Post-translational processing of chicken bone phosphoproteins

Identification of bone (phospho)protein kinase

Yuko MIKUNI-TAKAGAKI and Melvin J. GLIMCHER

Laboratory for the Study of Skeletal Disorders and Rehabilitation, Harvard Medical School, The Children's Hospital, 300 Longwood Avenue, Enders 11, Boston, MA 02115, U.S.A.

We have detected a protein kinase which phosphorylates bone phosphoproteins (BPPs) in the detergent extract of the membranous fractions in the periosteal bone strips of 12-day-embryonic-chick tibia. This enzyme, tentatively named BPP kinase, has a catalytic subunit of $M_r \sim 39000$, utilizes GTP as well as ATP as a phospho-group donor, is inhibited by 2,3-bisphosphoglycerate and heparin, and is therefore similar to casein kinase II. The enzyme can phosphorylate dephosphorylated proteins such as casein, phosvitin and chicken BPPs, but the last-named are preferred substrates. The *in vitro*-phosphorylation-assay products of this enzyme in the extract were indistinguishable on an SDS/polyacrylamide gel from the major [^{32}P]phosphoproteins metabolically labelled in the embryonic-chick bone tissue. The regulatory mechanisms of the phosphorylation process of BPPs by BPP kinase as well as the potential role of this enzyme in mineralization are discussed.

INTRODUCTION

The phosphoproteins of avian [1,2] and mammalian bone (sialoprotein I (osteopontin) and II [3–5]), which are all extensively phosphorylated, belong to a class of non-collagenous glycosylated sialoproteins [6]. It has been postulated that they may be directly involved in the nucleation and growth of the hydroxyapatite crystals [6,7]. Recent reports show that the osteopontin gene is expressed [8] and the message translated [9] in many other non-mineralizing tissues such as kidney, where the protein seems to be little if at all phosphorylated. Therefore post-translational processing of these proteins may in part determine their functional roles in tissue mineralization or in cell binding [10–12].

To understand the mechanisms by which these phosphoproteins are phosphorylated, we studied protein kinases in strips of periosteal bone isolated from 12-day-embryonic-chick tibiotarsus in which bone phosphoproteins (BPPs) have already been characterized (see the preceding paper [1]).

MATERIALS AND METHODS

Materials

Phosphatidylserine, dephosphorylated casein (C4032), heparin (H5640), cyclic AMP, ATP, GTP and Nonidet P40 were purchased from Sigma Chemical Co. (Chicago, IL, U.S.A.). Chicken BPPs were purified in our laboratory from the diaphyses of 14-week-chicken metatarsals as reported [2]. Periosteal bone strips from 12-day-old-embryonic-chick tibiae [1] were used as a source for BPP kinase.

Protein kinase assay

Protein kinase activity was monitored as described by Kuo *et al.* [14], with minor modifications, to identify four different types of protein kinase, cyclic AMP (or GMP)-dependent, calmodulin/ Ca^{2+} -dependent, phospholipid-sensitive Ca^{2+} -dependent (C kinase) and independent kinases. Unless otherwise described, buffers were ice cold and materials were handled in

ice. In a typical experiment, 20 periosteal bone strips [1,15] were homogenized in 1 ml of homogenization buffer [15 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose/2.5 mM- MgCl_2 /2.5 mM-EGTA/50 mM-2-mercaptoethanol]. Homogenization for 5 s was repeated twelve times at 15 s intervals using the Tek-Mar homogenizer. The homogenates were first centrifuged for 5 min at 200 *g* to remove bone residues, followed by centrifugation for 1 h at 100 000 *g* to obtain the cytosolic fractions. The pellets were suspended in the original volume of the homogenization buffer and were incubated for 1 h in ice in the presence of 0.3 % Nonidet P40 (NP-40). Bone residues were also treated with NP-40 in a similar manner. Typically, cytosol and bone residue fractions contain approx. 4 mM- Ca^{2+} , whereas particulate fraction contains only approx. 0.7 mM- Ca^{2+} . Enzyme fractions were kept frozen in portions in liquid N_2 . These fractions were diluted 3–100-fold with the extraction buffer [20 mM-Tris/HCl (pH 7.5)/2 mM-EDTA/50 mM-2-mercaptoethanol] before assays were carried out. The above crude fractions were used to estimate the activity of protein kinases. Phospholipid-sensitive kinase was assayed in the presence of added CaCl_2 (0.5 mM) and phosphatidylserine (25 $\mu\text{g}/\text{ml}$) in a final volume of 200 μl with a substrate protein such as phosvitin and bone phosphoprotein (40 μg), which was partially dephosphorylated by the method described by Hathaway & Traugh [16]. Another substrate, dephosphorylated casein (C4032), was purchased from Sigma Chemical Co. Appropriate amounts of enzyme preparations were added to 25 mM-Tris/HCl buffer (pH 7.5)/10 mM- MgCl_2 /40 mM-NaF (for the particulate and bone residue fractions). The concentration of Ca^{2+} was adjusted depending on the endogenous level in each enzyme preparation so that the final concentration was 0.5 mM. Similarly, EGTA was added to chelate endogenous Ca^{2+} to assay factor-independent and cyclic AMP-dependent kinases. The reaction was started by adding [γ - ^{32}P]ATP (1 nmol, containing approx. 1×10^6 c.p.m.) and was allowed to proceed for 2–10 min at 30 °C. The phosphorylated substrate was precipitated with 5 % (w/v) trichloroacetic acid containing 0.25 % tungstate. The precipitate was dissolved in 50 mM-NaOH, re-precipitated with 2 % trichloroacetic acid, and washed with ethanol/diethyl ether

Abbreviations used: BPP, bone phosphoprotein; NP-40, Nonidet P40; I_{50} , concentration of inhibitor required to give 50 % inhibition; BPP kinase, bone (phospho)protein kinase.

(1:1) to remove free ATP and trichloroacetic acid. The Ca^{2+} -stimulated activity in the absence of added phospholipid represents calmodulin/ Ca^{2+} -dependent kinase, because of endogenous calmodulin usually present in crude tissue extracts. To measure cyclic AMP-dependent kinase, 0.5 μM -cyclic AMP was added instead of phospholipid and Ca^{2+} to activate the enzyme under conditions similar to that used for Ca^{2+} -dependent enzymes. In addition, 0.5 nM-theophylline was added to inhibit the enzymic degradation of cyclic nucleotides. In case of cyclic GMP kinase, purified stimulatory modulator has to be added to the assay mixture. Since it was reported that cyclic GMP kinase shows much lower activity than cyclic AMP-dependent kinase, except in fetal lung [14], we did not measure this enzyme. A unit of protein kinase activity was defined as 1 μmol of ^{32}P transfer from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ /min per g of protein under the conditions described. Phosphorylated proteins were analysed by SDS/PAGE and autoradiography.

Purification and characterization of BPP (casein) kinase

After removing the cytosolic fraction, which contains significant amounts of other kinases, by ultracentrifugation, we extracted the enzyme with NP-40 as described in the previous section. The extracts (particulate and bone-residue fractions) from approx. 300 tibia were combined and applied to a DEAE-Sephacel column (1.2 cm $\times$ 3 cm) equilibrated with 20 mM-Tris/HCl (pH 7.4)/2 mM-EGTA/5 mM-EDTA/10 mM-2-mercaptoethanol. Protein kinase activity in the NaCl eluate was assayed with dephosphorylated casein as a substrate.

Renaturation of bone phosphoprotein kinase in polyacrylamide gel

We have used the '*in situ*' (in the gel) renaturation method of Geahlen *et al.* [17] with some modification and obtained the apparent catalytic subunit size of BPP kinase in SDS/polyacrylamide gel. Gels were impregnated with 1 mg of dephosphorylated casein/ml before polymerization. After a 2 h incubation of the enzyme preparation at 37 °C with 2.5 % SDS/25 % (w/v) sucrose/25 mM-Tris/HCl buffer (pH 8.0)/2.5 mM-EDTA/1.5 % dithiothreitol/Phenol Red, samples were electrophoresed under standard SDS/PAGE conditions at 4 °C overnight. Prestained standard proteins were electrophoresed at the same time. After electrophoresis, proteins were renatured sequentially in 40 mM-Hepes (pH 7.4)/2.5 % NP-40 for 45 min,

40 mM-Hepes (pH 7.4)/0.3 % NP-40 for 15 min and 40 mM-Hepes for 1–2 h. To detect the kinase activity, the gel pieces were incubated in 50 ml of 25 mM-Hepes (pH 7.4)/10 mM- MnCl_2 /[$\gamma\text{-}^{32}\text{P}]\text{ATP}$ (250 μCi) at room temperature for 3 h. After rinsing the gel briefly with water, free [$\gamma\text{-}^{32}\text{P}]\text{ATP}$ was removed by incubating the gel overnight at 4 °C in 40 mM-Hepes (pH 7.4)/20 mM-NaF, containing 20 g of Dowex AG 1-X8 resin (Cl^- form) placed in a bag made with a polyethylene filter. Free [^{32}P]phosphate was then removed by 20 g of the same resin but in the OH^- form in the same buffer made without NaF. This latter step (for 1 h at room temperature) proved to be better than the method described (1 % sodium pyrophosphate) to reduce the background. Proteins were then fixed in the gel by a 1 h incubation in propan-2-ol/acetic acid/water (5:2:3, by vol.). The gels were dried and exposed to Kodak X-Omat AR film to detect radiolabelled bands.

RESULTS

Detection of protein kinase activity and endogenous products in bone

The periosteal bone strips were homogenized to extract protein kinases, which were assayed with dephosphorylated '32-kDa' BPP [2] and casein. Results of the assay are summarized in Table 1. It should be noted that the only detectable kinase in detergent extracts of 'particulate' and 'residue' fractions was independent of Ca^{2+} or cyclic AMP. In the 'cytosol' fraction, however, Ca^{2+} /phospholipid-dependent and cyclic AMP-dependent activities were detected as well as the independent kinase. The enzyme in the particulate and the bone-residue fractions appears to be a factor-independent kinase which 'prefers' BPP to casein as a substrate for phospho-group transfer. The phosphorylation assay products *in vitro* of this protein kinase were analysed by using the bone-residue fraction as a source of enzyme (Fig. 1). The endogenous phosphorylation products (Fig. 1, lane 3) appeared to be almost identical with those obtained by metabolically labelling the bones with $\text{Na}_2\text{H}^{32}\text{PO}_4$ (lane 1). Phosphorylation of these BPPs is abolished by including heparin (50 $\mu\text{g}/\text{ml}$) in the assay mixture (lane 2). Phosphorylation products of other substrates, such as semi-purified '32-kDa' and '60-kDa' BPPs [2] and casein, were also shown. Endogenously labelled proteins shown in lane 3 were hydrolysed in 4 M-HCl for 4 h to analyse the phosphoamino acids [1]. The ratio of [^{32}P]phosphoserine to [^{32}P]phosphothreonine was approx. 4:1.

Table 1. Protein kinase activities in cytosol, particulate and bone residue of periosteal strips of 12-day-old-chick tibiae

Kinase activities are expressed as net radioactivity (c.p.m.) (average for three assays). The values in parentheses represent the values for phosphorylation converted into $\mu\text{mol}/\text{min}$ per g of protein. —, not detectable; +, less than 200 c.p.m.

Preparation	Dependency	Substrate...	Kinase activity		
			Endogenous	Dephosphorylated '32-kDa' BPP	Dephosphorylated casein
Cytosol (100 000 g supernatant)	Independent	—	—	2900 (1.5)	1750 (0.9)
	Cyclic AMP	—	—	820 (0.42)	870 (0.45)
	Ca^{2+}	—	—	—	—
	Ca^{2+} /phospholipid	—	—	520 (0.27)	1020 (0.52)
Particulate (100 000 g ppt., NP-40 extract)	Independent	+	+	2880 (0.63)	2020 (0.44)
	Cyclic AMP	—	—	—	—
	Ca^{2+}	—	—	—	—
	Ca^{2+} /phospholipid	—	—	—	—
Bone residue (200 g ppt., NP-40 extract)	Independent	—	2540 (0.78)	3900 (1.2)	2910 (0.89)
	Cyclic AMP	—	—	—	—
	Ca^{2+}	—	—	—	—
	Ca^{2+} /phospholipid	—	—	—	—

Fig. 1. SDS/PAGE patterns of phosphorylation products in the periosteal bone dissected from 12-day-embryonic-chick tibia

Products of the kinase assay phosphorylated *in vitro* were compared with those in culture. Bone-residue extract was assayed for the independent kinase with [γ - 32 P]ATP under the conditions described in the Materials and methods section in the presence of 25 mM-EGTA, either without added substrate (lane 3) or with Sephacryl S-300 purified 14-week-old-chicken BPP components [2]; '32-kDa' (lane 4) and '60-kDa' (lane 5) or casein (lane 6). Lane 2 is similar to lane 3, except that heparin (50 μ g/ml) was added. Substrate proteins were dephosphorylated as described in the Materials and methods section. Phosphorylated matrix proteins were radiolabelled in the same tissue in culture with $\text{Na}_2^{32}\text{PO}_4$ (lane 1).

Fig. 2. DEAE-Sephacel chromatography of BPP kinase

The enzyme was eluted from DEAE-Sephacel by an NaCl gradient and assayed with dephosphorylated casein as a substrate in the presence (■) or absence (●) of heparin (50 μ g/ml). Conditions were as described in the Materials and methods section. The continuous line and broken lines show the protein (A_{280}) and conductivity respectively. Results of duplicate assays are depicted.

Characterization of bone (phospho)protein kinase (BPP kinase)

Purification of the BPP kinase was attempted by DEAE-Sephacel anion-exchange chromatography. The casein kinase, which was inhibited by heparin, was eluted from DEAE-Sephacel at about 0.2M-NaCl (Fig. 2). The peak was pooled and the pH profile and KCl-dependence were examined with portions of the pooled enzyme. As Fig. 3 shows, the maximum activity was obtained at pH 8–8.5 and at ~200 mM-KCl. Both heparin and

Fig. 3. Characteristics of BPP kinase

The enzyme was assayed as described in Materials and methods, except that Hepes buffer, pH 6.0–7.0, and Tris/HCl buffer, pH 7.0–9.5, were used for the pH-dependency study (a) and that Tris/HCl buffer, pH 8.0, was used for the KCl-dependency study (b). Vertical bars represent the S.D. ($n = 4$).

Fig. 4. Renaturation of BPP kinase

The NP-40 extract from approx. three bones was electrophoresed on a gel containing 1 mg of dephosphorylated casein/ml. Proteins were renatured *in situ* and the enzyme reaction was allowed to proceed as described in the Materials and methods section. Phosphorylated proteins were detected by autoradiography.

2,3-bisphosphoglycerate inhibited the enzyme significantly: I_{50} , the concentration of inhibitors which is required to give 50% inhibition, was 3 ± 1.1 μ g/ml for heparin and 1 ± 0.3 mM for 2,3-bisphosphoglycerate under standard assay conditions with casein as a substrate. A comparable enzyme activity was detected when [γ - 32 P]ATP was replaced by [γ - 32 P]GTP (results not shown).

In the renaturation experiment, we detected the radiolabelled band which resulted from phosphorylation of the substrate casein included in the gel (Fig. 4). The starting material for DEAE-Sephacel chromatography was used as a source of the enzyme. As judged from its relative mobility compared with those of prestained standard proteins in the same gel, the apparent molecular size of the catalytic subunit of BPP kinase is 39 kDa. The radioactivity in the band was significantly reduced by

incubating the gel with either heparin (M_r 4000–6000; 50 μ g/ml) or 100 mM non-radioactive ATP (results not shown). Lower- M_r heparin was used to ensure the penetration of heparin into the gel. Also, the above subunit band was not phosphorylated if the gel was prepared without casein (results not shown), thus eliminating the possibility of autophosphorylation of the subunit.

DISCUSSION

Many extracellular-matrix components, such as collagen [18,19], cartilage proteoglycan [20,21] and fibronectin [22], have been found to contain phosphoamino acid residues, but little is known about their biological significance or about the protein kinases which phosphorylate them *in vivo*. In mineralized tissues there is a group of proteins which are highly phosphorylated compared with the aforementioned proteins. BPPs contain 10–20 residues of phosphoserine and phosphothreonine in their molecules. Protein kinase activity was detected in bone and enamel in previous studies [23], but no studies have been undertaken in relation to the matrix phosphoproteins. The present study has demonstrated that the protein kinase that we have detected in the detergent extract of periosteal bone strips dissected from 12-day-embryonic-chick tibia is independent of cyclic AMP, Ca^{2+} or phosphatidylserine. The specific activity of this independent kinase in the bone extract is extremely high compared with those of other kinases found in other tissues [14]. The major phosphoproteins which were metabolically labelled in culture with $\text{Na}_2\text{H}^{32}\text{PO}_4$ in this embryonic bone tissue were indistinguishable from the 'in vitro' kinase assay products in this tissue which were radiolabelled with [γ - ^{32}P]ATP (Fig. 1). No significant labelling was observed when heparin was added to the assay mixture, indicating that this bone protein kinase which phosphorylates BPPs is similar to the casein kinase II [16,24]. Also shown in Figs. 3 and 4 are certain characteristics of the enzyme which are in agreement with those reported for the casein kinases [16,24–26]. The apparent M_r of the enzyme, namely 39000, is quite similar to those reported for casein kinases I and II [16,17,25]. It appears that the casein kinases lose their activity under physiological conditions [16] in a manner similar to what we have observed with the chicken bone enzyme. Finally, the inhibitory effects of heparin and 2,3-bisphosphoglycerate, the utilization of GTP as well as ATP by the enzyme, and the substrate specificity towards threonine residues all indicate that the BPP kinase is quite similar to casein kinase II [16,24,27,28]. Casein kinases are multipotential, cyclic nucleotide-independent and Ca^{2+} -independent protein kinases which phosphorylate a variety of acidic substrates, including casein. They are present in nuclei and cytoplasm, and are found associated with membranes, ribosomes and mitochondria [16,24]. Casein kinase II, which is the most-studied casein kinase, is a ubiquitous enzyme for which a clear mode of control or cellular function has yet to be identified [24,25]. It has been suggested that phosphorylation of cellular proteins such as clathryn and tubulin by this enzyme [29,30] may regulate polymerization of these proteins [30]. Apparently the enzyme is evolutionarily related to the cell-cycle-control protein kinases [31]. The enzyme is unique among the serine/threonine kinases in its ability to modify threonine residues to an extent comparable with its ability to modify serine [27]; this is in agreement with the results obtained with embryonic and 14-week-old chicken BPPs [1,2] and with the results from kinase phosphorylation studies described here. It has been reported that serine and threonine residues followed by a series of acidic amino acid residues in substrates are the phosphorylation sites for this enzyme [27,28]. In the cDNA sequence of rat bone sialoproteins I (osteopontin) and II there are many Ser(Thr)-Asp(Glu)-Glu(Asp) sequences in the N-terminal side of the molecule

[12,32,33]. In another sialoprotein, BAG-75, found in rat calvaria [34], there is a sequence Thr-Glu-Glu-Glu-Glu near the N-terminal end. One of us has also identified Ser(Thr)-Asp(Glu)-Asp(Glu) sequences in chicken BPPs (Y. Mikuni-Takagaki, unpublished work). All these serine/threonine residues are potential phosphorylation sites for casein kinase II. It was suggested that one of the requirements for optimal phosphorylation by casein kinase II is that these sites be located within β -turns [35]. It was reported previous from this laboratory that smaller BPPs containing 38% aspartic acid/asparagine + glutamic acid/glutamine residues show almost no α -helix, but some β -turns [36]. Also, in the reported cDNA sequence of osteopontin, there are ten potential phosphorylation sites by casein kinase II (Thr/Ser-Asp/Glu-Asp/Glu), which are clustered at the N-terminal side of the molecule between residues 26 and 183 [12]. Many of these threonine and serine residues can be within β -turns. It is interesting that this segment of the molecule is also extremely acidic, containing 45 residues of aspartic acid/glutamic acid and only a few basic amino acid residues. Especially between residues 71 and 152 there is a region which lacks basic amino acids completely. Studies of these polyanionic proteins in fact supported their ability to facilitate mineralization when complexed with bone collagen fibrils [37], especially in a solid phase [38]. The stretches of the molecule which are acidic and phosphorylated by bone phosphoprotein kinase may be the functional domain of bone phosphoproteins involved in mineralization. At least in an experiment *in vitro* it was shown that phosphate residues were essential to the nucleation of hydroxyapatite crystals by collagen-phosphoprotein complexes [37]. The osteopontin gene has been found to be expressed and the message translated in several other non-mineralizing tissues, such as the kidney and intestine, where the protein seems to be little or not at all phosphorylated (D. Heinegård, personal communication) [39]. Therefore post-translational modification of these proteins by phosphorylation may determine some of their functional and biological roles in each of the tissues. Osteopontin, which is the 2ar-gene product [8], was also identified as transformation-associated phosphoprotein [33], although the phosphorylation mechanisms may be different in bone and in transformed cells. The cell-binding sequence probably plays a more general biological role for these proteins in all tissues, including bone. It was suggested in the preceding paper [1] that only the molecules proteolytically processed to certain sizes may become functional BPPs. If that is the case, tissue-specific proteolytic cleavages, which are known for prohormone processing (for a review, see [40]), may be an important regulatory step for this phosphorylation. Also, it should be noted that basic peptides, which were suggested to be released by the proteolytic cleavage of M_r -68000 BPP component [1], are known to activate casein kinase II [16,24]. All of the reported bone sialoproteins contain significant amounts of galactose, N-acetylgalactosamine and glucosamine compared with their mannose contents, suggesting that they have as many as twenty O-glycosidic chains, which also modify serine and threonine residues. Further studies of the phosphorylation mechanisms in relation to these processing enzymes, such as proteinases [40] and N-acetylgalactosaminyltransferase [41], which initiates O-glycosidic linkages, should shed light on the role of this unique group of proteins and, it is to be hoped, help us to understand the potential role of bone phosphoprotein kinase in the initiation of normal and pathological connective-tissue mineralization.

This work was supported by grants from the National Institutes of Health (AR34078) and the Peabody Foundation Inc., and an Institutional Grant from the Orthopaedic Research and Education Foundation, which is funded by the Bristol-Myers/Zimmer

Corporation. We thank Perdue Farms, Salisbury, MD, U.S.A., for supplying the chicken bones. We also thank Dr. Yumiko Takahashi and Mr. Alfredo Tiu for their technical assistance, Ms. Joan Hill and Mr. Bruce Lucier for their skilled photography, and Mr. Everett Carlson and Ms. Kismet McDonough for their assistance in manuscript preparation.

REFERENCES

- Mikuni-Takagaki, Y. & Glimcher, M. J. (1990) *Biochem. J.* **268**, 585–591
- Uchiyama, A., Suzuki, M., Lefteriou, B. & Glimcher, M. J. (1986) *Biochemistry* **24**, 6363–6369
- Prince, C. W., Oosawa, T., Butler, W. T., Tomana, M., Bhowm, A. S., Bhowm, M. & Schrohenloher, R. E. (1987) *J. Biol. Chem.* **262**, 2900–2907
- Franzen, A. & Heinegård, D. (1985) *Biochem. J.* **232**, 715–724
- Fisher, L. W., Hawkins, G. R., Tuross, N. & Termine, J. D. (1987) *J. Biol. Chem.* **262**, 9702–9708
- Glimcher, M. J. (1984) *Philos. Trans. R. Soc. London B* **304**, 479–508
- Veis, A. & Sabsay, B. (1983) in *Biomineralization and Biological Metal Accumulation* (Westbrooke, P. & deJong, E. W., eds.), pp. 273–284, Reidel Publishing Co., Dordrecht, The Netherlands
- Nomura, S., Wills, A. J., Edwards, D. R., Heath, J. K. & Hogan, B. L. (1988) *J. Cell Biol.* **106**, 441–450
- Mark, M. P., Prince, C. W., Gay, S., Austin, R. L. & Butler, W. T. (1988) *Cell Tissue Res.* **251**, 23–30
- Pierschbacher, M. D., Ruoslahti, E., Sundelin, J., Lind, P. & Peterson, P. A. (1982) *J. Biol. Chem.* **257**, 9593–9597
- Pytela, R., Pierschbacher, M. D., Ginsberg, M. H., Plow, E. F. & Ruoslahti, E. (1986) *Science* **231**, 1559–1562
- Oldberg, A., Franzén, A. & Heinegård, D. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8819–8823
- Reference deleted
- Kuo, J. F., Anderson, R. G., Wise, B. C., Mackerlova, L., Salomonsson, I., Brackett, N. L., Katoh, N., Shofi, M. & Wrenn, R. W. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7039–7043
- Mikuni-Takagaki, Y. & Chen, Y. S. (1987) *Arch. Biochem. Biophys.* **259**, 576–588
- Hathaway, G. M. & Traugh, J. A. (1983) *Methods Enzymol.* **99**, 317–331
- Geahlen, R. L., Amostario, M., Jr., Low, P. S. & Harrison, M. L. (1986) *Anal. Biochem.* **153**, 151–158
- Urushizaki, Y. & Seifter, S. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3091–3095
- Fisher, L. W., Robey, P. G., Tuross, N., Otsuka, A. S., Tepen, D. A., Esch, F. S., Shimasaki, S. & Termine, J. D. (1987) *J. Biol. Chem.* **262**, 13457–13463
- Anderson, R. S. & Schwartz, E. R. (1984) *Arthritis Rheum.* **27**, 58–71
- Oegema, T. R., Kraft, E. L., Jourdan, G. W. & van Valen, T. R. (1984) *J. Biol. Chem.* **259**, 1720–1726
- Etheredge, R. E., Han, S., Fossel, E., Tanzer, M. L. & Glimcher, M. J. (1985) *FEBS Lett.* **186**, 259–262
- Krane, S. M., Stone, M. J. & Glimcher, M. J. (1965) *Biochim. Biophys. Acta* **97**, 77–87
- Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) *Annu. Rev. Biochem.* **56**, 567–613
- Takio, K., Kuenzel, E. A., Walsh, K. A. & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4851–4855
- Bingham, E. W. & Groves, M. L. (1979) *J. Biol. Chem.* **254**, 4510–4515
- Kuenzel, E. A., Mulligan, J. A., Sommercorn, J. & Krebs, E. G. (1987) *J. Biol. Chem.* **262**, 9130–9140
- Pinna, L. A., Donella-Deana, A. & Meggio, F. (1979) *Biochem. Biophys. Res. Commun.* **87**, 114–120
- Bar-Zvi, D. & Branton, D. (1986) *J. Biol. Chem.* **261**, 9614–9621
- Serrano, L., Diaz-Nido, J., Wandosell, F. & Avila, J. (1987) *J. Cell Biol.* **105**, 1731–1739
- Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241**, 42–52
- Oldberg, A., Franzén, A. & Heinegård, D. (1988) *J. Biol. Chem.* **263**, 19430–19432
- Craig, A. M., Smith, J. H. & Denhardt, D. T. (1989) *J. Biol. Chem.* **264**, 9682–9689
- Gorski, J. P. & Shimizu, K. (1988) *J. Biol. Chem.* **263**, 15938–15945
- Pinna, L. A., Donella-Deana, A. & Meggio, F. (1979) *Biochem. Biophys. Res. Commun.* **87**, 114–120
- Renugopalakrishnan, V., Uchiyama, A., Horowitz, P. M., Papaka, R. S., Suzuki, M., Lefteriou, B. & Glimcher, M. J. (1986) *Calcif. Tissue Int.* **39**, 166–170
- Endo, A. & Glimcher, M. J. (1989) *Calcif. Tissue Res.* **21**, 179–196
- Linde, L. & Lussi, A. (1989) *Connect. Tissue Res.* **21**, 197–203
- Reinholt, F. P., Hultenby, K., Oldberg, A. & Heinegård, D. (1990) *Calcif. Tissue Res.* **22**, 286
- Pontremoli, S. & Melloni, E. (1986) *Annu. Rev. Biochem.* **55**, 455–481
- Sugiura, M., Kawasaki, T. & Yamashina, I. (1982) *J. Biol. Chem.* **257**, 9501–9507

Received 27 July 1989/8 December 1989; accepted 17 January 1990