of mutants are phosphorylated by rMEK, and have reduced MBP phosphorylating activity, the third class are neutral, in that the mutation has no effect on biological activity of the ERK2, the fourth class being completely inactive mutants and the fifth class being mutants which are highly phosphorylated by rMEK.

2. Materials and methods

2.1. Materials

pGEX2T-ERK2 was a kind gift from Dr. Tim Bird (Immunex Corp. Seattle, Washington). Myelin basic protein (MBP), sodium orthovanadate, β-glycerolphosphate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the protease inhibitors were from Boehringer Mannheim Corp. (Indianapolis, IN, USA) Glutathione-Sepharose, Fast-flow Q Sepharose, MonoQ HR 10/10 were from Pharmacia (Piscataway, NJ, USA). All the oligonucleotides for the mutagenesis studies were synthesized by National BioSciences (Plymouth, MN, USA).

2.2. Recombinant DNA methods

Human ERK2 was mutagenized by polymerase chain reaction using the plasmid pGEX-2T/ERK2 (a kind gift from T. Bird, Immunex Corp.) as template. Oligonucleotides were designed to introduce *SalI* and *NotI* sites at 5′ and 3′ ends of the amplified fragments to enable cloning into the pGEX-4T vector. With this technique, two complementary mutagenic primers were required. Two separate amplifications were required to generate the complete 1,072 kb ERK2 insert. For the first amplification, plasmid DNA containing the wild type insert was used as the template DNA. In general, one tube contained the reverse mutagenic primer plus the primer with the *SalI* (4TP1-1; CCAAAA-TCGGATCTGGTTCCGCGTGGTCGACCGGTCCGCGGGCAG) and the other tube contained the forward mutagenic primer and the primer with the *NotI* restriction site (4TP2-1; GTCAGTCACGATG-

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SSDI 0014-5793(94)01017-X

CGGCCGCTTATTAAGATCTGTATCCTGGCT). After amplification, the PCR products were isolated on a 4% polyacrylamide gel. For the second amplification, the two mutagenic fragments were combined along with the 4TP1-1 and 4TP2-1 to produce the 1,072 bp ERK2 fragment. The insert DNA was next digested with *SalI* and *NotI* followed by ligation to pGEX-4T digested with *SalI* and *NotI*. The ligation mixture was then transformed into *E. coli* DH5α. Individual colonies were selected for plasmid and DNA sequencing analysis to confirm the presence of the desired mutation. All cloning, bacterial plasmid isolation and recombinant DNA methods were followed as described in [18].

2.3. Expression and purification of wild type and mutant GST-ERKs

The GST-ERKs were purified by the method of Smith and Johnson [19] with a few modifications. The pGEX-4T vectors harboring the GST-ERK2s were transformed into *E. coli* DH5α. The *E. coli* were grown at 37°C in Luria broth until the O.D. at 600 nm = 0.5–0.6 at which time isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM) was added and growth continued for an additional 2 h. The cells were then pelleted, washed once with PBS and then frozen at −80°C. The pellet was then suspended in sonication buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml antipain, 0.2 mM PMSF), disrupted by an ultrasonication disruptor for 1 min and sedimented. After addition of Triton X-100 to 1%, the lysate was loaded on a 10 ml glutathione-Sepharose column (Pharmacia). The column was washed with 10 bed volumes of sonication buffer +1% Triton, until OD 280 nm was 0. The GST-ERK2s were eluted with 5 mM glutathione in sonication buffer (pH 8.0) and analyzed by SDS-PAGE electrophoresis. The purity of the proteins isolated was greater than 95%. Approximately 2–5 mg of purified GST-ERK2 were recovered from 1 l of bacteria. The fractions containing GST-ERK2 were pooled, concentrated in Centriprep 3 and dialyzed overnight against storage buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 10 mM EGTA, 0.1 mM sodium orthovanadate, 1 mM DTT, 50 mM NaCl, 20% glycerol).

2.4. Purification of ERK activator (MAP kinase kinase or MAPKK or rMEK) from rabbit skeletal muscle

Extracts were prepared from 750 g of rabbit skeletal muscle and purified as described previously [20,21] but with the following modifications. Firstly, the homogenate was subjected to batchwise chromatography on Fast Flow Q-Sepharose and the MAP kinase activator was eluted with buffer (50 mM β-glycerophosphate, 2 mM EGTA, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM benzamidine, 0.1 mM PMSF, 25 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.1% 2-mecaptoethanol) containing 0.4 M NaCl. The enzyme was then precipitated with 60% ammonium sulfate, dialyzed extensively and then loaded on a MonoQ (HR 10/10) column. After washing with equilibration buffer (25 mM Tris-HCl, pH 8.9, 1 mM EDTA, 5% (v/v) glycerol, 0.02% (w/v) Brij 35, 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol), the column was developed with 300 ml of linear gradient to 1 M NaCl and 3 ml fractions were collected at 2 ml/min. The active fractions which eluted as a single peak were pooled, pH adjusted to pH 7.5 and then frozen in aliquots at −80°C.

2.5. Assay of MBP kinase activity of GST-ERK2 activated by MAP kinase kinase

The conditions for the GST-ERK2 kinase assay were as described previously [20]. Briefly, inactive, recombinant GST-ERK2 (2.5 μl; 0.1–1.0 μg) was incubated for 3 min at 30°C with 2.5 μl of MAP kinase activator (diluted 15-fold; 0.66 μg) fraction and the activation of GST-ERK2 was initiated with 2.5 μl of 20 mM magnesium acetate/0.8 mM unlabelled ATP. Dilution buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5% (v/v) glycerol, 0.02% (w/v) Brij 35) replaced MAPKK in control incubations. After 20 min, the assay of MAP kinase was initiated with 40 μl of 10 mg/ml myelin basic protein (MBP; 20 μg) in 30 mM Tris-HCl, pH 7.4, 0.125 mM EGTA, 0.125 mM sodium orthovanadate and 12.5 mM magnesium acetate/0.25 mM [γ-^{32}P]ATP (3×10^5 cpm/nmol). Assays were terminated by spotting 40 μl aliquots on to 2.5 cm Whatman P81 phosphocellulose paper. The papers were washed 4 times (5 min) with 180 mM phosphoric acid and then in acetone before counting in a scintillation counter.

2.6. SDS-PAGE assay of ERK2 activation by MAPKK

The activation reaction was carried out as above except that MBP was omitted from the assay and 1 μM okadaic acid and 0.1 mM sodium orthovanadate were included. The reaction was terminated by the addition of 4 × sample buffer, and proteins were separated on 12% SDS-PAGE gels [22]. The proteins were immunoblotted onto nitrocellulose membranes and counted in a phosphorimager.

3. Results and discussion

3.1. Properties of purified GST-ERK2 protein

The GST-ERK2 protein migrated as a 68 kDa protein on a 12% SDS-PAGE gel. The GST-ERK2 mutants were recovered from this procedure with equal yields and purity. The recombinant GST-ERK2 isolated from *E. coli* is inactive as a kinase and thus has very low MBP phosphotransferase activity. The incubation of GST-ERK2 for 20 min with partially purified MAP kinase kinase from rabbit skeletal muscle (rMEK) resulted in a 100-fold increase in phosphorylation of the GST-ERK2 compared with the autophosphorylation of GST-ERK2 in its absence. This MEK dependent phosphorylation has been shown to occur within the sequence Thr-Glu-Tyr on Map kinase (amino acid position 183 to 185) and it has been shown that phosphorylation of both the threonine as well as the tyrosine residue are necessary for full kinase activity [23,24]. Under the reaction conditions chosen, the activation of GST-ERK2 by rMEK as assessed by the SDS-PAGE assay (section 2) is linear for 80 min and the kinase activity of the activated GST-ERK2 is linear for 30 min (Data not shown).

3.2. Properties of the mutant GST-ERK2 proteins

As shown in Fig. 1 and Table 1, 14 mutants localized to the amino terminal of MAP kinase were constructed either singly or in combination. These mutant proteins, along with the wild type protein, were purified on glutathione Sepharose beads and phosphorylated in vitro with FPLC purified rabbit skeletal muscle rMEK as described above. As shown in Fig. 2, of the 14 mutants analyzed by the kinase assay and SDS-PAGE assay to evaluate the ability of ERK mutant proteins to be phosphorylated by rMEK, the mutants G30A/G32A, A50D and R65A/R68A/E69A are phosphorylated 44%, 53% and 72% of the wild type and with diminished MBP kinase activity (4%). The consensus Gly-X-Gly-X-X-Gly is found in many nucleotide binding proteins in addition to protein kinases (25,26] and substitution of the first two glycines with a hydrophobic group such as alanine results in reduced activities thus confirming the importance of the glycines in ATP binding. Also the introduc-

Table 1
Mutations in human ERK2

Wild type	Mutant
G30/G32	AA
Y34	A
D42/K46/R48 (−9aa[40–48])	AAA
A50	D
K53	A
E58/H59	AA
R65/R68/E69	AAA
E94/K97/D98	AAA
D104/E107/D109	AAA
K112/K115	AA
D122/H123	AA
R133/K136	AA
N142	A

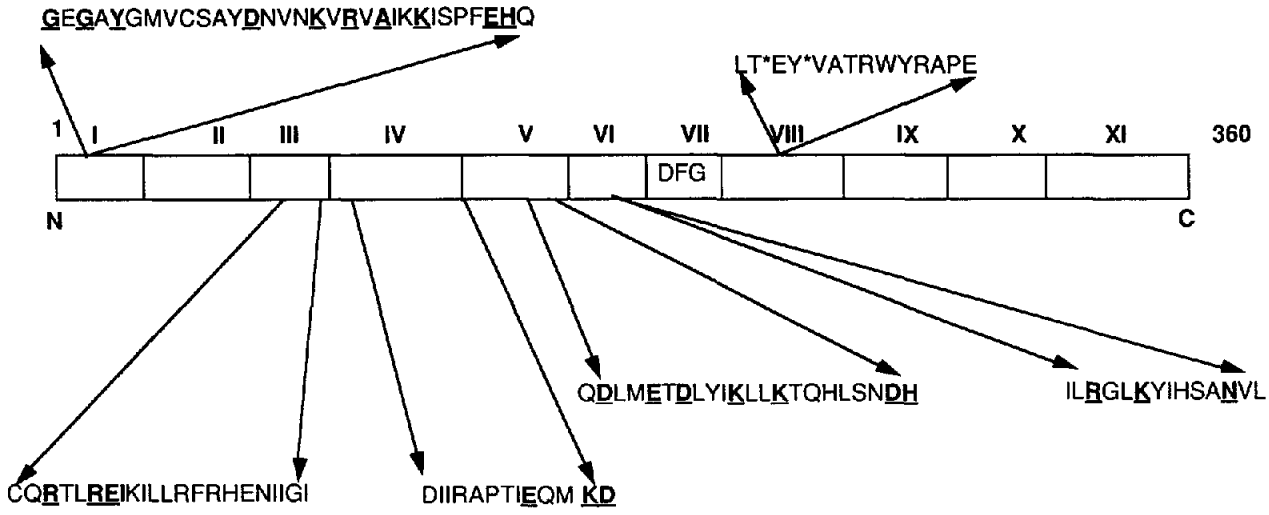

Fig. 1. Mutations in human ERK2. Amino acid sequence of human ERK2 is shown. The catalytic domains are shown in Roman numerals. The residues that were mutated are underlined and shown in bold lettering. The phosphorylation sites in ERK2 are indicated by asterisks.

tion of a charge with no change in the size, that is, substituting the invariant alanine with aspartic acid (A50D), close to the ATP binding lysine (K52), reduces phosphorylation by 50% with loss of kinase activity. A similar mutation, that is, introduction of charge in the case of rat ERK2 (S39D) in the ATP binding site, as shown by Robbin et al. [11] led to an increase in phosphorylation by rMEK with slightly reduced MBP kinase activity. With reference to the triple mutant, R65A/R68A/E69A, it has been proposed, based on the X-ray crystal structure of the unphosphorylated ERK2 that R65 and R68 would interact with phosphorylated Thr^{183} thus causing domain closure leading to relief of the steric constraints to substrate binding [27]. It would thus be expected that replacing them with alanine would prevent domain closure and subsequent substrate binding and therefore loss of MBP kinase activity. Our results that the mutant R65A/R68A/E69A which is still

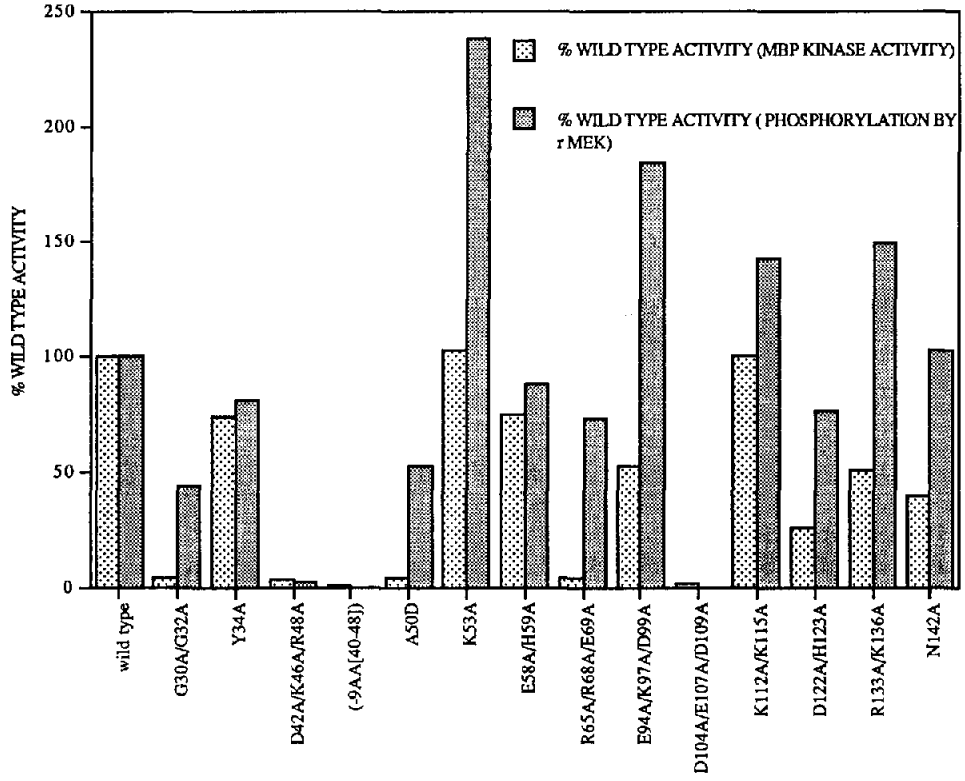

Fig. 2. Comparison of MBP kinase and phosphorylation by rMEK activities of GST-ERK2 mutants (0.1 μg). Wild type GST-ERK2 (0.1 μg) activities is 100%. In case of MBP kinase activity it is nmol of phosphate incorporated and activation of ERK2 with rMEK was measured by SDS-PAGE assay and counted on a phosphorimager.

phosphorylated and therefore not denatured and has no kinase activity, confirms the presence of R65 and R68 at the active site of ERK2. The second class of mutants, D122A/H123A and N142A show reduced kinase activity with almost no change in their activation by rMEK. The above data also indicates that the domains required for phosphorylation by rMEK and for kinase activity are separable which would have important implications for inhibitor design.

The third class of mutants include Y34A and E58A/H59A which show only 20–30% reduction in either activity compared to wild type ERK2. The fourth class of mutants are the completely inactive mutants, D42A/K46A/R48A, a deletion mutant in the same region (−9aa(40–48)) and D104A/E107A/D109A. The complete loss of activity of the deletion mutant shows the importance of the distance between the GEGAYG box and the invariant Lysine 52. In addition, the nature of the amino acids within the spacing is equally critical as changing the amino acids to alanine (D42A/K46A/R48A) completely destroys the activities also. The X-ray crystal structure of the nucleotide bound ERK2 (MgATP-ERK2) [27] shows that the amino group (N6) of the adenine ring is hydrogen bonded to the backbone carbonyl group of Asp^{104}. The ribose O2′ and O3′ hydroxyls form a hydrogen bonding network to Asp^{109} and Lys^{112}. So the loss in activity may be due to the changes in Asp^{109} and not Asp^{104}. Clearly this mutation (D109A) eliminates these linkages such that rMEK is unable to phosphorylate ERK2 and therefore has no kinase activity.

Finally the mutants, K53A, E94A/K97A/D98A, K112A/K115A and R133A/K136A exhibit increased phosphorylation by rMEK and either lower (50%) or similar kinase activity as wild type ERK2.

Based on these data obtained from the mutagenesis studies described above, we constructed a series of peptides based on the ERK2 amino acid sequence. The peptides are currently being tested for their ability to inhibit rMEK activation of GST-ERK2 and GST-ERK2 dependent MBP phosphorylation. Smaller peptides (4–8 amino acids) based on these will be tested in this assay to determine the minimal length required to maintain kinase inhibitory activity. After defining this relationship in more detail a peptidomimetic approach to design a more 'drug' like molecule will be initiated.

In conclusion, we have defined five classes of mutants in human ERK2 by classical mutagenesis studies. Additional mutants are in the process of being constructed in the remaining 70% of the molecule. This will allow us to define other domains of ERK2 that may be amenable to inhibition by peptides or other molecules. Furthermore, some of the mutants described above, which were generated by substituting the amino acid alanine for the cognate wild type amino acid, will be further mutagenized by substituting other amino acids. This will allow us to determine if the mutant ERK2 biochemical phenotype is dependent on alanine substitution or if other (e.g. more bulky or charged amino acids) have the same effect on the activity and presumably on the structure of ERK2.

Acknowledgements: We thank Dr. Tim Bird for kindly providing the plasmid pGEX2T-ERK2, Dr. Peter Bohlen and Dr. Y. Gluzman for a useful discussion, Dr. Doug Kitchen for help in interpreting the X-ray data and Mr. Bob Jeyseelan for assistance in obtaining the skeletal muscle from a rabbit.

References

[1] Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827.
[2] Bos, J.L. (1989) Cancer Res. 49, 4682–4689.
[3] Feramisco, J.R., Gross, M., Komata, T., Rosenberg, M. and Sweet, R.W. (1984) Cell 38, 109–117.
[4] Muroya, K., Hattori, S. and Nakamura, S. (1992) Oncogene 7, 277–281.
[5] Moodie, S.A., Willumsen, B.M., Weber, M.J. and Wolfman, A. (1993) Science 260, 1658–1660.
[6] Van Aelst, L., Barr, M., Marcus, S., Polverino, P. and Wigler, M. (1993) Proc. Natl. Acad. Sci. USA 90, 6213–6217.
[7] Vojtek, A.B., Gollenberg, S.M. and Cooper, J.A. (1993) Cell 74, 205–214.
[8] Zhang, X.F., Settleman, J., Kyriakis, J.M., Takeuchi, S.E., Elledge, S.J., Marshall, M.S., Bruder, J.T., Rapp, U.R. and Avruch, J. (1993) Nature 364, 308–313.
[9] Warne, P.H., Viciana, P.R. and Downward, J. (1993) Nature 364, 352–355.
[10] Hall, A. (1994) Science 264, 1413–1414.
[11] Robbins, D.J., Zhen, E., Owaki, H., Vanderbilt, C.A., Ebert, D., Geppert, D.J. and Cobb, M.H. (1993) J. Biol. Chem. 268, 5097–5106.
[12] Hoshi, M., Nishida, E. and Sakai, H. (1993) Mol. Cell. Biol. 13, 4679–4690.
[13] Ray, L.B. and Sturgill, T.W. (1987) Proc. Natl. Acad. Sci. USA 84, 1502–1506.
[14] Pelech, S.L., Tombes, R.M., Meijer, L. and Krebs, E.J. (1988) Dev. Biol. 130, 28–36.
[15] Seth, A., Gonzalez, F.A., Gupta, S., Raden, D.L. and Davis, R.J. (1992) J. Biol. Chem. 267, 24796–24804.
[16] Baker, S.J., Kerppula, T.K., Luk, D., Vandenberg, M.T., Marshak, D.R., Curran, T. and Abate, C. (1993) Mol. Cell. Biol. 12, 4697–4705.
[17] Gibbs, C.S., Knighton, D.R., Sowadski, J.M., Taylor, S.S. and Zoller, M.J. (1992) J. Biol. Chem. 267, 4806–4814.
[18] Sambrook, J., Fritsch, E.R. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual.
[19] Smith, D.B. and Thomas, K.S. (1988) Gene 67, 31–40.
[20] Nakielny, S., Campbell, D.G. and Cohen, P. (1992) FEBS Lett. 308, 182–189.
[21] Nakielny, S., Cohen, P., Wu, J. and Sturgill, T. (1992) EMBO J. 11, 2123–2129.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Ray, L.B. and Sturgill, T.W. (1988) Proc. Natl. Acad. Sci. USA 85, 3753–3757.
[24] Anderson, N.G., Maller, J.L., Tonks, N.K. and Sturgill, T.W. (1990) Nature 343, 651–653.
[25] Wirenega, R.K. and Hol, W.G.J. (1983) Nature 302, 842–844.
[26] Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) Science 24, 42–51.
[27] Zhang, F., Strand, A., Robbin, D., Cobb, M.H. and Goldsmith, E.J. (1994) Nature 367, 704–711.