A 180-kDa Protein Kinase Seems to Be Responsible for the Phosphorylation of Prothymosin α Observed in Proliferating Cells*

(Received for publication, October 11, 1996)

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Prothymosin α (ProTα) is an acidic protein involved in cell proliferation. Its phosphorylation status is correlated with proliferative activity. Here we report the isolation and characterization of a ProTα-phosphorylating kinase (ProTαK) from mouse splenocytes that seems to be responsible for the in vivo phosphorylation of ProTα and that differs from other protein kinases reported to date. This enzyme, mainly located in the cytosol, has an molecular mass of 180 kDa and appears to be made up of two proteins of 64 and 60 kDa. Its activity was markedly enhanced by mitogenic activation of cells. The ProTα residues phosphorylated by the enzyme in vitro are a Thr at position 7 and another Thr at positions 12 or 13, which differs from other protein kinases reported to date. The new enzyme shows a number of clear structural and catalytic differences from CK-2. It phosphorylates histones H2B and H3, although with weaker activity than ProTα. An enzyme with the same characteristics was also found in other murine tissues and cell lines.

Although the precise function of ProTα remains unclear (for review, see Ref. 20), there is strong evidence to suggest a nuclear role; its C terminus region bears a karyophilic signal (21) and ProTα migrates to the nucleus in proliferating cells (22, 23). Recent work in our laboratory has shown that ProTα binds histones and cooperates in nucleosome assembly in vitro (24), suggesting that the putative nuclear function may be related to chromatin remodeling; this possibility is consistent with the structural similarities between ProTα and various nuclear proteins known to be involved in chromatin activity (25, 26).

Phosphorylation of ProTα has recently been reported by us (27) and corroborated by other authors (28). This post-translational modification constitutes an additional clue to its biological role. The available data indicate that the extent of phosphorylation of ProTα is positively correlated with cell proliferation activity (27, 28). The phosphorylation sites are included in the first 14 amino acids of the sequence: however, there is controversy as to whether the residues which undergo phosphorylation are Thr residues (our findings; Ref. 27), or Ser residues (Sburlati et al. (28)).

Regardless of whether the residues phosphorylated are Thr or Ser, the N-terminal sequence of ProTα (AcSer-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys) suggests that the in vivo phosphorylation sites are casein kinase 2 (CK-2) consensus sites (29). This is in accordance with our previous results which show that CK-2 is able to phosphorylate the 14-residue N-terminal fragment of ProTα in vitro (30). However, phosphoamino acid analysis showed that in vitro phosphorylation with CK-2 leads to phosphorylation of both Thr and Ser residues, in approximately equal proportion.

In the work reported here, we aimed to characterize the enzyme responsible for the phosphorylation of ProTα in proliferating cells. At the same time, we set out to resolve the controversy as to which amino acid residues undergo phosphorylation. Fractionation of cell extracts by affinity chromatography on a ProTα-Sepharose column and then by ion-exchange HPLC yielded a protein kinase, different from CK-2, which phosphorylated the N-terminal 14-mer of ProTα. Analysis of the sites phosphorylated both in vivo and by the purified enzyme in vitro confirmed that the residues phosphorylated in the N-terminal peptide of ProTα are Thr residues, and indicate that the purified enzyme is probably responsible for the phosphorylation of ProTα observed in vivo.

EXPERIMENTAL PROCEDURES

Materials

The triethylammonium salts of adenosine 5′-[γ-32P]triphosphate ([γ-32P]ATP, 3000 Ci/mmol) and guanosine 5′-[γ-32P]triphosphate ([γ-32P]GTP, 5000 Ci/mmol) were purchased from Amersham International. [32P]Orthophosphate (1 Ci/mmol) was from DuPont NEN. TPCK-treated trypsin, alkaline phosphatase from bovine intestinal mu-
cosa, poly-L-lysine, hydrobromide, dephosphorylated \( \beta \)-casein from bovine milk, protamine (grade IV) from salmon, and concanavalin A were from Sigma. Thin-layer cellulose plates (0.1 mm) were from Merck. Interleukin-2, DNase I, RNase I, histones, the artificial casein kinase II from Sigma. Thin-layer cellulose plates (0.1 mm) were from Merck.

Methods

Cell Culture and Subcellular Fractionation—Splenocytes, thymocytes, and hepatocytes were obtained from 45-day-old female BALB/c mice. Cells were isolated by pressing a suspension of the freshly excised and minced tissue in RPMI 1640 medium through a 65-μm mesh stainless steel screen. In all cases the resulting suspension was treated with erythrocyte lysis buffer (0.75% \( \text{NH}_2\text{Cl}, 0.21\% \text{Tris-Cl}, \text{pH 7.2} \) and washed twice in RPMI 1640 before use. Splenocytes and thymocytes (5 × 10⁶ cells/ml) were grown for various times in RPMI 1640 containing 10% fetal calf serum, 100 units ml⁻¹ of penicillin, and 100 μg ml⁻¹ of streptomycin.

Subcellular fractionation of the different cell types was as follows. Cells were collected from culture, washed twice, suspended (about 5 × 10⁵ cells/ml) in ice-cold lysis buffer (50 mM Tris-Cl, pH 7.4, containing 150 mM NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40, 0.5 mM dithiothreitol) to yield the cytosol fraction. Pellet II was suspended in buffer A containing 0.5M NaCl and 0.5% sodium deoxycholate, incubated at 0 °C for 10 min; the supernatant was then dialyzed against buffer A and concentrated to final volumes of 0.5 ml.

Isolation and Purification of \( 32P \)-ProTα from Metabolically Labeled Cells—\( 32P \)-Labeled ProTα was obtained from proliferating cells following modification of the procedure of Haritos et al. (32), as we have described previously (27). A subline of the murine splenic lymphocytes (strain 32PN) obtained with concanavalin A and interleukin-2 were incubated with \( [32P] \)orthophosphate (50 μCi/ml), then frozen in liquid nitrogen, powdered, and suspended in boiling 0.15 μl NaCl. After cooling, the suspension was centrifuged, and the supernatant brought to pH 2.5, clarified by centrifugation, made up to 50 m Tris-Cl (pH 8) and 70 mM NaCl, and applied to a DEAE-cellulose column. The first peak was eluted with 3 volumes of boiling buffer (50 mM Tris-Cl, pH 8) containing 0.2 mM NaCl, followed by 6 volumes containing 0.5 mM NaCl. The components eluted with the latter buffer were purified by reverse-phase HPLC. Phosphorylated ProTα, which co-purified with calf ProTα, was characterized by SDSPAGE and isoelectric focusing, and on the basis of immunoreactivity with an antibody raised against the first 28 amino acids of ProTα (27). This procedure has previously been shown by us (10) and by others (5, 9) to yield 43–130 μg of ProTα/g of cell, depending on tissue. In the present study, the yields of radioactive ProTα after HPLC purification were about 4 × 10⁶ cpm/10⁶ mitogen-activated T-cells.

Peptide Mapping and Phosphoamino Acid Analysis—Peptide mapping on the basis of tryptic digestion of \( 32P \)-labeled phosphorylated ProTα and phosphoamino acid analysis of the ProTα fragments were as described (27). Briefly, \( 32P \)-labeled, either purified or naturally labeled cells or from kinase-assay reaction mixtures, was digested with TPCK-trypsin for 10 h at 37 °C. Tryptic peptides were then separated by reverse-phase HPLC. Aliquots of the radioactive peak coeluting with the 14-residue N-terminal fragment of calf ProTα were then subjected to phosphoamino acid analysis, by hydrolysis with 6 N HCl, mixing with nonradioactive phosphoamino acid standards, thin-layer electrophoresis, and autoradiography. Alternatively, aliquots of radioactive peaks (of the HPLC-separated tryptic digests, whether from metabolically labeled cells or from kinase-assay reaction mixtures) were further digested with endoproteinase V8 (Boehringer Mannheim) (protease:peptide ratio 1:10) in 25 mM ammonium carbonate (pH 7.8). The resulting peptides were separated by reverse-phase HPLC in an UltraphaseODS column (5 μm, 4.6 × 250 mm), and phosphoamino acid analysis was performed as above. The amino acid compositions of the peptides derived from V8 digestion of the N-terminal fragment were determined after acid hydrolysis in a Biochrom 20 analyzer (Pharmacia).

Kinase Activity Assays—Assays of the ProTα phosphorylating activity of cell extracts or rat liver CK-2 were performed in 25-μl reaction mixtures containing 50 mM Tris-Cl (pH 7.4), 150 mM KCl, 25 mM MgCl₂, 1.6 mM EGTA, 1 mM EDTA, 3.3 mM dithiothreitol, 80 ng/ml bovine serum albumin, 83 mM β-glycerol phosphate, and 100 μM \( \gamma \)-ATP. Phosphorylated calf thymus ProTα (5 μg), or that indicated under "Results," was used as substrate. After 30 min at 37 °C, the reaction was stopped with 5 μl of 100 mM ATP. Activity was quantified (a) by separating the components of the reaction mixture by SDS-PAGE (33), with autoradiography to estimate \( \gamma \)-ProTα concentration and kinase activity subsequently expressed in arbitrary units, or (b) by separating the components by reverse-phase HPLC (as described above), then quantifying the radioactivity co-eluting with ProTα, with activity expressed as amount of \( 32P \) incorporated into the substrate. Assays of immunoreactivity with antibodies raised against the first 28 amino acids of ProTα (see above) were also used to characterize \( 32P \)-ProTα in the various reaction mixtures.

Immunodepletion assays were carried out in 50-μl aliquots of reaction mixture buffer, without ATP, containing 30 μl of the purified kinase and different concentrations of antibody to the α subunit of CK-2. The mixture was incubated overnight at 4 °C with gentle shaking. Forty μl of Sepharose-bound protein A in reaction mixture buffer was then added, and the mixture gently shaken for 2 h at 4 °C. Immuno complexes were collected by centrifugation and kinase activity was assayed in the supernatant after adding 100 μM \( \gamma \)-ATP.

RESULTS

ProTα Phosphorylating Activity in Splenic Lymphocytes—Phosphorylation of ProTα is particularly marked in mitogen-activated splenic lymphocytes, as compared with non-activated lymphocytes and other proliferating cell types (27, 28). We thus searched for the ProTα kinase (ProTαK) in extracts of these cells. The first step was to isolate cellular components which show affinity for ProTα by using a ProTα-Sepharose column.
that we have previously shown to be effective for this purpose (24). ProTα phosphorylation activity in the various affinity chromatography fractions was assayed under conditions equivalent to those used for assaying CK-2 activity, since the in vivo phosphorylation sites of ProTα are similar to those of this enzyme (27, 28). Subcellular fractions (cytosol, nucleoplasm, microsome, and nuclear envelope) from mitogen-activated splenic lymphocytes were chromatographed on ProTα-Sepharose columns and ProTα phosphorylation activity was assayed in the different eluates. As shown in Fig. 1, components with high affinity for ProTα-Sepharose (lanes 4) in the cytosol (Fig. 1A), nuclear envelope (Fig. 1B), and nucleoplasm (Fig. 1C) fractions all showed ProTα phosphorylation activity, as judged by the radioactivity migrating with ProTα in the SDS-PAGE analysis, whereas no such activity was observed when ProTα was omitted from the reaction mixtures (Fig. 1, lanes 3). Components obtained from the microsome fraction showed no detectable ProTα phosphorylation activity (data not shown). Affinity chromatography appears to be efficient for isolating the ProTα phosphorylating activity, since no phosphorylated product migrating with ProTα was detected in the flow-through fractions (Fig. 1, lane 1), and since the phosphorylated product was absent (Fig. 1, B and C, lanes 2) or present in only low concentration (Fig. 1A, lane 2) in the moderate-affinity fractions. ProTα phosphorylation activity was imperceptible in the various crude subcellular fractions (not shown).

Similar results were obtained when an HPLC-based assay (see “Methods”; results not shown) was used for determination of ProTα phosphorylation activity in the different fractions. That the radioactive peaks co-eluting with calf ProTα in HPLC were indeed radiolabeled ProTα was confirmed by immunoprecipitation with the anti-28-mer antibody (see “Methods”); results not shown.

Densitometric scanning of the gels shown in Fig. 1 indicates that ProTα phosphorylation activity is highest in the high-affinity fraction (HAF, lane 4) of the cytosol extract. Activities in the HAFs of the nuclear envelope and nucleoplasm extracts were about 25 and 5.9%, respectively, of that in the HAF of the cytosol extract. Activity in the moderate-affinity fraction of the cytosol extract was about 4.8% of that in the HAF of the cytosol extract. Total protein concentrations were 15, 9, and 4 μg/10⁸ cells in the HAFs of cytosol, nucleoplasm, and nuclear envelope extracts, respectively.

ProTα phosphorylation activity in the HAF of the cytosol extract from non-activated splenic lymphocytes was about 14% of that in the HAF of the cytosol extract from mitogen-activated splenic lymphocytes (Fig. 2). As is also shown in Fig. 2, activity in the HAF of the nuclear envelope extract from non-activated cells was about 35% of that in this fraction from activated cells, whereas activity in the HAF of the nucleoplasm extract from non-activated cells was scarcely detectable.

Taken together, these results indicate that the ProTαK activity in splenic lymphocytes is influenced by proliferation status and is largely contained in the cytosol. Some activity is contained in nuclear envelopes, while activity in the nucleoplasm is negligible. To characterize this activity, we further purified components of the cytosol, nuclear envelope, and nucleoplasm extracts of activated splenic lymphocytes with affinity for ProTα-Sepharose by ion-exchange HPLC. ProTα phosphorylation activity in the HAFs of the cytosol and nuclear envelope fractions showed different elution patterns, but was in both cases concentrated in single clearly defined peaks (Fig. 3A). Kinase activity eluting at the same position as that in the HAFs from cytosol was scarcely detectable either in the eluate fractions obtained by ion-exchange HPLC of the HAFs of the nucleoplasm fraction or in eluate fractions obtained by ion-exchange HPLC of moderate-affinity fractions (see Fig. 3A, lane 2) from cytosol (result not shown). The specific activity of the HPLC-purified ProTαK from cytosol extract was about 300-fold higher than in the extract prior to HPLC. No such assessment of the effectiveness of the second purification step is possible for the nuclear envelope extract, since protein content in the peak-activity HPLC fraction was below detection limits. Gel filtration (Fig. 3B) indicated that the HPLC-purified kinase from the cytosol extract has a molecular mass of about 180 kDa, while the molecular mass of the kinase from the nuclear envelope extract was about 130 kDa.

Identification of Sites of Phosphorylation—To investigate whether the purified kinases are responsible for the phosphorylation of ProTα observed in vivo, we performed experiments aimed at more detailed characterization. In various animal cells ProTα has been shown to be phosphorylated in its 14-residue N-terminal region. However, controversy remains as to
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Fig. 3. Chromatographic separation of ProTα binding components in subcellular fractions of splenic lymphocytes. Components in the subcellular fractionates showing high affinity for Sepharose-ProTα were further separated first by ion-exchange HPLC and then by gel filtration. Panel A shows the elution pattern of the ProTα kinase activity (PK) separated by ion-exchange HPLC from the high-affinity fractions (0.4 ml) of the indicated subcellular fractionates from 4 × 10^6 mitogen-stimulated splenic lymphocytes, in the conditions described under “Methods.” ProTα phosphorylation activities were assayed in 20-μl aliquots of each HPLC eluate fraction, and are shown in the figures as arbitrary units (determined as indicated in the legend to Fig. 2). The two fractions with the highest activity were collected and concentrated to 0.3 ml. Panel B shows the results of separation by gel filtration on a Sephacryl S-200 column (40 × 1.2 cm) of 0.1-ml aliquots of the maximum activity fractions from ion-exchange HPLC. The column was equilibrated and eluted with 50 mM Tris-HCl (pH 7.6), containing 5% glycerol at a flow rate of 12 ml/h. ProTα phosphorylation activity was assayed in 20-μl aliquots of each 1-ml fraction collected, and is represented as in panel A. Positions of protein markers are indicated.

whether the residues phosphorylated are Thr, as we have found (27), or Ser, as proposed by Sburlati et al. (28). We thus set out to identify sites phosphorylated in vivo and sites phosphorylated in vitro by the kinases purified from cytosol and nuclear envelopes. To this end, 32P-ProTα purified by reverse-phase HPLC (see “Methods”) from metabolically labeled mouse splenocytes, or from kinase activity assay reaction mixtures, was trypsin-digested, and the resulting peptides were separated by reverse-phase HPLC. The elution pattern of the peptides derived from the tryptic digestion of 32P-ProTα phosphorylated in vitro by the cytosol-fraction ProTαK is shown in Fig. 4A. The observed pattern is identical to those obtained with ProTα phosphorylated in vitro by the nuclear envelope fraction ProTαK or with ProTα phosphorylated in vivo (results not shown). As can be seen from Fig. 4A, all the radioactivity co-eluted with the 14-residue N-terminal fragment of ProTα. Phosphoamino acid analysis of the radioactive tryptic peptides derived from ProTα phosphorylated in vivo or in vitro by the purified enzymes are shown in Fig. 4B. This result indicates that only Thr residues were phosphorylated by the cytosolic enzyme and in the ProTα phosphorylated in vivo, whereas both Ser and Thr residues, in similar proportion, were phosphorylated by the kinase from nuclear envelopes. This result confirms our previous findings as regards the phosphorylation of ProTα in vivo (27), and at the same time indicates that the cytosolic ProTαK, which phosphorylates only Thr residues, is probably that responsible for the phosphorylation observed in vivo. To investigate this hypothesis, we next performed a structural study of the radioactive tryptic fragments of ProTα phosphorylated in vivo and in vitro which co-purified with the N-terminal fragment.

The sequence of the first 14 amino acid residues of ProTα (see Fig. 4) forms part of the most conserved region of the protein and is identical in all mammals studied to date (5). Protease V8 (in ammonium carbonate buffer, pH 7.8) specifically cleaves this fragment between residues Glu-10 and Ile-11, giving rise to a decapeptide (AcSDAAVDTSES) and a tetrapeptide (ITTK). Since only one of these peptides contains Ser residues, V8 digestion is a useful tool for identification of the sites phosphorylated by the purified enzymes. V8 treatment of the N-terminal peptide from ProTα phosphorylated by the cytosolic kinase (Fig. 4C), the nuclear envelope kinase (Fig. 4D), or from ProTα phosphorylated in vivo (Fig. 4E), in all cases efficiently cleaved the N-terminal fragment to yield a decapeptide and a tetrapeptide, the sequences of which were confirmed by amino acid analysis (results not shown). The coincidence of the radioactive peaks in Fig. 4C–E, with those derived from V8 cleavage of the N-terminal fragment of ProTα (in the same figures) provides further confirmation that both the in vitro (Fig. 4C, D) and in vivo (Fig. 4E) phosphorylation sites are contained within the N-terminal fragment. Moreover, these elution patterns again indicate that the cytosolic enzyme phosphorylates the same sites as in vivo. Specifically, the Thr at position 7 in the fragment AcSDAAVDTSES, and, to judge from the amount of radioactivity, the Thr in the fragment TTTK at positions 12 or 13 (or both partially) are phosphorylated both by the cytosolic enzyme (Fig. 4C) and in vivo (Fig. 4E). By contrast, the nuclear envelope kinase (Fig. 4D) phosphorylates Thr residues at positions 12 or 13 (or both) in the tetrapeptide, as well as the Thr at position 7, and either one or two Ser residues in the decapeptide; the latter conclusion is inferred from the fact that the results of phosphoamino acid analysis of the decapeptide phosphorylated by the nuclear envelope kinase (not shown) are similar to those of analysis of the whole 14-mer phosphorylated by this enzyme (Fig. 4B). These data support our contention that the 180-kDa cytosolic ProTαK is that responsible for the phosphorylation of ProTα in vivo.

Characterization of the Purified Kinases—We next carried out experiments aimed at characterizing the cytosolic ProTαK and also the kinase from the nuclear envelope fraction which,
although apparently not responsible for the phosphorylation of ProT in vivo, shows similar specificity to that of the cytosolic enzyme. Since the specificities of both enzymes implied a consensus phosphorylation site identical to that of CK-2 (29), the first step was to investigate the behavior of the two enzymes with CK-2 substrates and effectors. The results of kinase activity assays performed with dephosphorylated casein and with the synthetic peptide RRRDDDSDDD, an artificial CK-2 substrate, are shown in Fig. 5A. The two kinases clearly differ in substrate specificity, since the nuclear envelope kinase phosphorylated both substrates while the cytosolic ProT kinase did not phosphorylate either. Experiments involving immunodepletion with antibodies to the α subunit of CK-2 (Fig. 5B) confirmed the difference between the two kinases, since only the activity of the nuclear envelope kinase was blocked. Moreover, the behavior of the nuclear envelope enzyme in these experiments, together with the molecular size data, suggest that this enzyme is in fact CK-2. Phosphopeptide mapping and phosphoamino acid analysis of ProT phosphorylated by CK-2 from rat liver (data not shown) gave identical results to those obtained with the nuclear envelope kinase (see Fig. 4), which corroborates this conclusion. The antibody to the α subunit of CK-2 was likewise ineffective for depleting the minor ProT phosphorylation activity present in the HAF of nucleoplasm and in the fraction of the cytosolic extract with moderate affinity for ProT-Sepharose (see Fig. 1, nucleoplasm panel, lane 4, and cytosol panel, lane 2); as indicated above, these activities were undetectable after ion-exchange HPLC. This suggests that this minor kinase activity may correspond to the main 180-kDa ProT kinase.

The response of the cytosolic ProT to CK-2 effectors was, however, quite similar to that of CK-2; it was markedly inhibited by heparin (at 0.2–0.4 μg/ml) and activated up to 4-fold by protamine or polylysine at 0.04–0.08 μg/ml. Kinetic study indicated that the cytosolic ProT kinase has a $K_m$ for ProT of 50 μM and a $V_{max}$ of 235 pmol/min/mg. The enzyme uses ATP (with a $K_m$ of 55 μM) as phosphate donor; GTP is an inefficient donor.

Determination of cytosolic ProT activity in extracts obtained at different periods (0–24 h) after the start of mitogenic activation of non-synchronized splenic lymphocytes indicated a
quantifying the amount of [32P]orthophosphate incorporated into the enzymes with casein and the synthetic peptide as substrates. Activity in no case exceeded 15%.

Means of values obtained in three replicate assays; interassay variation was less than 8%.

The phosphorylation activities were, respectively, 2 and 4.5 times lower in vivo than in vitro.

We have recently reported that ProTα kinase is quite stable in the fractions from affinity chromatography (about 2 months at 4 °C, 7 months at −70 °C), but shorter-lived after purification by ion-exchange HPLC (about 8 days at 4 °C, 3 weeks at −70 °C).

**ProTα Kinase Activity in Different Cell Types**—The ProTα kinase was isolated from other cell types following the same procedures as for mouse splenocytes. Subcellular fractionates obtained from hepatocytes and mitogen-activated thymocytes (both from mouse), semiconfluent HeLa cell cultures, and NC37 cells were subjected to ProTα-Sepharose affinity chromatography, and ProTα phosphorylation activity was assayed in the resulting eluate fractions. Kinase activity, only detected in components with high affinity for ProTα, was found to be appreciable in cytosolic fractions from all the cell types tested. ProTα kinase activity was also detected in nuclear envelope extracts of NC37 cells (results not shown), although substrate specificity and immunodepletion with anti-CK-2 indicated that the enzyme was CK-2, as in nuclear envelope extracts of mouse splenocytes. ProTα kinase activities in cytosolic fractions from the different cell types are shown in Fig. 7. As can be seen from this figure, activities appear to be dependent on the cell's proliferation activity. Cells with moderate proliferation activity (hepatocytes, mitogen activated thymocytes, and semiconfluent HeLa cells) showed activity which was about 26–40% of that observed in mitogen-activated splenocytes, while activity in extracts from NC37 cells was in the range of that in mitogen-activated splenocytes. Fractions from unstimulated thymocytes and confluent HeLa cells showed negligible ProTα activity (results not shown). Ion-exchange HPLC separation of the cytosolic components with high affinity for ProTα from NC37, semiconfluent HeLa, and mitogen-activated mouse thymocytes gave kinase activity elution patterns similar to those observed with cytosolic components from mouse splenocytes. Peptide mapping and phosphoamino acid analysis of the HPLC-purified enzymes, following the same procedure as for cytosolic ProTαK from splenocytes, indicated that the cytosolic kinases from NC37, semiconfluent HeLa, and mitogen-activated thymocytes show the same specificity for ProTα as the spleenocyte enzyme.

**DISCUSSION**

We have isolated and purified a 180-kDa protein kinase whose specificity for ProTα suggests that it is responsible for the phosphorylation of this protein observed in vivo in mouse splenic lymphocytes and other cells (27, 28). The data presented in this study, particularly the affinity of the enzyme for Sepharose-bound ProTα, and the results of peptide mapping and phosphoamino acid analysis of ProTα phosphorylated in vitro and in vivo by the purified enzyme, support this hypothesis.

Affinity chromatography on a ProTα-Sepharose column has been shown to be effective for isolating components which can interact with ProTα, including core histones (24). The results presented here corroborate the efficiency of this chromatogram.
Fig. 7. ProTK kinase activity in different cell types. Cytosolic fractionates from about 0.3 g of the different cell types were chromatographed on ProTK-Sepharose columns, and the ProTK phosphorylation activity in aliquots (10 μl) of the diverse eluates was assayed as described under “Methods.” The figure shows kinase activity in arbitrary units (upper panel) of the cytosolic ProTK kinase obtained from the different cell types, deduced on the basis of densitometric estimation of the amount of radioactive product migrating with ProTK in the SDS-PAGE gels, as indicated in the legend to Fig. 2 (lower panel). Activities shown are the means of values obtained in four (thymocyte and NC37) or three (hepatocyte and HeLa) replicate experiments; interassay variation was generally less than 18%.

The protein kinase isolated from the cytosol of the various mammalian cells is responsible for the phosphorylation of ProTK in vivo. It can be assayed either by the purified enzyme in vitro or by the purified enzyme in vivo.

Phosphorylation sites of the 180-kDa ProTK match consensus motifs for CK-2 (29). Furthermore, the ProTK kinase is, like CK-2, highly sensitive to inhibition by heparin and to activation by polyamines. However, the purified enzyme cannot be included in the CK-2 family in view of its structure (molecular size and subunit composition), and given that it does not phosphorylate substrates of CK-2, it is not inhibited by anti-CK-2 antibodies (Fig. 5) and is unable to use GTP as phosphate donor. There are also differences as regards subcellular location (34–36). On the other hand, the ability of CK-2 to phosphorylate acidic nuclear proteins structurally related to ProTK, such as HMG-14 (37), protein P1 (38), and nucleolin (39), suggests that this enzyme is related to ProTK.

Interestingly, the ProTK phosphorylates histone H2B and, to a lesser extent, histone H3 (Fig. 6). Although no biological significance can at present be inferred from this result, it should be noted that these proteins are not substrates of CK-2 (29) and that the pattern of phosphorylation of ProTK by the ProTK does not bear any resemblance to that reported for kinases which phosphorylate H2B (40, 41). In addition, the kinases that phosphorylate this histone are cAMP or cGMP-dependent (40); by contrast, signal transduction experiments performed in our laboratory indicate that ProTK is not directly phosphorylated by protein kinase A or C, and its phosphorylation in proliferating splenic lymphocytes seems to be dependent on protein kinase C activity. The present data thus suggest that the purified 180-kDa ProTK does not fall into any of the protein kinase categories described to date (41). That this is a new enzyme should be confirmed by further investigation of its structure and substrate specificity. It should be noted that the widespread distribution of ProTK in mammalian cells, together with its high concentration (0.03–0.15 pg/per cell), imply a central role in the cell proliferation; it is thus quite reasonable to hypothesize that there is a specific kinase for this protein.

An important question is whether the phosphorylation of ProTK is biologically significant. In view of the present data, we would argue that it is, at least in proliferating cells, for two main reasons: first, the existence of the protein kinase reported herein, which appears to be highly specific for ProTK; second, the high concentration of phosphorylated ProTK in proliferating cells. The amount of [32P]orthophosphate incorporated into ProTK after 20 h of metabolic labeling of mitogen-stimulated mouse splenocytes (in the conditions indicated in “Methods”) was about 0.3 nmol/10^8 cells. As ProTK concentration in splenic lymphocytes was about 0.8 nmol/10^8 cells (in accordance with estimates reported by other authors; Refs. 5 and 9), and since peptide mapping suggests that only two Thr residues are phosphorylated in ProTK, the above finding suggests that about 18% of ProTK molecules are phosphorylated in mitogen-stimulated mouse splenocytes (assuming no phosphorylation before labeling and a 100% yield in the recovery of [32P]-ProTK from labeled cells). Finally, the direct relationship between the activity of the ProTK and the concentration of phospho-ProTK in proliferating cells (present results; and Ref. 27) argues for a significant biological role.

Phosphorylation makes ProTK more similar to other structurally related acidic nuclear proteins which undergo phosphorylation in vivo, such as nucleoplasm (42), nucleolin (39), P1 (37), and HMGs (37). However, the putative effects of phosphorylation on the behavior of ProTK are unknown. Interestingly, the recently demonstrated capacities of ProTK to interact with histones and to enable nucleosome assembly activity in vitro (24) do not appear to be affected by phosphorylation. Although this does not rule out the possibility that phosphorylation affects in vivo interactions between ProTK and histones or other molecules in the cell, it seems probable that phosphorylation is involved in some other aspect of ProTK function, such as degradation to yield thymosins α (10) or nuclear import. In this connection, it should be noted that the rate of nuclear import of some proteins is regulated by phosphorylation in putative CK-2 sites (43). In this sense, ProTK could be included in the group of proteins (nucleoplasm, lamins, etc.) whose nuclear activity is regulated by phosphorylation of CK-2 consensus site motifs (44). Clearly, both structural and biochemical characterization of the 180-kDa ProTK.
together with elucidation of the effects of phosphorylation on the behavior of ProTα may help to shed light on the function of this protein.

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J. Biol. Chem. 1997, 272:10506-10513.
doi: 10.1074/jbc.272.16.10506

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