Presence in many mammalian tissues of an identical major cytosolic substrate (M_r 100000) for calmodulin-dependent protein kinase

H. Clive Palfrey

Department of Pharmacological and Physiological Sciences, University of Chicago, 947 E. 58th Street, Chicago, IL 60637, USA

Received 12 May 1983

Incubation of cytosol fractions from a variety of mammalian tissues (heart, liver, lung, adrenal, spleen and skeletal muscle) with Ca^{2+} (0.5 mM) in the presence of γ-[^{32}P]ATP resulted in the phosphorylation of a prominent substrate of M_r ~100000 (100 kDa). One-dimensional peptide maps and two-dimensional tryptic fingerprints of the phosphoprotein from these sources were identical. A single major phosphopeptide was generated by trypsin and was determined to contain exclusively phosphothreonine. The 100 kDa substrate could be distinguished from glycogen phosphorylase (M_r ~97000) by a number of criteria including phosphopeptide mapping and by its failure to bind either to glycogen or to a specific antiphosphorylase antibody. The Ca^{2+}-dependent protein kinase responsible for phosphorylation of the 100 kDa protein appeared to be a calmodulin (CaM)-requiring enzyme in that it could be inhibited in cytosol extracts by trifluoperazine (IC_{50} 6–16 μM) and that exogenous CaM was necessary for 100 kDa phosphorylation in CaM-depleted cytosol. These results suggest that a rise in intracellular Ca^{2+} resulting in an activation of CaM-dependent protein kinase leads to the phosphorylation of a common 100 kDa substrate in many tissues.

Calcium *Calmodulin* *Protein kinase* *Phosphorylase*

1. INTRODUCTION

Ca^{2+}-activated protein phosphorylation is a common feature of mammalian tissues and may play an important role in regulating the responsiveness of tissues to intracellular Ca^{2+} signals. The protein kinases responsible for this activity appear to comprise two major types: those involving calmodulin (CaM) as an obligatory cofactor [1–7], the Ca–CaM complex then becoming the activator, and a second type involving the participation of lipid cofactors [8,9]. The CaM-activated protein kinases are evidently heterogeneous; two enzymes have been identified on the basis of their extreme substrate specificity: myosin light-chain kinase [2] and phosphorylase kinase [3]. Recently, however, CaM-dependent protein kinases that are distinct from these two enzymes have been identified in *Torpedo* electric organ [7], avian erythrocytes ([10] and H.C. Palfrey, Y. Lai and P. Greengard, in preparation), and brain [11]. In addition, CaM-dependent glycogen synthase kinases that differ from phosphorylase kinase have been isolated from mammalian liver [4,5] and skeletal muscle [6]. Preliminary results suggest that some members of this group may have certain properties in common, for example, their substrate specificity and physical characteristics [7].

The widespread distribution of CaM-dependent protein phosphorylation in membrane fractions

Abbreviations: CaM, calmodulin; 100 kDa, major phosphoprotein of M_r 100000; EGTA, ethylene glycol bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; SAP, *Staphylococcus aureus* protease; SAC, *Staphylococcus aureus* cells

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

from various tissues was established in [12]. Subsequently, others also noted the presence of various substrates that are phosphorylated in a Ca^{2+}- and CaM-dependent manner in specific tissues (e.g., [7,10,13–15]). However, apart from a few notable examples (myosin light chains, glycogen phosphorylase, glycogen synthase, phospholamban), these substrates have not been characterized. Here, the presence of an identical Ca^{2+}-dependent phosphorylated substrate of M_r ~100000 in the cytosol of various mammalian tissues is described. This protein appears to be the major soluble substrate for a CaM-dependent protein kinase in many tissues.

A preliminary account of this work has appeared: H.C. Palfrey (1983) Fed. Proc. 42, 567, abs.1611. The M_r of the major substrate has been corrected from 97000 to 100000 in the current work.

2. MATERIALS AND METHODS

CaM was purified from bovine brain by minor modifications of the methods in [17] and [18]. Phosphorylase *b*, phosphorylase kinase, DCC-treated trypsin, *O*-phosphoserine, *O*-phosphothreonine and M_r-markers were from Sigma. *Staphylococcus aureus* V8 protease (SAP) was from Miles. *Staphylococcus aureus* cells (SAC; Pansorbin) was from Calbiochem. Cellulose thin-layer chromatography (TLC) plates were from Eastman. γ-[^{32}P]ATP was from ICN.

Cytosol fractions were obtained from various rat tissues by homogenization (1:5, w/v) in a buffer containing 25 mM Tris–HCl (pH 7.4), 5 mM 2-mercaptoethanol, 1 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride. Homogenates were centrifuged at 1000 × *g* for 10 min to remove debris, then at 100000 × *g* for 30 min to yield the final cytosol preparations. This material was stored at −70°C in small aliquots that were thawed only once prior to assay.

Ca^{2+}-dependent endogenous phosphorylation was assayed in a final volume of 100 μl containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 20 μM [^{32}P]ATP (2500 cpm/pmol), 30–60 μg cytosol protein and either 1 mM EGTA ('$-Ca^{2+}$') or 0.5 mM free $CaCl_2$ ('$+Ca^{2+}$'). (The high concentration of $CaCl_2$ was used to optimise activity of any phospholipid-dependent protein kinase in the preparation.) Assays were initiated by the addition of ATP, carried out at 30°C for 1 min, and terminated by addition of 50 μl of SDS–'stop' solution [10]. Proteins were separated on SDS–6% or 7.5% polyacrylamide gels and phosphoproteins identified by autoradiography. Quantitation of ^{32}P incorporated into protein was achieved by excising the relevant bands from gels with the aid of the autoradiograph, and counting the pieces by liquid scintillation.

One-dimensional peptide maps of phosphoproteins were generated as in [10,19]. Two-dimensional tryptic fingerprinting as well as phosphoaminoacid analysis followed established procedures (see [10] for details). Immune precipitation of phosphorylase, subsequent to phosphorylation assays as described above, was carried out by the method in [16] using a final concentration of 0.25% NP40 and no SDS. Goat anti-rabbit skeletal muscle phosphorylase antibodies [20] were kindly provided by Dr Donald Graves (Iowa State University); 2–5 μl of this material was used per assay tube. These antibodies have been shown to cross-react with phosphorylase from heart (D. Graves, personal communication) and liver (H.C. Palfrey, unpublished).

3. RESULTS

When cytosolic preparations from a number of rat tissues were phosphorylated in vitro a protein of M_r ~100000 (100 kDa), whose phosphorylation was stimulated upon the addition of Ca^{2+}, was visible following gel electrophoresis and autoradiography (fig.1). In heart, liver, lung, adrenal, and spleen cytosol, this protein was the major species phosphorylated with Ca^{2+}, and exhibited a 5–10-fold increase in ^{32}P-incorporation under these conditions. The phosphoprotein was less prominent in skeletal muscle and absent from kidney cytosol. In the case of skeletal muscle, the major Ca^{2+}-dependent phosphorylation occurred on a protein of M_r ~97000 (97 kDa), subsequently identified as glycogen phosphorylase (see below). In fig.1, the phosphorylation of the 100 kDa protein in skeletal muscle is obscured by the intense ^{32}P-incorporation into the 97 kDa band; these two species are resolved more distinctly on SDS–6% polyacrylamide gels (see fig.5). In other experiments, the 100 kDa protein has been identified as the most

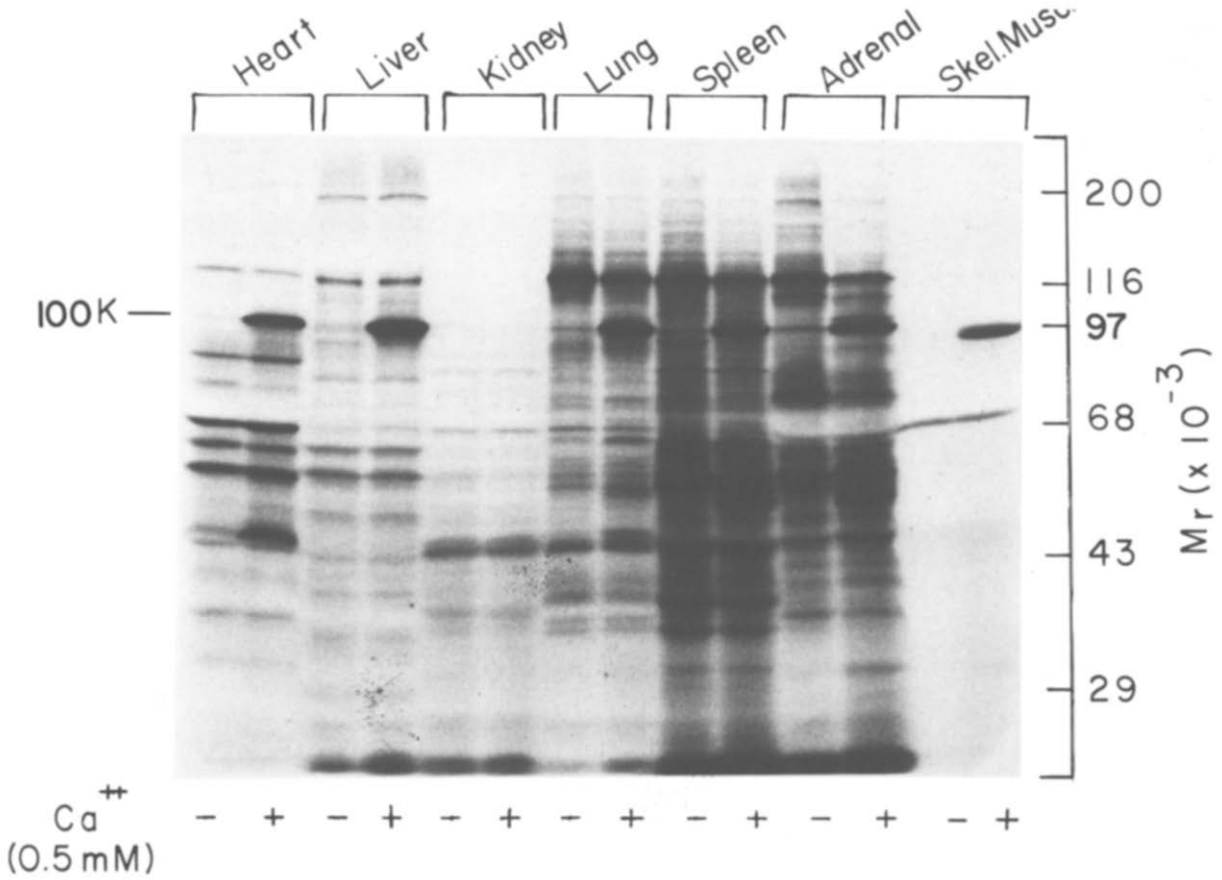

Fig.1. Autoradiogram of an SDS–7.5% polyacrylamide gel showing proteins (30 μg/lane) phosphorylated in the absence (1 mM EGTA) and presence of Ca^{2+} (0.5 mM added Ca^{2+}) in cytosolic extracts from 7 rat tissues. The M_r-markers ($M_r \times 10^{-3}$) are myosin (200), β-galactosidase (116), phosphorylase *b* (97.4, [26]), bovine serum albumin (68), ovalbumin (43) and chymotrypsinogen A (29).

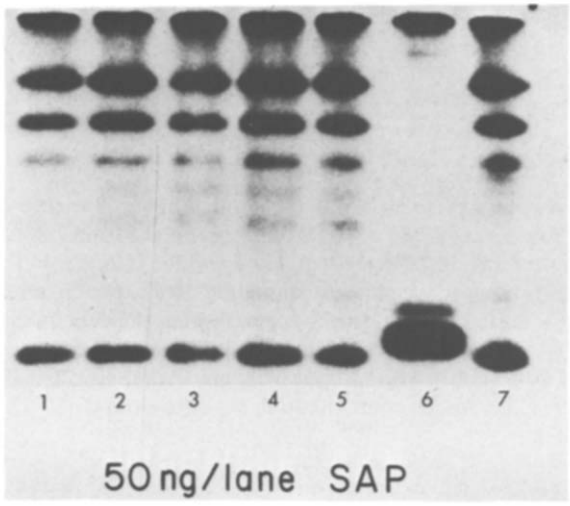

Fig.2.

prominent Ca^{2+}-dependent phosphoprotein in epithelial tissue cytosol [21] and in a number of mammalian cell lines (H.C. Palfrey and N.E. Owen, in preparation). No effect of cyclic

Fig.2. One-dimensional phosphopeptide maps of 100 kDa following phosphorylation of the protein in the presence of Ca^{2+}. Dried gel pieces corresponding to phosphoproteins were cut out from SDS–6% polyacrylamide gels, re-swollen and subjected to proteolysis as in [19] using SAP (50 ng/lane). Samples were 100 kDa phosphoproteins from: (1) heart; (2) liver; (3) lung; (4) spleen; (5) adrenal; (7) skeletal muscle. Lane (6) was the 97 kDa phosphoprotein from skeletal muscle. Gels were loaded such that about the same number of counts was applied per sample.

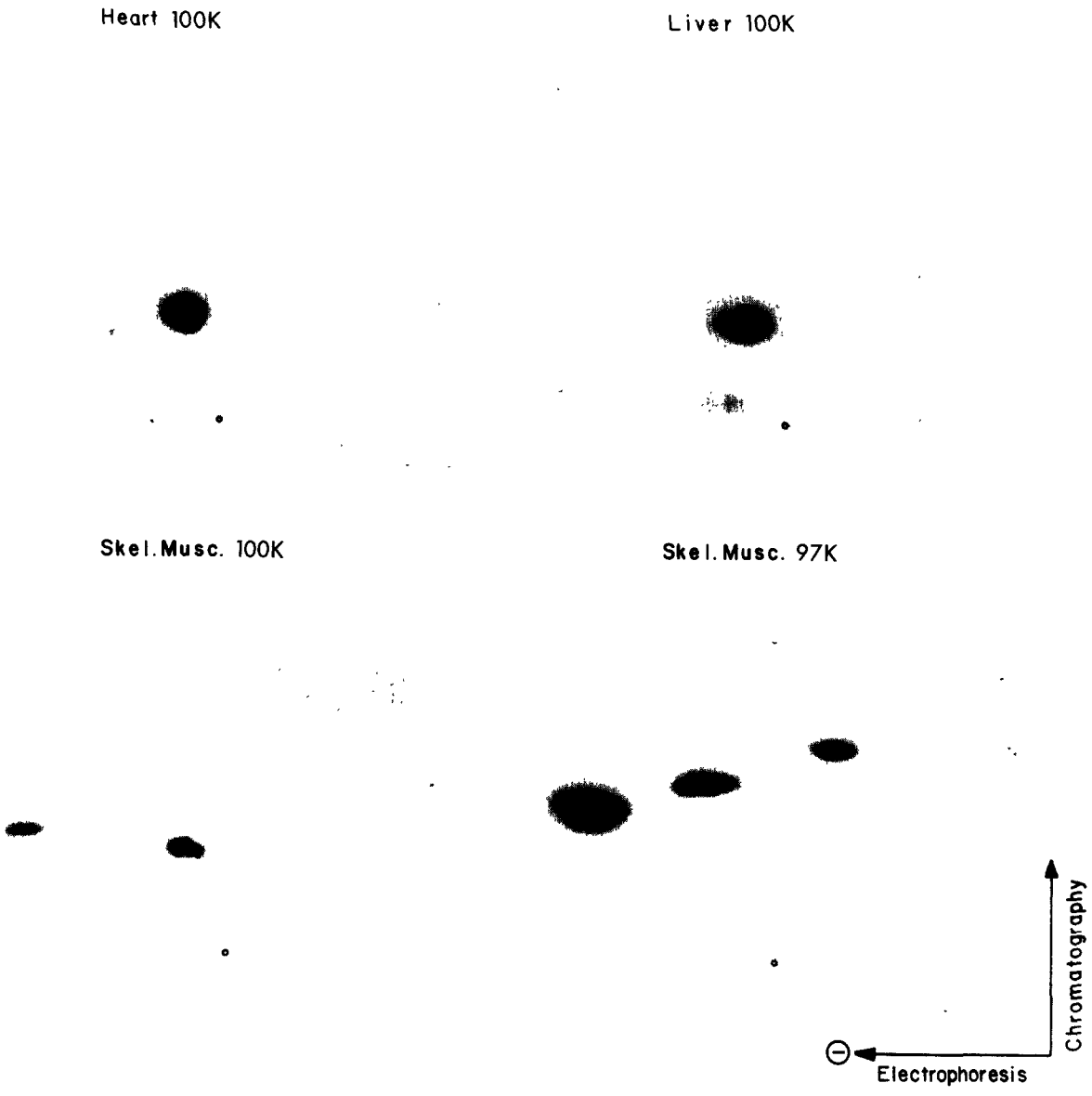

Fig.3. Two-dimensional tryptic fingerprints of 100 kDa following phosphorylation of the protein in the presence of Ca^{2+} using: (A) heart; (B) liver; (C) skeletal muscle cytosol; (D) fingerprint of M_r ~97000 phosphoprotein from skeletal muscle cytosol included for comparison. Dried gel pieces were re-swollen in 25% methanol and washed in this solution over a period of 24 h. The pieces were then lyophilized and re-swollen in a solution containing 50 μg trypsin/ml of 50 mM NH_4HCO_3 (pH 8.3) + 0.5 mM DTE. Digestion was allowed to continue for 24 h after which the supernatant was removed, lyophilized and redissolved in a small volume of electrophoresis buffer (pH 3.5). This material was spotted onto cellulose plates (O, Origin) and chromatographed as described [10]. About 600 cpm (A,B), 400 cpm (C) and 850 cmp (D) were applied. In (C) the 3 faint spots seen are probably due to contaminating peptides from the 97 kDa phosphoprotein.

nucleotides on the phosphorylation of 100 kDa was found in any of the tissues examined. On examination of washed membrane preparations from various tissues, no protein of comparable M_r was phosphorylated in a Ca^{2+}-dependent manner (cf. [12]). Other proteins that became phosphorylated on incubation of cytosol samples in the presence of Ca^{2+} included those of M_r ($\times 10^{-3}$) 78, 53, 51 and 43 in heart (cf. [14] where CaM-dependent phosphorylation of proteins of 94, 78, 57, 48, 34 and 26 in guinea pig heart cytosol was observed); 39, 29 in liver; 125, 85 and 14.5 in skeletal muscle; 68, 59, 49 and 36 in adrenal; 105, 56 and 48 in lung and 68, 56, 50 and 28.5 in spleen. Rat erythrocyte cytosol was found to be devoid of Ca^{2+}-dependent protein kinase activity (not shown). Rat brain cytosol has previously been shown to contain a large number of Ca^{2+}-dependent substrates (e.g., [15]), but further studies are needed to assess whether 100 kDa is present.

The possibility of homology between the 100 kDa species in the different tissues under consideration was investigated by one- and two-dimensional phosphopeptide mapping. One-dimensional maps generated by the method in [19] using SAP 50 ng/lane; fig.2), α-chymotrypsin or cyanogen bromide cleavage (not shown) revealed extensive similarities between the 100 kDa phosphoproteins from the tissues indicated above. Moreover, using the same degradative procedures, the 97 kDa phosphoprotein from skeletal muscle had a totally different digestion pattern (e.g., fig.2). Identity between the 100 kDa species was also established by tryptic fingerprinting, examples of which are shown in fig.3 (heart, liver and skeletal muscle), similar results being obtained with 100 kDa from other tissues. In all cases, a single major phosphorylated peptide with a characteristic, slightly cathodic mobility at pH 3.5 was identified, with several minor peptides also visible, especially on longer film exposures. In contrast, the 97 kDa protein from skeletal muscle showed 3 distinct phosphopeptides, none of which comigrated with the major phosphopeptide from the 100 kDa protein, consistent with the identification of the 97 kDa species as phosphorylase [22]; purified phosphorylase phosphorylated with commercial phosphorylase kinase exhibited the same 3 peptides (not shown).

The major phosphopeptide derived from the 100 kDa species was eluted from tryptic chromatograms and subjected to acid hydrolysis and phosphoaminoacid analysis (fig.4, lanes 2,4,5). This peptide was found to contain exclusively phosphothreonine, although some stimulation of ^{32}P-incorporation into serine residues was noted when the entire 100 kDa gel band was analyzed (fig.4, lanes 1,6; such incorporation may be into very minor 100 kDa-derived phosphopeptides or could be due to contaminating phosphopeptides derived from proteins of similar M_r extracted from the SDS gel or both). Two-dimensional phospho-

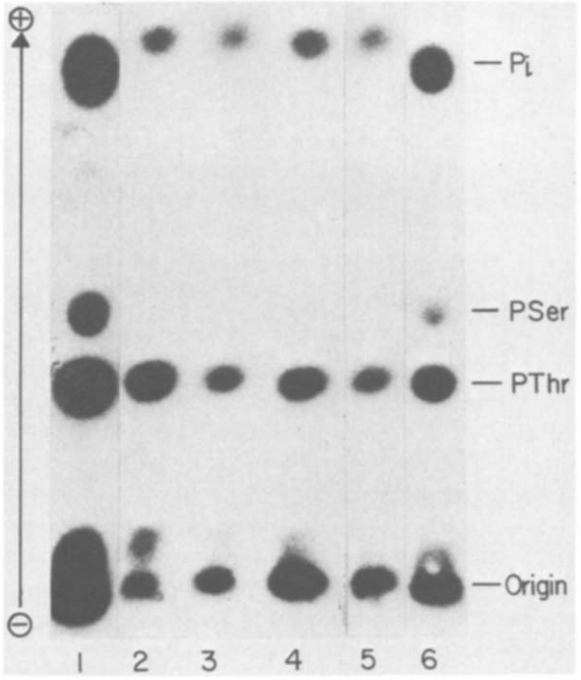

Fig.4. Phosphoaminoacid analysis of acid hydrolysates of various samples derived from phosphorylated 100 kDa. The 100 kDa band was cut out from SD–6% polyacrylamide gels and trypsinized; the tryptic hydrolysate was lyophilized and then either directly hydrolyzed with 6 N HCl at 110°C (lanes 1,6) or fingerprinted (see fig.3), and the individual spots eluted from the cellulose plate prior to acid hydrolysis (lanes 2–5). Lanes: (1) liver – entire 100 kDa band; (2) liver – 100 kDa major phosphopeptide; (3) liver – 100 kDa minor phosphopeptide (see fig.3; seen just below major phosphopeptide); (4) lung – 100 kDa major phosphopeptide; (5) heart – 100 kDa major phosphopeptide; (6) skeletal muscle – entire 100 kDa band.

aminoacid analysis, performed to distinguish phosphothreonine from phosphotyrosine [23], revealed no incorporation into the latter species (not shown).

Because the mobility of 100 kDa was similar to that of phosphorylase, and heart and liver are known to contain large quantities of this enzyme, it was considered important to unequivocally demonstrate that 100 kDa was not related to this protein. In addition to the divergent peptide maps described above, 100 kDa was unable to bind to glycogen under conditions where the 97 kDa pro-

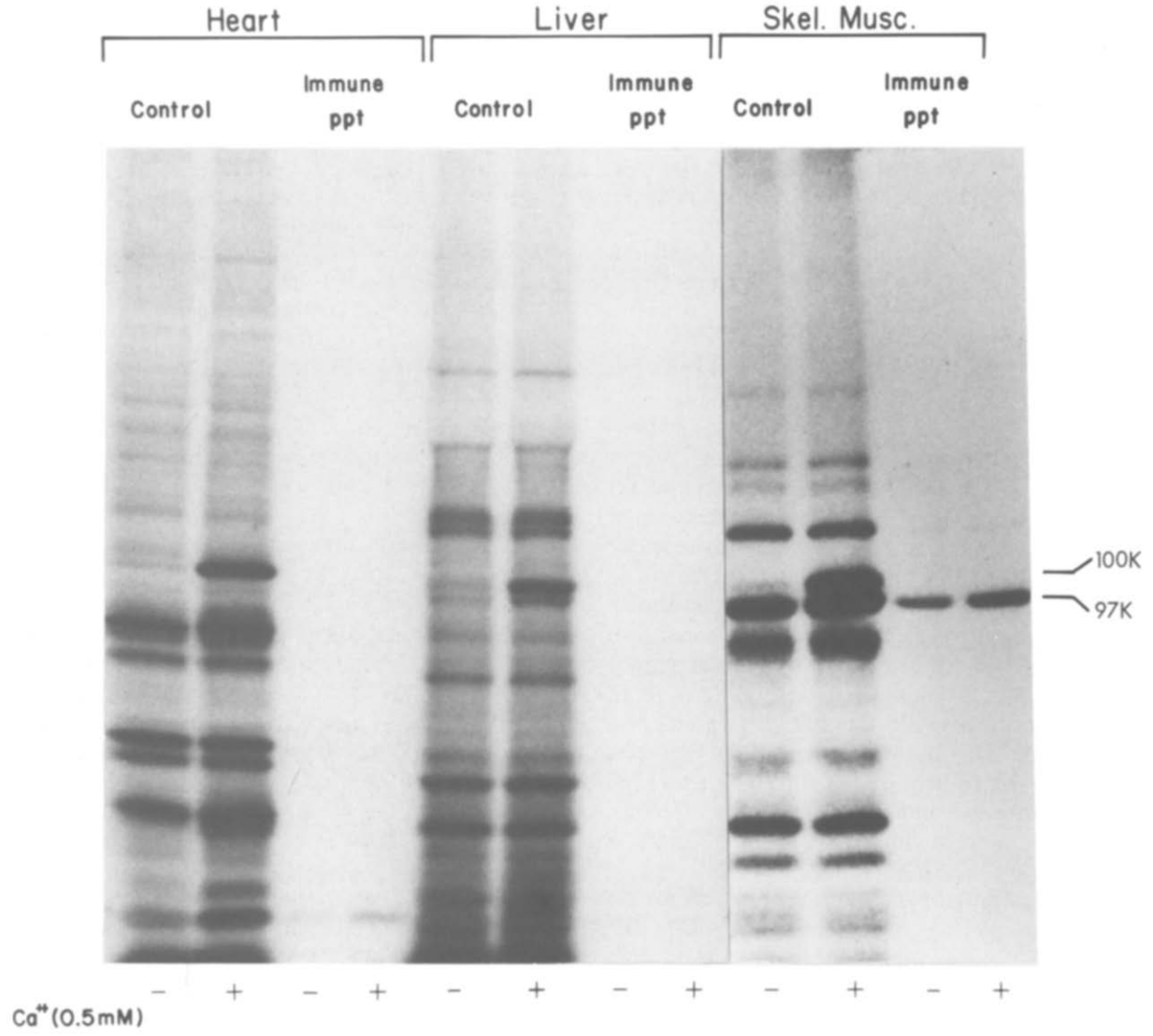

Fig.5. Distinction between 100 kDa and phosphorylase; autoradiograms of SDS–6% polyacrylamide gels showing immune precipitates with anti-phosphorylase antibodies. Cytosol extracts (30 μg protein) from 3 tissues were phosphorylated in the absence or presence of Ca^{2+} (see fig.1) and then subjected to immune precipitation, using goat anti-skeletal muscle phosphorylase antibodies [20] and SAC, as in [16]. Note the precipitation of the major 97 kDa phosphoprotein but not the 100 kDa phosphoprotein in skeletal muscle (also seen in Coomassie-stained gels – not shown); a faint 97 kDa Ca^{2+}-dependent phosphoprotein in heart and liver cytosol was also seen on longer exposures of similar autoradiographs. No precipitation of a stainable protein in the 100 kDa region was ever observed.

tein from skeletal muscle clearly did (not shown). Moreover, 100 kDa was not recognized by anti-phosphorylase antibodies which precipitated the 97 kDa protein from skeletal muscle in the same assay (fig.5). These experiments rule out the possibility that 100 kDa is in any way related to phosphorylase, and confirm the notion that the skeletal muscle 97 kDa protein corresponds to phosphorylase.

The 100 kDa kinase appears to belong to the group of Ca^{2+}-dependent protein kinases that depend on CaM for their activity. Although addition of exogenous CaM to cytosol from most tissues enhanced Ca^{2+}-dependent phosphorylation by insignificant amounts (e.g., fig.6), 100 kDa phosphorylation could be inhibited by low concentrations of trifluoperazine, a known CaM antagonist [24]. Half maximum inhibition was obtained at concentrations of 6–16 μM of this drug in different tissues (not shown). Moreover, phosphorylation of 100 kDa could be made dependent on exogenous CaM if tissue cytosol fractions were first treated to deplete them of endogenous CaM. An example of such an experiment is shown in fig.6 for heart cytosol. Proteins were precipitated with 55% $(NH_4)_2SO_4$, leaving the majority of the CaM in the supernatant (cf. [15]). Subsequent phosphorylation of the redissolved, dialyzed $(NH_4)_2SO_4$ pellet revealed that 100 kDa kinase activity was dependent on the addition of exogenous CaM. As with crude cytosol, phosphatidylserine readdition to samples fractionated by $(NH_4)_2SO_4$ had no effect on 97 kDa phoshorylation.

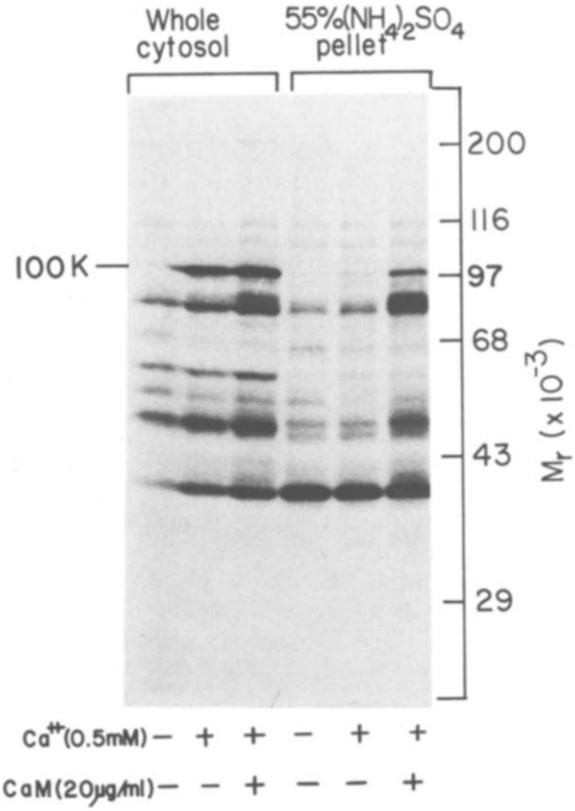

Fig.6. Dependence of 100 kDa kinase activity on CaM in heart cytosol; autoradiogram of SDS–6% polyacrylamide gel. Phosphorylation of 100 kDa in cytosol and in 55% $(NH_4)_2SO_4$ precipitates (after redissolution and dialysis) derived from cytosol were compared. Note that CaM (20 μg/ml) has very little effect on 100 kDa phosphorylation in crude cytosol (20% greater than Ca^{2+} alone), but has a much greater effect in the CaM-depleted $(NH_4)_2SO_4$ pellet (250% greater than Ca^{2+} alone). The apparent increase in phosphorylation of an M_r ~80000 substrate relative to that of 100 kDa in the $(NH_4)_2SO_4$ precipitate is due to the relative lability of the 100 kDa kinase during dialysis.

4. DISCUSSION

A major cytosolic substrate for CaM-dependent protein kinase has been identified in several mammalian tissues. The widespread distribution of the 100 kDa phosphoprotein suggests that it may play an important function in mediating Ca^{2+} signals in the cytoplasm. The purification of this protein, currently in progress, should allow us to determine its function.

An overwhelming body of evidence suggests that 100 kDa is unrelated to glycogen phosphorylase, a closely migrating protein (M_r ~97000) which is phosphorylated in a Ca^{2+}-dependent manner by the enzyme phosphorylase kinase. Thus, 100 kDa and phosphorylase could be distinguished by:

(i) Their migration on one-dimensional gels (fig.5) and two-dimensional gels (not shown);
(ii) Phosphopeptide mapping using 4 different methods;
(iii) Presence of phosphothreonine rather than phosphoserine (cf. [22]);
(iv) Failure of 100 kDa to bind to glycogen;

(v) Failure of 100 kDa to be recognized by anti-phosphorylase antibodies.

It also appears unlikely that the enzyme phosphorylating 100 kDa is phosphorylase kinase as the enzymatic activity did not bind to glycogen as does phosphorylase kinase, and 100 kDa phosphorylation could be made dependent on exogenous CaM by simple $(NH_4)_2SO_4$ fractionation, whereas intrinsic CaM (i.e., the δ-subunit of the enzyme) is not dissociated from phosphorylase kinase by such a treatment (e.g., [3]). The nature of the CaM-dependent protein kinase that phosphorylates 100 kDa is currently under investigation.

It is likely that the protein described here is identical to that studied in [25], termed S97, in the cytosol of GH_3 pituitary adenoma cells. These authors showed slight stimulation of S97 phosphorylation in cytosolic extracts by exogenous CaM and inhibition by TFP (although their IC_{50}-value of 40 μM was somewhat higher than that found here). They also demonstrated that S97 phosphorylation in intact cells was stimulated by conditions leading to an elevation of intracellular Ca^{2+} such as depolarizing agents and A23187, as well as by a physiological stimulus, thyrotropin-releasing hormone, thought to release Ca^{2+} from intracellular stores in these cells. We have recently shown the presence of 100 kDa in a variety of cultured cell lines, and are currently evaluating the phosphorylation of this protein in response to physiological stimulation of intact cells.

ACKNOWLEDGEMENTS

I thank Marilyn Donahue for her expert technical assistance and Dr D. Graves for the gift of anti-phosphorylase antibody.

REFERENCES

[1] Schulman, H., Huttner, W.B. and Greengard, P. (1980) in: Calcium and Cell Function (Cheung, W.Y. ed) vol.1, pp.220–251, Academic Press, New York.
[2] Adelstein, R. and Klee, C.B. (1981) J. Biol. Chem. 256, 7501–7509.
[3] Shenolikar, S., Cohen, P.T., Cohen, P., Nairn, A.C. and Perry, S.V. (1979) Eur. J. Biochem. 100, 329–337.
[4] Payne, M.E., Schworer, C.M. and Soderling, T.R. (1983) J. Biol. Chem. 258, 2376–2382.
[5] Ahmad, Z., DePaoli Roach, A. and Roach, P. (1982) J. Biol. Chem. 257, 8348–8355.
[6] Woodgett, J., Tonks, N.K. and Cohen, P. (1982) FEBS Lett. 148, 5–11.
[7] Palfrey, H.C., Rothlein, J.E. and Greengard, P. (1983) J. Biol. Chem., in press.
[8] Takai, Y., Kishimoto, A., Isawa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695.
[9] Wise, B.C., Rayner, R.L. and Kuo, J.F. (1982) J. Biol. Chem. 257, 8481–8488.
[10] Alper, S.L., Palfrey, H.C., DeRiemer, S.A. and Greengard, P. (1980) J. Biol. Chem. 255, 11029–11039.
[11] Fukunaga, K., Yamamoto, H., Matsui, K., Higashi, K. and Miyamoto, E. (1982) J. Neurochem. 39, 1607–1617.
[12] Schulman, H. and Greengard, P. (1978) Proc. Natl. Acad. Sci. USA 75, 5432–5436.
[13] Campbell, K.P. and MacLennan, D.H. (1982) J. Biol. Chem. 257, 1238–1246.
[14] Katoh, N., Wrenn, R.W., Wise, B.C., Shoji, M. and Kuo, J.F. (1981) Proc. Natl. Acad. Sci. USA 78, 4813–4817.
[15] Walaas, S.I., Nairn, A.C. and Greengard, P. (1983) J. Neurosci. 3, 302–311.
[16] Palfrey, H.C., Schiebler, W. and Greengard, P. (1982) Proc. Natl. Acad. Sci. USA 79, 3780–3784.
[17] Watterson, D.M., Harrelson, W.G., Keller, P.M., Sharief, F. and Vanaman, T.C. (1976) J. Biol. Chem. 251, 4501–4513.
[18] Gopalakrishna, R. and Anderson, W.B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836.
[19] Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[20] Janski, A.M. and Graves, D.J. (1977) FEBS Lett. 82, 232–236.
[21] Rao, M., Nash, N. and Palfrey, H.C. (1982) J. Cell Biol. 95, 254a.
[22] Fischer, E.H., Graves, D.J., Crittenden, E.R.S. and Krebs, E.G. (1959) J. Biol. Chem. 234, 1698–1704.
[23] Hunter, T.A. and Sefton, B. (1980) Proc. Natl. Acad. Sci. USA 77, 1311–1315.
[24] Weiss, B., Prozialeck, W.C. and Wallace, T.L. (1982) Biochem. Pharmac. 31, 2217–2226.
[25] Drust, D.S. and Martin, T.F.J. (1982) J. Biol. Chem. 257, 7566–7573.
[26] Titani, K., Koide, A., Hermann, J., Ericsson, L.H., Kumar, S., Wade, R.D., Walsh, K.A., Neurath, H. and Fischer, E.H. (1977) Proc. Natl. Acad. Sci. USA 74, 4762–4765.