Identification of MYPT1 and NIPP1 as subunits of protein phosphatase 1 in rat liver cytosol

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Received 1 June 1999

Abstract Various studies have provided evidence for the existence of spontaneously active cytosolic species of protein phosphatase 1, but these enzymes have never been purified and characterized. We have used chromatography on microcystin-Sepharose and Resource Q to purify cytosolic protein phosphatases from rat liver. Two of the isolated enzymes were identified by Western analysis and peptide sequencing as complexes of the catalytic subunit of protein phosphatase 1 and either the inhibitor NIPP1 or the myosin-binding subunit MYPT1, which reportedly is not present in chicken liver. In contrast, PCR cloning revealed the expression of two MYPT1 splice variants in rat liver.

Key words: Dephosphorylation; Protein phosphatase; NIPP1; MYPT

1. Introduction

Protein phosphatase (PP) 1 and PP2A represent two major subfamilies of Ser/Thr protein phosphatases [1,2]. With phosphorylase *a* as substrate they account together for nearly all the protein phosphatase activity in crude tissue and cell fractions. Protein phosphatases of types 1 and 2A contain a structurally related catalytic subunit, but they can easily be differentiated biochemically by their distinct sensitivity to toxins (e.g. microcystin-LR) and protein inhibitors (e.g. inhibitor-2). The catalytic subunits of PP1 and PP2A do not exist freely in the cell but are associated with polypeptides that control the activity, substrate specificity and intracellular location of the phosphatase. PP2A holoenzymes consist of the catalytic subunit ($PP2A_C$), a constant A-subunit (PR65) and a variable third subunit or B-subunit. The type-1 holoenzymes usually contain, in addition to the catalytic subunit ($PP1_C$), only one regulatory subunit.

While most PP2A is soluble after homogenization and tissue fractionation, a large fraction of PP1 is sedimentable. This can be explained by the anchoring of specific PP1 holoenzymes to glycogen particles, ribosomes, endoplasmic reticulum, contractile elements and nuclei [1–4]. However, cytosolic species of PP1 have also been described. One of these represents an inactive heterodimeric complex between the catalytic subunit and inhibitor-2 [1]. In addition, several reports described spontaneously active species of PP1 in the cytosol, varying in size between 68 and 260 kDa [5–8]. The latter enzymes have never been characterized in more detail, however, largely because of problems with proteolysis during further purification.

Significant progress in the purification of PP1 holoenzymes has been made by the use of microcystin affinity chromatography [9,10]. This affinity chromatography is based upon the covalent binding of $PP1_C$, either free or associated with regulatory subunits, to microcystin [11] and the subsequent disruption of this bond by KSCN [9,12]. Haystead and co-workers developed a variant of this method whereby PP1 holoenzymes were eluted from avidin-Sepharose as a complex with microcystin-biotin [10]. We report here the use of microcystin-Sepharose for the purification of PP1 holoenzymes from rat liver cytosol. Furthermore, we have identified the regulatory subunits of two of these enzymes as MYPT1 and NIPP1, which were originally described as myosin-associated and nuclear regulators of PP1, respectively [4,13].

2. Materials and methods

2.1. Materials and assays

$PP1_C$ [14] and phosphorylase *b* [15] were purified from rabbit skeletal muscle. Inhibitor-2 was purified from heat-treated lysates from BL21(DE3) cells transformed with pET8d-inhibitor-2 plasmid (kindly provided by Dr. A.A. DePaoli-Roach), using chromatography on blue Sepharose [16]. Rabbit muscle myosin was obtained from Sigma. Phosphorylase and myosin were phosphorylated by phosphorylase kinase and myosin light-chain kinase, respectively, in the presence of [γ^{32}P]ATP. The digoxigenin protein labelling and detection kit was purchased from Roche Diagnostics. Microcystin-LR from ICN was coupled to CH-Sepharose as described [9].

A synthetic peptide comprising the carboxy-terminal 10 residues of the δ-isoform of human $PP1_C$ was coupled to bovine serum albumin, using glutaraldehyde [17], and used to raise antibodies in rabbits. The antibodies were purified by chromatography on Affi-T agarose. A peptide with the sequence of residues 341–351 of bovine NIPP1, plus an additional carboxy-terminal cysteine, was coupled to keyhole limpet hemocyanin and to bovine serum albumin, using the Pierce immunogen conjugation kit. Rabbit polyclonal antibodies against the hemocyanin-coupled peptide were affinity purified on albumin-coupled peptide linked to CNBr-activated Sepharose 4B. Rabbit antiserum against the N-terminal 38 residues of chicken MYPT1 was kindly provided by Dr. D. Hartshorne (University of Arizona, USA).

2.2. Purification of cytosolic protein phosphatases

The livers of five female Wistar rats (200 g) were homogenized with a Potter-Elvehjem device in two volumes of a buffer containing 50 mM Tris at pH 7.4, 0.5 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 0.25 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine and 5 μM leupeptin. After centrifugation for 10 min at $10\,000\times g$, the low-speed supernatant was re-centrifuged for 35 min at $170\,000\times g$. The second supernatant is further referred to as the 'cytosolic fraction'.

The cytosolic fraction (80 ml) was supplemented with 0.3 M NaCl

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Abbreviations: PP1, protein phosphatase 1; $PP1_C$, catalytic subunit of protein phosphatase 1; PP2A, protein phosphatase 2A; $PP2A_C$, catalytic subunit of PP2A

PII: S0014-5793(99)00875-3

and applied to a microcystin-Sepharose column (3 ml) that was equilibrated in buffer A (50 mM glycylglycine at pH 7.4, 0.5 mM dithiothreitol, 5 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine and 5 μM leupeptin) plus 0.3 M NaCl. The retained proteins were eluted with buffer A plus 3 M KSCN. After extensive dialysis against buffer A, the eluate (18 ml) was applied to a Resource Q column (1 ml; Amersham Pharmacia Biotech) that had been equilibrated in buffer A with 50 mM NaCl. The proteins were eluted with a 50 ml gradient of 50–1000 mM NaCl. The eluted fractions were assayed for spontaneous and trypsin-revealed phosphorylase phosphatase activities as described by Beullens et al. [18].

2.3. Peptide sequencing

Phosphatase pools B, C and D from Resource Q (see Fig. 1) were incubated overnight with either modified trypsin (Roche; 5 μg/ml) at 37°C or *Staphylococcus aureus* SV8 protease (25 μg/ml) at 22°C. After addition of 0.1% trifluoroacetic acid, the digest was applied to a C_2/C_{18} reversed-phase column (0.35 ml; Amersham Pharmacia Biotech), which was eluted with a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. The eluted peptides, as detected by absorbance measurements at 215 nm, were collected manually and sequenced with an Applied Biosystems Procise (model 492) protein sequencer in the pulsed liquid mode.

2.4. Northern and (far-) Western analysis

Total RNA was isolated from 100–500 mg of frozen cells or pulverized rat tissues by CsCl gradient centrifugation [19]. The RNA was fractionated by electrophoresis (30 μg/lane) in formaldehyde-agarose (1%) gels and transferred by capillary diffusion to nylon membranes. The membranes were hybridized with cDNAs encoding nt 430–1923 (A of initiation codon taken as nt 1) of isoform 3 of rat aorta MYPT1 [13] or with 18S ribosomal cDNA, labelled to a specific radioactivity of 2×10^9 cpm/μg by random priming (Life Technologies, Inc). Hybridizations were performed overnight at 65°C in 5×SSC, 20 mmol/l phosphate (pH 6.5), 1×Denhardt's solution, 8% dextran sulfate, and 100 μg/ml salmon sperm DNA. Subsequently, the membranes were washed twice for 15 min at room temperature in 2×SSC/0.1% SDS and twice for 15 min at 65°C in 0.2×SSC/0.1% SDS. The hybridized cDNA was visualized by phosphor image analysis.

The $PP1_{C\delta}$ and NIPP1 antibodies were used at a final concentration of 1 μg/ml. The MYPT1 antiserum was used at a final dilution of 1/10 000. The peroxidase-labelled secondary antibodies were detected by enhanced chemiluminescence. Far-Western blotting using digoxigenin-labelled $PP1_C$ was performed as described by Beullens et al. [18].

2.5. The cloning of rat liver MYPT1

A rat liver cDNA library (Stratagene) was used as a template for several PCR-reactions with r*Taq* DNA polymerase (TaKaRa Biomedicals) and different sets of primers that were deduced from the sequence of the rat aorta MYPT1 isoform 1 [13]. The sense-primers were: 5′-TCGCGATAAGAAGAGCCGGCGGCA-3′, 5′-GATATTGCAGAGGAGGAAGCAATG-3′, 5′-TTGTGGGCTGAGGATAGTACT-3′ and 5′-AAACGGAGGTCTACTGGAGT-3′. The antisense primers were: 5′-TTCCTCTTCTTTTCGAGCTGCTTC-3′, 5′-AGCACTTACAACAGTTGGAGCAA-3′, 5′-GGAGCCATCCTCGGTGTCTGACT-3′ and 5′-TCCTAGATAAGAGGGCGTTTGGCA-3′. The PCR products were analyzed by gel electrophoresis and Southern blotting, and the essential fragments were cloned into the pGEM-T easy vector (Promega). DH5α cells (Life Technologies) were transformed with these plasmids and the inserts were sequenced in an automated laser fluorescent DNA sequencer (Amersham Pharmacia Biotech).

3. Results

3.1. Purification of cytosolic protein phosphatases

Following chromatography of a rat liver cytosolic fraction on microcystin-Sepharose (not shown), the phosphorylase phosphatase activity in the eluate could be further fractionated into four peaks (A–D) by anion exchange chromatography on Resource Q (Fig. 1). The phosphatase activity in all four peaks was completely blocked by 0.7 μM inhibitor-2,

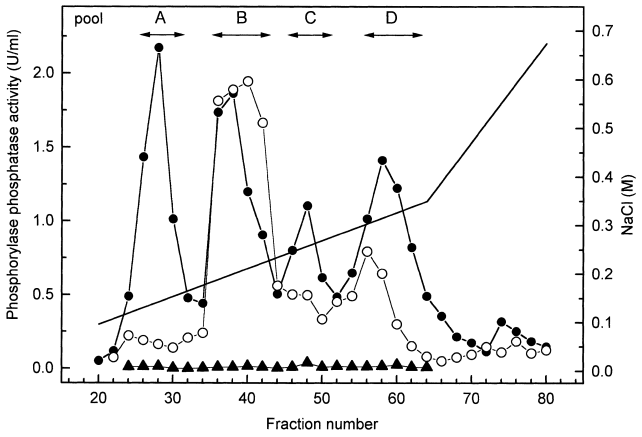

Fig. 1. Chromatography of cytosolic phosphorylase phosphatases on Resource Q. The dialyzed eluate of microcystin-Sepharose was applied to a Resource Q column (1 ml). The retained proteins were eluted with a salt gradient of 50 ml (solid line) and the fractions (0.5 ml) were assayed for phosphorylase phosphatase activities, as such (○), after preincubation of the fractions with trypsin (●), and in the presence of 0.7 μM inhibitor-2 (▲).

showing that the activity stemmed exclusively from PP1. The presence of $PP1_C$ in pools A–D was confirmed by Western analysis (not illustrated). The phosphorylase phosphatase activity in peaks A, C and D was stimulated severalfold by preincubation with trypsin (Fig. 1). Since trypsin barely affects the phosphorylase phosphatase activity of the free catalytic subunit [18], this suggested that the activity of $PP1_C$ in these fractions was restrained by associated regulatory subunits. By contrast, peak B behaved as free $PP1_C$; indeed, it migrated during gel filtration on Superdex 75 (Amersham Pharmacia Biotech) as a protein of about 40 kDa (not shown) and its phosphorylase phosphatase activity was somewhat inhibited by trypsin (Fig. 1). Furthermore, the N-terminal sequences of all six tryptic peptides that could be obtained from pool B were contained in $PP1_C$ (Table 1). The sequences obtained did not allow us, however, to identify the $PP1_C$ isoform(s) present in pool B.

The phosphatase in pool A showed a very low spontaneous activity (Fig. 1) and migrated during gel filtration as a protein of about 90 kDa (not shown). This was reminiscent of $PP1N_{NIPP1}$, which was identified in rat liver nuclei as a heterodimeric complex between $PP1_C$ and the inhibitory protein NIPP1 [4]. In further agreement with the presence of NIPP1 in pool A we found that a protein of the expected size (41 kDa on 10% Tricine-SDS-PAGE) could be detected by Western analysis with antibodies against the carboxy-terminus of NIPP1 and by far-Western analysis with digoxigenin-labelled $PP1_C$ (not shown). Furthermore, NIPP1 in pool A could also be shown to co-immunoprecipitate with $PP1_C$ (not illustrated), proving that both components formed a complex.

The fractions corresponding to pool C contained a protein of 65 kDa that appeared homogeneous by Coomassie staining (not illustrated). N-terminal sequencing of five peptides that were obtained by treatment of pool C with *S. aureus* SV8 identified the 65 kDa polypeptide as the A-subunit of PP2A (Table 1). Since no catalytic subunit of PP2A could be detected in these fractions by Coomassie staining or by phosphorylase phosphatase assays in the presence of inhibitor-2, we conclude that the A-subunit was eluted without associated

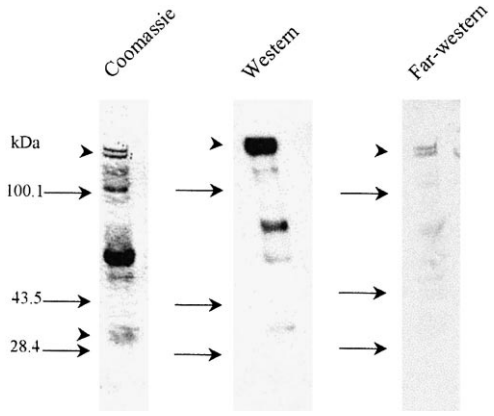

Fig. 2. Identification of MYPT1 as a subunit of cytosolic PP1. MYPT1 was visualized in pool D by Coomassie staining, by Western analysis and by far-Western analysis with the digoxigenin-labelled catalytic subunit. The arrows indicate the position of the marker proteins. The arrowheads indicate the position of migration of intact MYPT1 (110–115 kDa) and $PP1_C$ (35–38 kDa).

catalytic subunit, in agreement with findings of Campos et al. [10]. Apparently, the adopted elution procedure (3 M KSCN) disrupted the PP2A holoenzyme structure but did not affect the covalent interaction between microcystin and $PP2A_C$, which remained associated with the affinity column. On the other hand, it is well established that KSCN breaks the covalent bond between $PP1_C$ and microcystin [9,12]. We did not obtain any peptide sequence that enabled us to identify the subunit(s) of the type 1 phosphorylase phosphatase that eluted in pool C.

Sequencing of tryptic peptides revealed the presence of both $PP1_{C\delta}$ and the myosin-phosphatase binding subunit MYPT1 in pool D (Table 1). MYPT1, as well as some proteolytic fragments, could also be visualized in pool D by Western analysis with antibodies against the N-terminus of MYPT1 and by far-Western blot analysis with digoxigenin-labelled $PP1_C$ (Fig. 2). In further agreement with the presence of a myosin phosphatase in pool D, we found that this pool dephosphorylated myosin that had been phosphorylated by the myosin light chain kinase at a much faster rate than did free

Table 1
Amino acid sequence of proteolytic peptides derived from Resource Q pools B, C and D

Peptide sequence	Found in (residue number)
Pool B	
AHQVVEDGYEFFAK	$PP1_C$ (246–259)
LFSAPNYXGEF	$PP1_C$ (265–275)
IFXXHGGLSPDL	$PP1_C$ (168–179)
IYGFYDEXXR	$PP1_C$ (132–141)
GNHEXASIN	$PP1_C$ (122–130)
TFTDXFNXLPIAAXVDEK	$PP1_C$ (150–167)
Pool C	
EXMPLXAXQXGV	A-subunit of PP2A (473–484)
EAXFVPLV	A-subunit of PP2A (173–180)
EXVQLR	A-subunit of PP2A (71–76)
ELQKAV	A-subunit of PP2A (317–322)
ETVVRD	A-subunit of PP2A (149–154)
Pool D	
WIXSETDLEPXVVK	MYPT1 (17–30)
YQYTSLNSGRPVTP	$PP1_{C\delta}$ (304–317)

The numbers in parentheses show the corresponding residues of rat $PP1_{C\delta}$ (pools B and D), the human A-subunit (α-isoform) of PP2A, and isoform 1 of rat aorta MYPT1.

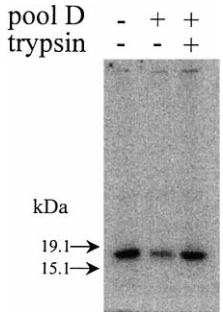

Fig. 3. Rat liver cytosol contains a myosin phosphatase. Myosin, phosphorylated by myosin light chain kinase, was incubated for 10 min as such, with an aliquot of pool D of the Resource Q column, or with the same amount of pool D that had been preincubated with trypsin to generate free $PP1_C$. Subsequently, the assay mixture was subjected to 7.5% Tricine-SDS-PAGE. The figure shows an autoradiogram of the dried gel.

$PP1_C$, obtained by trypsinolysis of the holoenzyme (Fig. 3). The specific phosphorylase phosphatase activity of the pool of purified $PP1M_{MYPT1}$ amounted to 897 U/mg, which is close to the value reported for the enzyme from chicken gizzard [20]. Thus, the Coomassie-stained bands in the pool of purified $PP1M_{MYPT1}$, in addition to that of intact MYPT1 (a doublet of 110–115 kDa) and $PP1_C$ (ca. 35 kDa), are likely to represent proteolytic fragments of MYPT1. In further agreement with this view, the major degradation product (55–60 kDa) corresponds in size to a previously characterized proteolytic fragment of MYPT1 in purified preparations of PP1M from smooth muscle [21].

3.2. The cloning of rat liver MYPT1

The detection of MYPT1 in pool D was unexpected since this 'smooth muscle' myosin-binding subunit was previously reported not to be expressed in the liver [21,22]. We wondered whether this discrepancy could perhaps be explained by the existence of liver-specific isoform(s) of MYPT1. Therefore, the hepatic MYPT1 isoforms were PCR-cloned using a rat liver cDNA library as template and primers based upon the cDNA sequences of rat aorta MYPT1. The sequencing of overlapping fragments covering the complete MYPT1 cDNA revealed that rat liver contains two isoforms of MYPT1 (not illustrated) that are identical to the rat aorta isoforms 1 and 3 [13]. These isoforms have calculated masses of 110 and 115

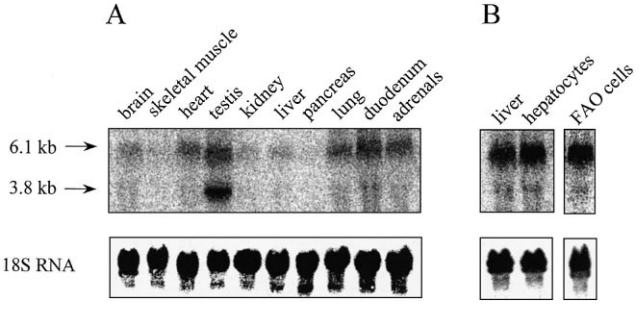

Fig. 4. Tissue distribution of MYPT1 in the rat. Total RNA (30 μg/lane) of the indicated rat tissues and cells was fractionated by electrophoresis and subsequently blotted onto nylon membranes. The membranes were hybridized with cDNAs encoding 18S RNA or a fragment (nt 430–1923) of rat aorta MYPT1 mRNA (isoform 3). The figure shows an autoradiogram of the hybridized membrane.

kDa, respectively, and only differ by the absence (isoform 1) or presence (isoform 3) of a 56 residue insert in the central domain.

Northern blot analysis showed a major MYPT1 transcript of 6.1 kb in all rat tissues investigated, except pancreas and skeletal muscle (Fig. 4A). In addition, a 3.8 kb MYPT1 transcript was detected that was particularly abundant in testis. The MYPT1 transcripts were equally abundant in rat liver and rat hepatocytes and in FAO hepatoma cells (Fig. 4B), showing that the detection of MYPT1 in liver cytosol cannot be attributed to its expression in non-parenchymal cells. MYPT1 could also be detected by Western blotting in the cytosolic fraction from rat liver (not shown).

4. Discussion

Both cytosolic holoenzymes of PP1 that we have isolated were originally described in a different context. $PP1N_{NIPP1}$ has previously been characterized as a nuclear complex of $PP1_C$ and NIPP1 [4]. By immunoblotting of hepatic fractions at the same protein concentration, NIPP1 could be readily detected in the nuclear fraction but not in the cytosolic fraction [23]. Given the larger volume and protein concentration of the cytosolic fraction these findings do not rule out the possibility, however, that a significant fraction of hepatic NIPP1 is cytosolic. It cannot be excluded either that the detection of a 'cytosolic' $PP1N_{NIPP1}$ (this work) is an artefact, resulting from nuclear leakage during tissue fractionation.

The myosin-associated phosphatase $PP1M_{MYPT1}$ was known to be present in several chicken tissues [21] except for skeletal muscle, which contains a distinct type of PP1M [24], and liver, which reportedly did not contain MYPT1 protein [21] or mRNA [22]. Unexpectedly, our results show that $PP1M_{MYPT1}$ is indeed present in rat liver, and that the concentration of MYPT1 transcripts in liver parenchymal cells is at least as high as that in total liver.

In non-muscle cells phosphorylation of the myosin light chains by a specific Ca^{2+}/calmodulin-activated kinase enables myosin to interact with actin and to assemble into microfilaments (also known as stress fibers or focal adhesions) that play a role in phagocytosis, cytokinesis and organelle movements. By its ability to dephosphorylate myosin light chains $PP1M_{MYPT1}$ causes the disruption of microfilaments and the release of myosin as a free cytosolic protein. Since myosin is expected to be rapidly dephosphorylated by $PP1M_{MYPT1}$ after tissue homogenization, for lack of an opposing kinase action, both myosin and $PP1M_{MYPT1}$ are expected to be present in the cytosolic fraction, which agrees with our observations.

In conclusion, we have identified two species of PP1 in rat liver cytosol as $PP1M_{MYPT1}$ and $PP1N_{NIPP1}$. We propose that these holoenzymes account, at least in part, for the previously described 'high-molecular-weight' cytosolic phosphatases [5–8].

Acknowledgements: This work was supported by the Fund for Scientific Research-Flanders (Grant G.036.98). M. Beullens is a postdoctoral fellow and E. Waelkens a research associate of the Fund for Scientific Research-Flanders. C. Stefan and A. Van Eynde are acknowledged for preparing the Northern blots from rat tissues and for advice on PCR cloning, respectively. N. Sente provided expert technical assistance. We thank Drs. D. Hartshorne and A.A. DePaoli-Roach for the generous gifts of MYPT1 antibodies and pET8d-inhibitor-2 plasmid, respectively.

References

[1] Bollen, M. and Stalmans, W. (1992) Crit. Rev. Biochem. Mol. Biol. 27, 227–281.
[2] Shenolikar, S. (1994) Annu. Rev. Cell Biol. 10, 55–86.
[3] Beullens, M., Stalmans, W. and Bollen, M. (1996) Eur. J. Biochem. 239, 183–189.
[4] Jagiello, I., Beullens, M., Stalmans, W. and Bollen, M. (1995) J. Biol. Chem. 270, 17257–17263.
[5] Killilea, S.D., Mellgren, R.L., Aylward, J.H., Metieh, M.E. and Lee, E.Y.C. (1979) Arch. Biochem. Biophys. 193, 130–139.
[6] Khandelwal, R.L., Zinman, S.M. and Ng, T.T.S. (1980) Biochim. Biophys. Acta 626, 486–493.
[7] Goris, J. and Merlevede, W. (1988) Biochem. J. 254, 501–507.
[8] Bollen, M., Vandenheede, J.R., Goris, J. and Stalmans, W. (1988) Biochim. Biophys. Acta 969, 66–77.
[9] Moorhead, G., MacKintosh, R.W., Morrice, N., Gallagher, T. and MacKintosh, C. (1994) FEBS Lett. 356, 46–50.
[10] Campos, M., Fadden, P., Alms, G., Qian, Z. and Haystead, T.A.J. (1996) J. Biol. Chem. 271, 28478–28484.
[11] MacKintosh, R.W., Dalby, K.N., Campbell, D.G., Cohen, P.T.W., Cohen, P. and MacKintosh, C. (1995) FEBS Lett. 371, 236–240.
[12] Beullens, M., Van Eynde, A., Stalmans, W. and Bollen, M. (1992) J. Biol. Chem. 267, 16538–16544.
[13] Hartshorne, D.J., Ito, M. and Erdödi, F. (1998) J. Muscle Res. Cell Motil. 19, 325–341.
[14] DeGuzman, A. and Lee, E.Y.C. (1988) Methods Enzymol. 159, 356–368.
[15] Fischer, E.H. and Krebs, E.G. (1958) J. Biol. Chem. 231, 65–71.
[16] Park, I.-K., Roach, P., Bondor, J., Fox, S.P. and DePaoli-Roach, A.A. (1994) J. Biol. Chem. 269, 944–954.
[17] Briand, J.P., Muller, S. and Van Regenmortel, M.H.V. (1985) J. Immunol. Methods 78, 59–69.
[18] Beullens, M., Stalmans, W. and Bollen, M. (1998) Methods Mol. Biol. 93, 145–155.
[19] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[20] Alessi, D., Macdougall, L.K., Sola, M.M., Ikebe, M. and Cohen, P. (1992) Eur. J. Biochem. 210, 1023–1035.
[21] Okubo, S., Ito, M., Takashiba, Y., Ichikawa, K., Miyahara, M., Shimizu, H., Konishi, T., Shima, H., Nagao, M., Hartshorne, D.J. and Nakano, T. (1994) Biochem. Biophys. Res. Commun. 200, 429–434.
[22] Shimizu, H., Ito, M., Miyahara, M., Ichikawa, K., Okubo, S., Konishi, T., Naka, M., Tanaka, T., Hirano, K., Hartshorne, D.J. and Nakano, T. (1994) J. Biol. Chem. 269, 30407–30411.
[23] Van Eynde, A., Wera, S., Beullens, M., Torrekens, S., Van Leuven, F., Stalmans, W. and Bollen, M. (1995) J. Biol. Chem. 270, 28068–28074.
[24] Moorhead, G., Johnson, D., Morrice, N. and Cohen, P. (1998) FEBS Lett. 438, 141–144.