THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 267, No. 23, Issue of August 15, pp. 16538-16544, 1992
Printed in U.S.A.

The Isolation of Novel Inhibitory Polypeptides of Protein Phosphatase 1 from Bovine Thymus Nuclei*

(Received for publication, March 2, 1992)

Monique Beullens, Aleyde Van Eynde, Willy Stalmans, and Mathieu Bollen‡

From the Afdeling Biochemie, Fakulteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Nuclei from bovine thymus contain a high level of partially latent protein phosphatase 1 (PP-1). More than 90% of this PP-1 is associated with the insoluble chromatin/matrix fraction and can be extracted with 0.3 M NaCl. The salt extract also contains three heat- and acid-stable inhibitory proteins of PP-1 that can be resolved on Mono Q. We have purified two of these nuclear inhibitors of PP-1 (NIPP-1a and NIPP-1b) until homogeneity. They are acidic proteins (pI = 4.4) with a molecular mass of 18 kDa (NIPP-1a) and 16 kDa (NIPP-1b) on SDS-PAGE. Judged from the larger molecular mass that was deduced from gel filtration (35 kDa), NIPP-1a and NIPP-1b appear to be asymmetric or dimeric proteins.

The nuclear inhibitors totally inhibited the phosphorylase phosphatase activity of PP-1, but even at a 250-fold higher concentration they did not affect the activities of the other major serine/threonine protein phosphatases (PP-2A, PP-2B, and PP-2C). NIPP-1a and NIPP-1b inhibited the catalytic subunit of PP-1 with an extrapolated K_i of about 1 pM, which is some three orders of magnitude better than the cytoplasmic proteins inhibitor 1/DARPP-32 and modulator. The nuclear inhibitors were not inactivated by incubation with protein phosphatases that inactivate inhibitor 1 and DARPP-32. Unlike modulator, they were not able to convert the catalytic subunit of PP-1 into a MgATP-dependent form.

Remarkably, the extent of inhibition of PP-1 by NIPP-1b depended on the nature of the substrate. The phosphorylase phosphatase and casein phosphatase activities of PP-1 were completely blocked by NIPP-1b, whereas the dephosphorylation of basic proteins was either not at all inhibited (histone IIA) or only partially (myelin basic protein). These data may indicate that the acidic NIPP-1b is inactivated through complexation by basic proteins. Indeed, nonphosphorylated histone IIA antagonized the inhibitory effect of NIPP-1b on the casein phosphatase activity of PP-1.

Our data show that the nucleus contains specific and potent inhibitory proteins of PP-1 that differ from earlier described cytoplasmic inhibitors. We suggest that these novel proteins may control the activity of nuclear PP-1 on its natural substrate(s).

Type-1 protein phosphatases constitute a well defined group of enzymes that play an essential role in the regulation of such diverse cellular processes as glycogen metabolism, calcium transport, muscle contraction, intracellular transport, protein synthesis, and cell division (1, 2). They can easily be differentiated from other serine/threonine-specific protein phosphatases by several criteria; *e.g.* their activity is specifically inhibited by cytoplasmic proteins, termed inhibitor 1 (and the isoform DARPP-32) and inhibitor-2 or modulator. It has also become apparent that all known type-1 protein phosphatases contain an isoform of the same catalytic subunit ($PP\text{-}1_C$).[1] They differ, however, in the noncatalytic subunit(s) that control the activity, the substrate specificity, and the subcellular location of the phosphatase.

Distinct species of PP-1 holoenzymes are bound with high affinity to glycogen particles, to the endoplasmic reticulum and to myosin (1, 2). There is also at least one cytosolic species of PP-1. In addition, it has been established that the nuclei of various cell types contain a high level of PP-1 (3–8). While some investigators detected only free $PP\text{-}1_C$ in the nucleus (4, 5), others provided evidence for an oligomeric structure of nuclear PP-1 (3, 7–9).

The physiological substrates of nuclear PP-1 (PP-1N) are not yet known. There is clear evidence, however, for an essential role of PP-1N in the progression of the cell cycle in fungi. For example, the isoform of $PP\text{-}1_C$ that is encoded by the *bimG*$^+$ gene of *Aspergillus nidulans* appears to be required for completion of the anaphase (10). A particular mutation in this gene (*bimG11*) causes an abnormally high level of nuclear phosphoproteins, indicating that the product of *bimG*$^+$ is a nuclear enzyme. A loss-of-function mutation of the *dis2*$^+$ gene (*dis2-11*) in the fission yeast *Saccharomyces pombe* has also been associated with a block in chromosome disjoining during mitosis (8, 9). The latter mutant could be complemented by genes, including *dis2*$^+$ itself, which encode isoforms of $PP\text{-}1_C$ that are preferentially located in the nucleus. The *dis2-11* mutant could, however, also be rescued by multicopy plasmids carrying the *sds22*$^+$ gene (11). This suppressor gene encodes a 30-kDa protein that is present in the insoluble nuclear fraction and that somehow appears to enhance the dephosphorylation by PP-1N. The *dis2*$^+$ gene is identical to the *bws*$^+$ gene, which has been shown to interfere with the timing for entry into mitosis (11). Finally, there is also conclusive evidence for an essential role of PP-1 during mitosis in *Drosophila* (13, 14). Indeed, mutants lacking a functional isoform of $PP\text{-}1_C$ that accounts for about 80% of the cellular activity of PP-1, die at a larval stage. This mutation is associated with

* This work was supported by Grant 3.0011.90 from the Belgian Fund for Medical Scientific Research and by a Concerted Research Action of the "Vlaamse Executieve." The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ "Bevoegdverklaard navorser" of the National Fund for Scientific Research (Belgium). To whom correspondence should be addressed: Afdeling Biochemie, Campus Gasthuisberg KULeuven, B-3000 Leuven, Belgium.

[1] The abbreviations used are: $PP\text{-}1_C$, catalytic subunit of PP-1; NIPP-1, nuclear inhibitor of PP-1; PP-1, protein phosphatase 1; PP-2A, protein phosphatase 2A; PP-2B, protein phosphatase 2B; PP-2C, protein phosphatase 2C; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

defects in mitosis, like a deficient spindle organization, an abnormal sister-chromatid segregation, hyperploidy, and an excessive degree of chromosome condensation.

It is not yet understood how the activity of PP-1N is controlled. Using immunological procedures, Kinoshita *et al.* (9) did not find any evidence for a fluctuation in the concentration of the product of *dis2*$^+$ during the cell cycle of fission yeast. This suggests that the activity of PP-1N, like that of the extranuclear species of PP-1, is controlled by interaction with other polypeptides. We have therefore started to look for regulatory polypeptides of PP-1 in the nucleus and report here the isolation of two novel proteins from bovine thymus nuclei that act as extremely potent and specific inhibitors.

EXPERIMENTAL PROCEDURES

Materials—Fresh calf thymus was collected on ice in a local abattoir and was stored within 2 h at −80 °C in pieces of 30–50 g. Microcystin-LR, histone IIA, myelin basic protein, and the catalytic subunit of cAMP-dependent protein kinase were purchased from Sigma. Heparin-Sepharose as well as columns of Mono Q, Mono P, and Superdex were obtained from Pharmacia LKB Biotechnology Inc. Polyvinylidene difluoride membranes (Immobilon) were purchased from Millipore. Casein was prepared according to the procedure of Mercier *et al.* (15). Modulator (16) and phosphorylase *b* (17) were prepared from rabbit skeletal muscle. Microcystin-Sepharose was prepared according to the recommendations of Pharmacia. 100 μg of microcystin-LR were dissolved in water at pH 12 and mixed with 0.3 g of epoxy-activated Sepharose 6B.

Purification of Protein Phosphatases—Except for PP-2C, which was prepared from rat liver (18), protein phosphatases were purified from rabbit skeletal muscle. These include PP-2B (19) as well as the catalytic subunits of PP-1 (20) and PP-2A (21). For the experiments illustrated in Figs. 4 and 5, it was important to have homogeneously purified PP-1_C. The adopted purification procedure (20) yielded an enzyme that showed only two bands on SDS-PAGE, of 37.6 and 32.7 kDa, corresponding to intact and C-terminally nicked PP-1_C, respectively (2, 20). However, the specific activity of this phosphatase could still be increased severalfold by affinity chromatography on microcystin-Sepharose. Due to its strong binding to microcystin-LR, the phosphatase had to be eluted with 3 M KSCN. After subsequent dialysis, the specific activity of PP-1_C amounted to 14,978 units/mg protein, when assayed with 10 μM phosphorylase.

Preparation of Phosphosubstrates—Phosphorylase *b* was fully converted to the active *a*-form by purified phosphorylase kinase (22). Casein, myelin basic protein, and histone IIA were phosphorylated by incubation at a final concentration of 2.5 mg/ml during 1 h at 37 °C with the catalytic subunit of cAMP-dependent protein kinase (1500 units/ml), 0.2 mM γ-^{32}P-labeled ATP and 1 mM $MgCl_2$. The phosphorylation mixture also contained 50 mM glycylglycine at pH 7.4, 0.5 mM dithiothreitol, 5 mM β-mercaptoethanol, and 0.02% (w/v) Brij (buffer A). The substrates were subsequently reisolated by precipitation with 10% (w/v) trichloroacetic acid. Remnants of the acid were removed by washing three times with ether, and the dried proteins were dissolved in buffer A. The phosphorylation stoichiometry amounted to 0.29 (casein), 0.6 (myelin basic protein), and 0.27 (histone IIA) mol phosphate/mol substrate.

Assays—Protein phosphatase activities were determined at 30 °C from the rate of dephosphorylation of substrates that were present at a final concentration of 10 μM (phosphorylase and histone IIA), 13 μM (casein), or 7 μM (myelin basic protein). In addition to buffer A (see above), the assay mixture also contained 5 mg/ml of bovine serum albumin and 5 mM caffeine. The extent of dephosphorylation was assessed from the released acid-soluble radioactivity. The phosphorylase phosphatase activity was either assayed as such ("spontaneous" activity) or after preincubation with trypsin (0.1 mg/ml) for 5 min at 30 °C ("total" activity). The action of trypsin was arrested by the addition of soybean trypsin inhibitor (1 mg/ml). One unit of PP-1 liberates 1 nmol of phosphate/min under the specified assay conditions.

NIPP-1 was assayed at the indicated dilutions as an inhibitor of protein phosphatase activity. Prior to the addition of the phosphosubstrate, NIPP-1 was preincubated during 5 min at 30 °C with the specified phosphatase. Unless indicated otherwise, the concentration of PP-1_C during the assay of NIPP-1 amounted to 0.5–1.5 nM. One unit of NIPP-1 is defined as the amount that inhibits one unit of the phosphorylase phosphatase activity of PP-1_C. During the initial stages of the purification of NIPP-1 (until heparin-Sepharose), the preparation also contained some nonprotein heat-stable inhibitor(s) of PP-1. Therefore, the activity of (trypsin-labile) NIPP-1 in these fractions was assessed from the comparison of the heat-stable inhibitory activity before and after preincubation with trypsin. The conditions for the incubation with trypsin were the same as for the assay of the total phosphorylase phosphatase activity.

Protein was measured with bovine serum albumin as standard (23). The recovery of protein in the last purification step of NIPP-1a and NIPP-1b (blotting and elution from Immobilon membranes) was calculated from scans of Coomassie-stained gels.

Results are means ± S.E. for the indicated number (n) of observations.

Preparation of Subnuclear Fractions—Nuclei were prepared from freshly thawed pieces of bovine thymus as described in method 7 of (24). As a mean, 1 kg of thymus yielded 238 ± 17 g (n = 10) of nuclei. These nuclei were resuspended in 1 liter of buffer B, containing 10 mM Tris-HCl at pH 7.5, 5 mM $MgCl_2$, 0.25 mM sucrose, 0.5 mM phenylmethanesulfonyl fluoride, and 0.5% Triton X-100, and resedimented by centrifugation (10 min at 5,000 × g). The latter concentration of Triton X-100 has been reported to dissolve the outer nuclear membrane without further disruption of the nuclei (25). The nuclei were washed once in 1 liter of buffer B without Triton X-100 and subsequently lyzed by resuspension in 1 liter of hypotonic buffer C, containing 10 mM Tris-HCl at pH 7.5, 5 mM $MgCl_2$, and 0.5 mM phenylmethanesulfonyl fluoride. Upon centrifugation (10 min at 5,000 × g) a supernatant (nucleoplasm) and a particulate fraction, termed "chromatin/matrix fraction" were obtained. The latter fraction was resuspended in 500 ml of buffer C plus 0.3 M NaCl, incubated during 30 min at 0 °C and resedimented. The chromatin/matrix fraction was extracted two times more with 250 ml of the same buffer, and the three supernatants were combined ("0.3 M NaCl extract"). In the experiments illustrated in Table I, the remaining particulate fraction was additionally extracted, twice with 500 ml of buffer C plus 1 M NaCl, and then twice with 500 ml of buffer C plus 0.5% Triton X-100.

Purification of NIPP-1a and NIPP-1b from the 0.3 M NaCl Extract—Crystalline ammonium sulfate was slowly added to the first salt extract of the nuclei from 1 kg thymus until 55% saturation. The pH was raised to 7.4 with NaOH and after standing for 30 min on ice, the flocculated proteins were sedimented by centrifugation during 30 min at 10,000 × g. The pellet was washed once with 1 liter of a buffer, containing 50 mM Tris-HCl at pH 7.5, 5 mM β-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, 0.3 mM benzamidine (buffer D), which was 55% saturated with ammonium sulfate. The washed pellet was dissolved in 100 ml of buffer D and dialyzed overnight against 5 liters of 5 mM Tris at pH 7.5. After 5-fold dilution with 10 mM Tris at pH 7.5, the dialyzed fraction was rapidly (~10 min) heated in a water bath until 90 °C and then incubated for an additional 10 min at the same temperature. Subsequently, the fraction was cooled on ice until 10 °C, and the denatured proteins were sedimented by centrifugation during 20 min at 10,000 × g. The supernatant was supplemented with 2% (w/v) trichloroacetic acid, kept during 90 min on ice, and the precipitated proteins were sedimented during 30 min at 10,000 × g. The pellet was successively washed once with 100 ml of 2% (w/v) trichloroacetic acid and three times with 20 ml acetone and resedimented each time by centrifugation during 10 min at 3,000 × g.

The pellet was dissolved in 25 ml of buffer A. Denatured proteins were removed by centrifugation and the soluble fraction was loaded onto a heparin-Sepharose column (15 × 1 cm) equilibrated in the same buffer. The column was eluted with a linear 150-ml gradient of 0–0.7 M NaCl in buffer A. Fractions containing NIPP-1 activity (elution peak at about 0.3 M NaCl) were pooled and concentrated until 1 ml by ultrafiltration (Amicon Centriprep 10). After a 10-fold dilution with water the sample was applied to a Mono Q column (5 × 0.5 cm) equilibrated in buffer A plus 50 mM NaCl. The column was subsequently washed with 10 ml of buffer A plus 50 mM NaCl, and developed with a 45-ml linear gradient of 50–500 mM NaCl in buffer A. Three species of NIPP-1 were identified and termed NIPP-1a, NIPP-1b, and NIPP-1c, according to their order of elution.

NIPP-1a and NIPP-1b were pooled separately, dialyzed against buffer A, and concentrated by lyophilization until 200 μl. Either fraction was applied to a C_{18} column (Econosil, Alltech, 25 × 0.46 cm) equilibrated in 0.1% (v/v) trifluoroacetic acid. The inhibitory proteins were eluted with a 40-ml linear gradient of 25–50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The lyophilized frac-

TABLE I

Subnuclear distribution of PP-1 and NIPP-1

The phosphorylase phosphatase activity was measured in several ways, as indicated in the experimental section. Modulator was added at a final concentration of 0.2 μM. NIPP-1 was assayed after heating the fractions during 10 min at 90 °C, by the difference in inhibitory power before and after treatment with trypsin. Results represent the means ± S.E. for three to four experiments.

Nuclear fraction	Phosphorylase phosphatase			NIPP-1
	Spontaneous	Plus modulator	After trypsin	
	units/g thymus			
Nucleoplasm	0.4 ± 0.1	0.1 ± 0.01	2.3 ± 0.2	0.5 ± 0.2
Chromatin/matrix fraction				
0.3 M NaCl extract	16.8 ± 3.4	2.2 ± 0.4	40.5 ± 4.9	8.8 ± 1.4
1.0 M NaCl extract	2.6 ± 1.2		4.9 ± 2.2	—[a]
0.5% Triton X-100 extract	0.8 ± 0.2		1.2 ± 0.3	0

[a] Cannot be reliably measured (see text).

tions were dissolved in 35 μl of buffer A, assayed for NIPP-1, and the peak fractions were subjected to SDS-PAGE in slab gels. Subsequently, the proteins were blotted overnight onto Immobilon membranes at 35 V. The blotted polypeptides were visualized by staining with Amido Black and cut out separately. The polypeptides were eluted from these membrane pieces with 70% formic acid, as described (26). Finally, the eluates were lyophilized, dissolved in 15 μl of buffer A and assayed for NIPP-1.

Electrophoresis—SDS-PAGE (27) was performed on 15% slab gels or on precast 10–15% gradient gels of the Phast system (Pharmacia). The apparent M_r was calculated by comparison with the following standard proteins: bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,200).

RESULTS

PP-1 in the Nuclei of Calf Thymus—Rupture of the outer nuclear membrane with 0.5% Triton X-100 did not result in the release of any measurable phosphorylase phosphatase activity (not shown). Upon further subnuclear fractionation, about 2% of the recovered spontaneous phosphorylase phosphatase activity was present in the nucleoplasm (Table I). The remainder of the phosphatase activity (98%) was associated with the insoluble nuclear fraction, which consists of chromatin and elements of the nuclear matrix (28). Most of the particulate phosphorylase phosphatase activity (16.8 units/g thymus) was solubilized by incubation with 0.3 M NaCl. The subsequent treatment of the chromatin/matrix fraction with 1 M NaCl and with 0.5% Triton X-100 only caused the additional release of 3.4 units phosphatase/g thymus.

Preincubation of the nucleoplasm and of the 0.3 M salt extract with saturating concentrations of modulator decreased the spontaneous phosphorylase phosphatase activity by 75 and 87%, respectively (Table I). This indicates that the phosphorylase phosphatase activity in these fractions stems largely from type-1 protein phosphatases.

A preincubation with trypsin is often used to measure the "total" phosphorylase phosphatase activity of PP-1 (2). This assay is based on findings that trypsin generates free PP-1_C by destruction of the noncatalytic subunits, that are often inhibitory. Trypsin also removes the C-terminal region of PP-1_C, and this causes a slight increase of the phosphorylase phosphatase activity. In contrast, the activity of PP-2A, which also possesses phosphorylase phosphatase activity, is considerably decreased by trypsin. Table I shows that trypsinolysis increased the phosphorylase phosphatase activity in the nucleoplasm and in the 0.3 M salt extract 5.8- and 2.4-fold, respectively.

Since the activity of PP-1N was increased more by trypsinolysis than could be accounted for by the removal of the C-terminal domain of PP-1_C, we investigated the possibility that the activity of this phosphatase was additionally suppressed by inhibitory polypeptides. Table I shows that the nuclei indeed contain a heat-stable inhibitory activity of PP-1_C that is, however, destroyed by trypsin. The nuclear inhibitor(s) of PP-1 (NIPP-1) displayed a subnuclear distribution similar to that of PP-1N, in that 95% of the recovered inhibitory activity was present in the 0.3 M NaCl extract. The heat-stable inhibitory activity that was extracted with 0.3 M NaCl was enough to inhibit about 20% of the endogenous trypsin-revealed phosphorylase phosphatase activity. It is possible that more NIPP-1 can be extracted at higher salt concentrations. This possibility was difficult to explore, however, due to the release of histones from the chromatin fraction by NaCl concentrations above 0.35 M (29). Histones interfere with the assay of NIPP-1, because they are themselves heat-stable inhibitors of PP-1 under some assay conditions (2, 30), and because they block the inhibitory effect of NIPP-1 (see below). The fact that other investigators (4–6) used high salt concentrations to extract the particulate fraction may thus explain why they did not detect NIPP-1.

Purification of NIPP-1—We have used the extreme resistance of NIPP-1 to denaturing procedures, like heating at 90 °C and exposure to strong acids, for a rapid initial 20-fold purification (Table II). NIPP-1 could be precipitated with 2% (w/v) trichloroacetic acid, which ensured its separation from the so-called high-mobility-group proteins that remain soluble in these conditions (31).

For unknown reasons, most chromatographic steps that have been explored for the further purification of NIPP-1 had to be abandoned because of an extremely low recovery. These include chromatography on DEAE-cellulose, phosphocellulose, Mono S, phenyl-Sepharose, and blue Sepharose. An acceptable recovery and extent of purification were consistently obtained, however, with heparin-Sepharose, Mono Q, and reversed-phase chromatography (Table II). The binding of NIPP-1 to heparin-Sepharose was especially important because soluble fragments of chromatin are not retained, as indicated by the high value (1.5) of the absorbance ratio (260:280 nm) in the flow-through (not illustrated).

On Mono Q, the NIPP-1 activity was resolved into three different peaks that were termed NIPP-1a, NIPP-1b, and NIPP-1c, according to their order of elution (Fig. 1). In general, NIPP-1b made the largest contribution to the overall heat-stable inhibitory activity. Since NIPP-1c eluted from Mono Q at variable ionic strengths, no efforts were undertaken to purify this inhibitor any further.

Both NIPP-1a (not shown) and NIPP-1b (Fig. 2) eluted

TABLE II

Purification scheme of NIPP-1a and NIPP-1b

NIPP-1 was assayed by its capacity to inhibit PP-1C. During the first four purification steps, NIPP-1 was assessed from the difference in inhibitory power before and after treatment with trypsin. The data represent the means of three purifications, as described under "Experimental Procedures," each with 1 kg of thymus as starting material.

Purification step	Proteins	Recovery	Purification
	μg/kg thymus	%	*-fold*
0.3 M NaCl extract	3,132,000	100 [8,800 units]	1
$(NH_4)_2SO_4$ precipitate	2,718,000	72	
Incubation at 90 °C	286,000	101	11
Acid precipitation	127,000	81	20
Heparine-Sepharose	19,000	30	49
NIPP-1a			
Mono Q	510	4	245
Reversed-phase chromatography	29	2	2,154
Elution from Immobilon membranes	0.25	0.24	30,067
NIPP-1b			
Mono Q	480	5	320
Reversed-phase chromatography	16	3	5,760
Elution from Immobilon membranes	0.5	0.30	18,792

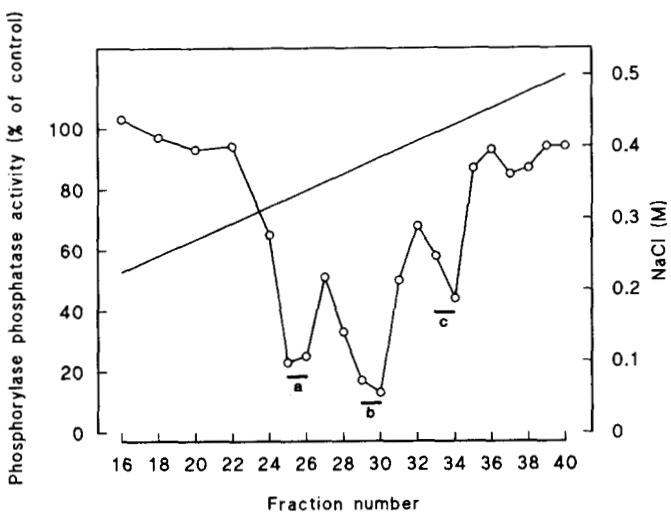

FIG. 1. **Separation of different species of NIPP-1 on Mono Q.** NIPP-1 was partially purified from the nuclei of 1 kg of thymus, as indicated under "Experimental Procedures." After chromatography on heparin-Sepharose, NIPP-1 was applied to Mono Q and eluted with a linear salt gradient. The fractions (0.5 ml) were assayed for NIPP-1 at a final 1000-fold dilution by their ability to inhibit PP-1C (O). The *straight line* shows the NaCl gradient. The initial 15 fractions (not shown) did not contain measurable NIPP-1.

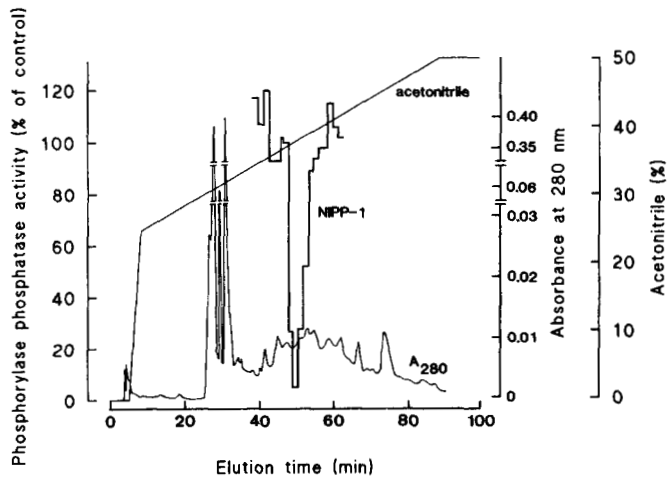

FIG. 2. **Reversed-phase chromatography of NIPP-1b.** The peak fractions of NIPP-1b from the Mono Q column were applied to a C_{18} column that was eluted with a linear gradient of acetonitrile, as described under "Experimental Procedures." The absorbance at 280 nm was continuously monitored and fractions were collected, lyophilized, and dissolved in 35 μl of buffer D. NIPP-1 was assayed at a final 5000-fold dilution by its capacity to inhibit the phosphorylase phosphatase activity of PP-1C.

during reversed-phase chromatography on a C_{18} column at 38 ± 1 % (n = 6) acetonitrile. At this stage, the concentrated peak fraction of NIPP-1a (not shown) and NIPP-1b (Fig. 3*B*, *lane 2*) still showed several bands on SDS-PAGE.

The last purification step, with a recovery of barely 10% (Table II), consisted consecutively of SDS-PAGE, blotting onto Immobilon membranes, and elution of the proteins from these membranes with 70% formic acid. Assay of the eluted polypeptides enabled us to identify NIPP-1b directly as a protein of 16 kDa (Fig. 3*A*). In *lane 1* of Fig. 3*B* it is shown that the active fraction shows a single band on Coomassie-stained gels. Using the same procedure, we have been able to identify NIPP-1a as a protein of 18 kDa (not shown).

The overall purification of NIPP-1a and NIPP-1b from the 0.3 M NaCl extract amounted to 30,000- and 19,000-fold, respectively (Table II). Typically, about 0.25 μg of NIPP-1a was obtained from 1 kg of thymus with an overall recovery of 0.2%. Twice as much NIPP-1b was obtained, with a slightly higher recovery (0.3%).

NIPP-1 Is Not Generated by Boiling—Even during the initial purification steps, NIPP-1 was always assayed after incubation at 90 °C, in order to destroy the endogenous phosphorylase phosphatase activity. We have considered the possibility that these inhibitory proteins were artefactually generated during denaturing conditions. For that purpose, we compared the activity of NIPP-1 in the 0.3 M salt extract before and after boiling. In these experiments the endogenous phosphorylase phosphatase activity was irreversibly blocked by a preincubation during 2 h at 30 °C with 50 mM fluoride and 5 mM pyrophosphate (2). After an overnight dialysis, the amount of trypsin-sensitive inhibitor was the same in both experimental conditions (not illustrated).

Sensitivity of PP-1C to Inhibition by NIPP-1b—Fig. 4 shows that the concentration of NIPP-1b required to inhibit PP-1C depended very much on the concentration of the phosphatase during the assay. In the range of phosphatase concentrations between 0.3 and 15 nM, the concentration of NIPP-1b that caused 50% inhibition (IC_{50}) increased from 0.16 to 9.57 nM. For tightly bound inhibitors the $IC_{50} = K_i + E/2$, where E is

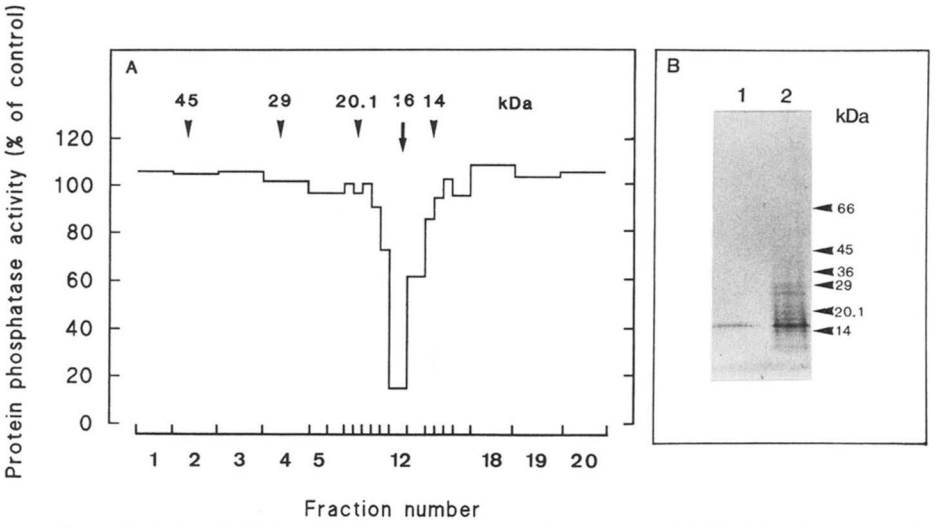

FIG. 3. **Purification of NIPP-1b by SDS-PAGE.** The redissolved peak fractions of NIPP-1b after reversed-phase chromatography were subjected to SDS-PAGE (see "Experimental Procedures"). After subsequent blotting onto an Immobilon membrane, the separated polypeptides were visualized by staining with Amido Black. The membrane was then cut in strips as shown, and the polyeptides were eluted from each strip with 70% formic acid. After lyophilization the corresponding fractions were dissolved in 15 μl of buffer A and assayed for NIPP-1 at a further 1500-fold dilution (*A*). *B* shows a Coomassie-stained gel (SDS-PAGE) of the peak fraction of NIPP-1b after reversed-phase chromatography (*lane 2*) and of the same preparation after elution of the peak fraction from Immobilon membranes (*lane 1*). These samples could be diluted 5250-fold (*lane 1*) and 16000-fold (*lane 2*) for half-maximal inhibition of the phosphorylase phosphatase activity of PP-1_C. The *small arrows* locate the position of marker proteins during SDS-PAGE. The *large arrow* in *A* indicates the position of NIPP-1b (16 kDa).

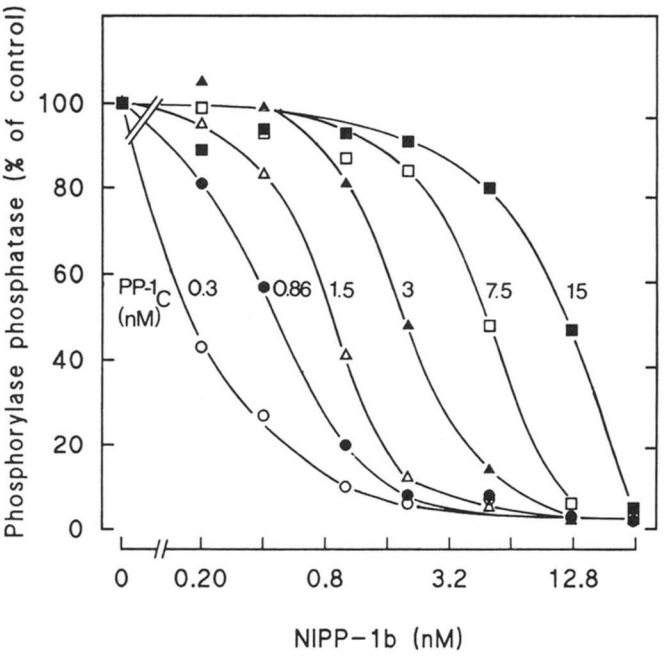

FIG. 4. **Sensitivity of PP-1_C to inhibition by NIPP-1b.** PP-1_C was assayed at the indicated final concentrations for spontaneous phosphorylase phosphatase activity in the presence of various concentrations of homogeneously purified NIPP-1b (shown on a log scale). The data represent the means for two experiments.

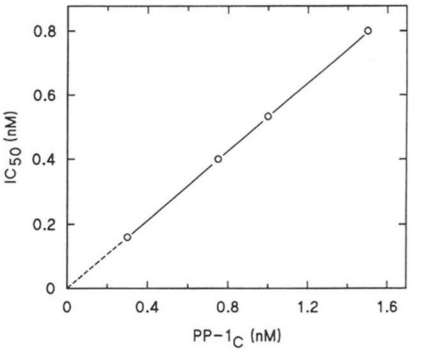

FIG. 5. **Correlation between IC_{50} and the concentration of PP-1_C.** The IC_{50} was derived from dose-response curves similar to those illustrated in Fig. 4. The results represent the means of two experiments. Extrapolation of the IC_{50} to infinite dilution of the phosphatase yielded a K_i value below 1 pM.

the total enzyme concentration (32, 33). At the lowest feasible phosphatase concentration during the assay (0.3 nM), a very approximate K_i of 0.01 nM can be calculated with this formula. Extrapolation of the IC_{50} to infinite dilution of the phosphatase yielded a true K_i below 1 pM (Fig. 5). NIPP-1a was an equally potent inhibitor of PP-1_C (not illustrated).

Lineweaver-Burk plots showed that NIPP-1b affected exclusively the V_{max} of the phosphorylase phosphatase reaction (not illustrated). However, with tightly bound inhibitors such data do not necessarily imply a noncompetitive inhibition, since the ratio between bound inhibitor and free inhibitor is not negligibly small (32). For inhibitor 1 and modulator, these problems have been circumvented by using concentrations of PP-1_C that are far below the K_i (34). A similar approach was not feasible for NIPP-1, however, because its K_i is at least a 100-fold lower than that of the cytoplasmic inhibitors.

Dependence on the Nature of the Substrate—NIPP-1b blocked totally the dephosphorylation of phosphorylase and of casein by PP-1_C (Fig. 6). On the other hand, the dephosphorylation of myelin basic protein was only 50% inhibited by saturating concentrations of NIPP-1b. With histone-IIA (10 μM) as a substrate for PP-1_C, NIPP-1b was even stimulatory. In Fig. 7 it is shown that similar concentrations of nonphosphorylated histone IIA (4 and 14 μM) also decreased the potency of NIPP-1b as an inhibitor of the casein phosphatase reaction.

Effect on Other Serine/Threonine Protein Phosphatases—NIPP-1b did not affect the activities of PP-2A, PP-2B, or PP-2C, even at a concentration that was 500 times the IC_{50} for inhibition of PP-1_C (Fig. 8). As for PP-2A, the same

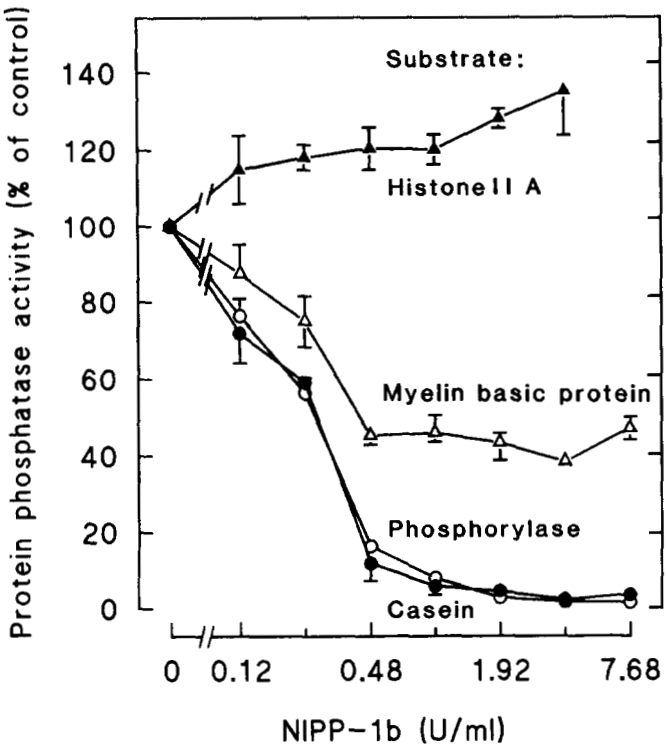

FIG. 6. **Role of the substrate in the inhibition of PP-1_C by NIPP-1b.** The effect of the indicated concentrations (on a log scale) of partially purified NIPP-1b (pool after reversed-phase chromatography) on the activity of PP-1_C was measured as indicated under "Experimental Procedures." The mean 100% values correspond to 0.65 units/ml (phosphorylase), 0.065 units/ml (casein), 0.228 units/ml (myelin basic protein), and 0.034 units/ml (histone IIA). In all assays the concentration of PP-1_C was the same. The results represent the means ± S.E. of three observations. 1 unit of NIPP-1b corresponds to 2 pmol.

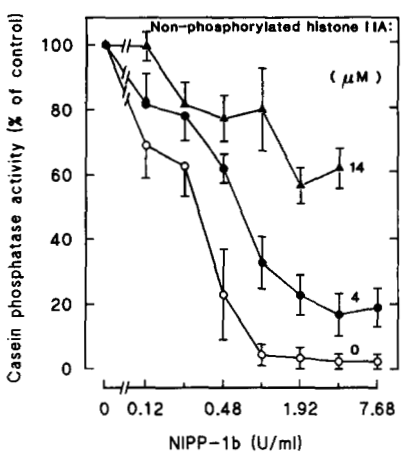

FIG. 7. **Effect of histone IIA on the inhibition of PP-1_C by NIPP-1b.** The casein phosphatase activity of PP-1_C was measured in the absence (○) or presence of 4 μM (●) or 14 μM (▲) nonphosphorylated histone IIA and with various concentrations of NIPP-1b, as indicated on a log scale. The addition of histones did not affect the casein phosphatase activity in the absence of NIPP-1b (100% value). The results represent the means ± S.E. of three observations. 1 unit of NIPP-1b corresponds to 2 pmol.

results were obtained with the free catalytic subunit (Fig. 8) and with the holoenzyme that is known as PP-$2A_2$ or PCS_L (35) (not illustrated).

Comparison with Inhibitor 1 and Modulator—NIPP-1a and NIPP-1b resemble inhibitor 1/DARPP-32 and modulator, in

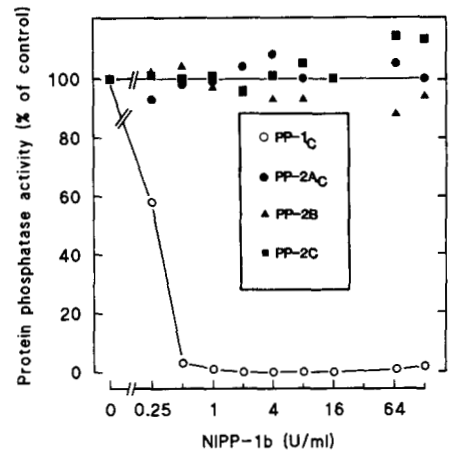

FIG. 8. **Effect of NIPP-1b on the activities of the major classes of serine/threonine protein phosphatases.** The activities of the catalytic subunits of PP-1 (○) and PP-2A (●) were measured with phosphorylase as substrate, while the activities of PP-2B (▲) and PP-2C (■) were determined from the rate of dephosphorylation of casein. PP-2B and PP-2C were assayed in the presence of 1 mM $MnCl_2$ and 5 mM $MgCl_2$, respectively. The NIPP-1b that was used in these experiments was purified until after reversed-phase chromatography. Its concentration is shown on a log scale. The results represent the means of four experiments. Standard errors have been omitted for clarity. 1 unit of NIPP-1b corresponds to 2 pmol.

that they are heat- and acid-stable proteins (Ref. 2 and Table II). From chromatofocusing on Mono P, a pI of 4.4 ± 0.1 (n = 3) was determined for NIPP-1a and NIPP-1b (not illustrated), which shows that they are, like the cytoplasmic inhibitors (36–38), very acidic proteins. During gel filtration on Superdex, NIPP-1a and NIPP-1b migrated with an apparent molecular mass of 35 kDa (not illustrated). This is much higher than the apparent M_r that was deduced from SDS-PAGE (Fig. 3) and could indicate that NIPP-1a and NIPP-1b, like inhibitor 1/DARPP-32 and modulator, are asymmetric proteins. An alternative possibility is that the native inhibitors are dimeric proteins.

The nuclear and cytoplasmic inhibitors differ considerably in their molecular mass and in their affinities for PP-1_C (see above). They can also be differentiated in other ways. Contrary to what has been described for inhibitor 1/DARPP-32 (1, 2), NIPP-1 could not be inactivated by incubation with PP-2A and/or PP-1_C plus 1 mM Mn^{2+} (not illustrated). Furthermore, unlike modulator, NIPP-1 did not convert PP-1_C into a MgATP-dependent form (not shown). Finally, the activity of NIPP-1a and NIPP-1b was not affected by polyclonal antibodies that cancelled the activity of modulator (not shown).

DISCUSSION

In agreement with reports on other cell types and tissues (3–7), we found that the nuclei from calf thymus contain high levels of PP-1 (Table I). Most of the recovered activity (95%) was associated with the chromatin/matrix fraction, from which it was released with 0.3 M NaCl. From the specific activity of PP-1_C (15,000 units/mg) under the adopted assay conditions and from the total phosphorylase phosphatase activity in the 0.3 M NaCl extract (Table I), it can be calculated that about 60 nmol of PP-1_C was extracted from the nuclei prepared from 1 kg of thymus. On the other hand, from the data in Table II one can calculate that the 0.3 M salt extract from the nuclei of 1 kg of thymus contains 6 and 10 nmol of NIPP-1a and NIPP-1b, respectively. These inhibitors, which together account for more than 80% of the heat-

stable inhibitory activity in the salt extract, can thus at most block about 25% of the endogenous PP-1_C in this fraction. This value is close to the 20% that is obtained by comparing the activities of total phosphorylase phosphatase and NIPP-1 in the 0.3 M NaCl extract (Table I). Thus, in spite of the exceedingly high affinity of NIPP-1 for PP-1_C, our observation that there is an excess of PP-1_C over NIPP-1 indicates that only part of the nuclear PP-1_C may be complexed with NIPP-1. This is in keeping with our findings that the phosphorylase phosphatase activity in the 0.3 M NaCl extract was only 2.4-fold increased by trypsin treatment and that it could be further inhibited by modulator (Table I).

It remains to be seen whether the inhibitory power of NIPP-1 is subject to regulation. Studies on fission yeast have provided evidence for the existence of a positive regulator of PP-1N (see Introduction). Perhaps the product of $sds22^+$ acts by antagonizing NIPP-1. If so, it would have a function similar to the deinhibitor protein that has been purified from the glycogen fraction of dog liver (40).

We do not yet understand the relationship between the three forms of NIPP-1 that we have identified. It is quite possible that NIPP-1b (16 kDa) originates from NIPP-1a (18 kDa) by proteolysis. Or NIPP-1a may represent a phosphorylated form of NIPP-1b and thus migrate slightly slower on SDS-PAGE.

A peculiar finding was that NIPP-1b did not or incompletely inhibit the dephosphorylation of basic proteins by PP-1_C (Fig. 6). One explanation is that the acidic NIPP-1b was partially or completely inactivated through complexation with these basic proteins. This view is substantiated by our observations that the casein phosphatase reaction was less efficiently inhibited by NIPP-1 when nonphosphorylated histones were added to the assay mixture (Fig. 7). Furthermore, it has been reported that myelin basic protein also blocks the modulator-induced inactivation of PP-1_C (39). A basic protein may also be involved in the association of NIPP-1 with the chromatin fraction.

Acknowledgments—M. Evens and N. Sente provided expert technical assistance. We thank V. Feytons for expert help with reversed-phase on HPLC, Dr. J. R. Vandenheede for the gifts of PP-$2A_C$ and polyclonal antibodies against modulator, and Dr. J. Goris for providing us with a sample of PCS_L.

REFERENCES

1. Cohen, P. (1989) *Annu. Rev. Biochem.* **58,** 453–508
2. Bollen, M., and Stalmans, W. (1992) *CRC Crit. Rev. Biochem. Mol. Biol.* **27,** 227–281
3. Doperé, F., and Stalmans, W. (1982) *Biochem. Biophys. Res. Commun.* **104,** 443–450
4. Jakes, S., Mellgren, R. L., and Schlender, K. K., (1986) *Biochim. Biophys. Acta* **888,** 135–142
5. Kuret, J., Bell, H., and Cohen, P. (1986) *FEBS Lett.* **203,** 197–202
6. Friedman, D. L. (1986) *Biochem. Biophys. Res. Commun.* **134,** 1372–1378
7. Jessus, C., Goris, J., Staquet, S., Cayla, X., Ozon, R., and Merlevede, W. (1989) *Biochem. J.* **260,** 45–51
8. Ohkura, H., Kinoshita, N., Miyatani, S., Toda, T., and Yanagida, M. (1989) *Cell* **57,** 997–1007
9. Kinoshita, N., Ohkura, H., and Yanagida, M. (1990) *Cell* **63,** 405–415
10. Doonan, J. H., and Morris, N. R. (1989) *Cell* **57,** 987–996
11. Ohkura, H., and Yanagida, M. (1991) *Cell* **64,** 149–157
12. Booher, R., and Beach, D. (1989) *Cell* **57,** 1009–1016
13. Axton, J. M., Dombrádi, V., Cohen, P. T. W., and Glover, D. M. (1990) *Cell* **63,** 33–46
14. Dombrádi, V., Axton, J. M., Barker, H. M., and Cohen, P. T. W. (1990) *FEBS Lett.* **275,** 39–43
15. Mercier, J. C., Maubois, J. L., Poznanski, S., and Ribadeau-Dumas, B. (1968) *Bull. Soc. Chim. Biol.* **50,** 521–530
16. Yang, S.-D., Vandenheede, J. R., and Merlevede, W. (1981) *FEBS Lett.* **132,** 293–295
17. Fischer, E. H., and Krebs, E. G. (1958) *J. Biol. Chem.* **231,** 65–71
18. Tsuiki, S., Hiraga, A., Kikuchi, K., and Tamura, S. (1988) *Methods Enzymol.* **159,** 437–446
19. Stewart, A. A., Ingebritsen, T. S., and Cohen, P. (1983) *Eur. J. Biochem.* **132,** 289–295
20. DeGuzman, A., and Lee, E. Y. C. (1988) *Methods Enzymol.* **159,** 356–368
21. Ramachandran, C., Goris, J., Waelkens, E., Merlevede, W., and Walsh, D. A. (1987) *J. Biol. Chem.* **262,** 3210–3218
22. Antoniw, J. F., Nimmo, H. G., Yeaman, S. J., and Cohen, P. (1977) *Biochem. J.* **162,** 423–433
23. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
24. Goodwin, G. H. (1989) in *Protein Purification Methods, a Practical Approach* (Harris, E. L. V., and Angel, S., eds) pp. 97–101, IRL Press, Oxford
25. Blobel, G., and Potter, V. R. (1966) *Science* **154,** 1662–1665
26. Vanfleteren, J. R. (1989) *Anal. Biochem.* **178,** 385–390
27. Laemmli, U. K. (1970) *Nature,* **227,** 680–685
28. de Jong, L., van Driel, R., Stuurman, N., Meijne, A. M. L., and van Renswoude, J. (1990) *Cell Biol. Int. Rep.* **14,** 1051–1074
29. Goodwin, G. H., Sanders, C., and Johns, E. W. (1973) *Eur. J. Biochem.* **38,** 14–19
30. Pelech, S., and Cohen, P. (1985) *Eur. J. Biochem.* **148,** 245–251
31. Goodwin, G. H., and Johns, E. W. (1973) *Eur. J. Biochem.* **40,** 215–219
32. Henderson, P. J. F. (1972) *Biochem. J.* **127,** 321–333
33. Takai, A., and Mieskes, G. (1991) *Biochem. J.* **275,** 233–239
34. Foulkes, J. G., Strada, S. J., Henderson, P. J. F., and Cohen, P. (1983) *Eur. J. Biochem.* **132,** 309–313
35. Merlevede, W. (1985) *Adv. Prot. Phosphatases* **1,** 1–18
36. Aitken, A., Bilham, T., and Cohen, P. (1982) *Eur. J. Biochem.* **126,** 235–246
37. Williams, K. R., Hemmings, H. C., Jr., LoPresti, M. B., Konigsberg, W. H., and Greengard, P. (1986) *J. Biol. Chem.* **261,** 1890–1903
38. Holmes, C. F. B., Campbell, D. G., Caudwell, F. B., Aitken, A., and Cohen, P. (1986) *Eur. J. Biochem.* **155,** 173–182
39. Vandenheede, J. R., Van Lint, J., Vanden Abeele, C., and Merlevede, W. (1987) *FEBS Lett.* **211,** 190–194
40. Goris, J., Waelkens, E., Camps, T., and Merlevede, W. (1984) *Adv. Enzyme Regul.* **22,** 467–484