Turnover of the Acyl Phosphates of Human and Murine Prothymosin \textit{\textit{\textbf{a}} in Vivo}\textsuperscript{*}

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Prothymosin \textit{\textit{\textbf{a}}} is a small, highly acidic, abundant, nuclear, mammalian protein which is essential for cell growth. Our laboratory has recently shown that primate prothymosin \textit{\textit{\textbf{a}}} contains stoichiometric amounts of phosphate on the glutamyl groups of the protein and that \textit{\textit{\textbf{in vitro}}} the phosphate undergoes rapid hydrolysis or transfer to a nearby serine residue. Here an assay for the presence of acyl phosphates \textit{\textit{\textbf{in vivo}}} has been developed by measuring stable phosphoserine and phosphothreonine \textit{\textit{\textbf{in vitro}}}.

The assay was used to determine the half-life of the acyl phosphates on prothymosin \textit{\textit{\textbf{a}} \textit{\textbf{in vivo}}} by pulse-labeling Hela cells with \textit{\textit{\textbf{[32P]P}}}orthophosphate and chasing using three different techniques: permeabilization with digitonin to allow extracellular ATP to equilibrate with the intracellular pool; electroporation in the presence of ATP to reduce the specific activity of \textit{\textit{\textbf{[32P]ATP}}} by expansion of the pool; and incubation with inorganic phosphate. Regardless of the method, the phosphate turned over with a half-life of 75–90 min.

The ability of cells to phosphorylate old prothymosin \textit{\textit{\textbf{a}}} molecules was established by demonstrating equivalent labeling of the protein with \textit{\textit{\textbf{[32P]P}}}orthophosphate in the presence and absence of cycloheximide. The half-life of the acyl phosphates was also studied in resting and growing NIH3T3 cells, with measured values of 30–35 and 70 min, respectively. Our data suggest that the “activity” of prothymosin \textit{\textit{\textbf{a}}} involves the turnover of its acyl phosphates and that it participates in a function common to all nucleated mammalian cells regardless of whether they are quiescent or undergoing rapid proliferation.

This is the first measurement of the stability of protein-bound acyl phosphates \textit{\textit{\textbf{in vivo}}}.

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protein (12) or to unspecified threonine residues located within the first 14 amino acids of the murine protein (28). The degree of accumulation of the stable component depends on the conditions experienced by prothymosin α at the moment of cell lysis. Here we demonstrate that the fortuitous, albeit inefficient, migration of the labile glutamyl phosphate to positions of stability on serine or threonine on the same molecule can form the basis of a quantitative assay. When this technique was used together with three independent pulse-chase methods, we found that both new and old prothymosin α molecules were indistinguishable targets for phosphorylation on glutamic acid and that all acyl phosphates were rapidly lost in vivo with half-lives slightly in excess of 1 h. We believe that turnover of prothymosin α’s phosphates might reflect a role that is continuously required by all cells, regardless of their metabolic state, and we present evidence for associating prothymosin α with processes required both for the maintenance of cells and for their growth.

**EXPERIMENTAL PROCEDURES**

**Cells, Culture Conditions, and Harvest Procedures**—HeLa S3 cells, African green monkey kidney cells (COS-1), and NIH3T3 cells were grown in modified Eagle’s medium (29) from Life Technologies, Inc. (catalog number 15240-030) or Biofluids (catalog number 172; Rockville, MD). Human and monkey cells were cultured with 10% heat-inactivated fetal calf serum (Life Technologies, Inc. or HyClone (Logan, UT)), whereas the murine cells grew in 10% calf serum. All cell cultures contained 2 mM glutamine, 90 units/ml of penicillin, 90 μg/ml of streptomycin, and 0.22 μg/ml amphotericin B, and all cells were maintained in an environment of 5% CO₂ at 37 °C. Quiescent NIH3T3 cells were obtained by incubating cells for 48 h in 0.25% calf serum. Cells were harvested from culture flasks by washing with Puck’s saline and treating with 0.05% trypsin in Hanks’ balanced salts containing 0.5% EDTA.

**Development of an Assay for Glutamyl Phosphate on Prothymosin α**—A two-stage approach was employed for relating 32P found in isolated prothymosin α on serine or threonine to the amount of glutamyl phosphate existing on the protein inside the cell. First, the relationship with samples analyzed on the same gel and subjected to the same massie Blue-stained band of protein and as a radioactive band on an autoradiograph was determined (20, 32, 33). The reaction is specific, but even in well defined solutions, amounts which are large relative to the amount of endogenous UTP in the reaction mix, to equilibrate with the interior in less than 1 h.

**In vivo**

**Determination of the Specific Activity of the ATP Pool**—The method of Lee et al. (33), based on the work of Sasvári-Székely et al. (34), was used to determine the specific activity of the ATP pool of HeLa cells. Briefly, the technique measures the amount of ATP in the reaction mix. More specifically, an aliquot of 1% of a neutralized perchloric acid extract from 5 × 10⁶ cells labeled for 2 h with 50 μCi/ml [3H]inulin (NEN Life Science Products; specific activity, 1000 mCi/mmol) was treated with 20–40 μg of poly(dA-dT) as template. The ATP, which is synthesized in vivo by the cells in the presence of [3H]Inulin, is supplied by the cell extract, whereas UTP of known specific activity is provided exogenously in amounts which are large relative to the amount of endogenous UTP in the same cell extract. Under these conditions, all of the ATP in the extract is used to generate polymer, with no free ATP remaining in the reaction mix. More specifically, an aliquot of 1% of a neutralized perchloric acid extract from 5 × 10⁶ cells labeled for 2 h with 50 μCi/ml [3H]Inulin (NEN Life Science Products; specific activity, 1000 mCi/mmol) was treated with 20–40 μg of poly(dA-dT) as template. The amount of radioactive UTP, and 3 units of RNA polymerase holoenzyme from Escherichia coli, the bacterial polymerase initiates synthesis of RNA at any location without the need for specific promoter sequences. Synthesis terminates when the limiting nucleotide, in this case ATP supplied by the extract, is exhausted. The specific activity of RNA is determined by the expression, (dpm in 6HdUMP in 4°C) × specific activity of UTP, was 6.3 × 10⁵ cpm/nmol. Thus, 10⁶ cells with an average cell volume of 1.6 μl contained 1.4 nmol of UTP at a concentration of 0.88 nmol.

**Pulse-Chase Experiments in Permeabilized Cells**—Conditions for the use of digitonin were selected after the evaluation of several parameters. Each dish of 5–6 × 10⁶ HeLa S3 cells in a variation of a solution termed “cytomix” (35) (120 mM KCl, 150 mM CaCl₂, 10 mM potassium phosphate at pH 7.6, 25 mM HEPES-KOH at pH 7.6, 2 mM EGTA, and 5 mM MgCl₂) or complete DMEM was treated with 20–40 μg/ml digitonin (Sigma) at either 0 or 37 °C for periods ranging from 5 to 30 min. In some experiments, the cells were labeled with [3H]glutamic acid or [3H]glutamate. After digitonin treatment, the permeabilized plasma membrane (see below). The bacterial polymerase initiates synthesis of RNA at any location without the need for specific promoter sequences. Synthesis terminates when the limiting nucleotide, in this case ATP supplied by the extract, is exhausted. The specific activity of RNA is determined by the expression, (dpm in 6HdUMP in 4°C) × specific activity of UTP, was 6.3 × 10⁵ cpm/nmol. Thus, 10⁶ cells with an average cell volume of 1.6 μl contained 1.4 nmol of UTP at a concentration of 0.88 nmol.

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Puck’s saline followed by cytoxin at 37 °C, permeabilized using the optimized conditions noted, and chased in 5 ml of DMEM (without serum and additives) containing 10 mM ATP and 10 mM MgCl2 at pH 7.5. Our methods were developed from published procedures (36). Permeabilized cells were maintained at 37 °C in the incubator during the chase. The cell suspension was then transferred to an Eppendorf tube, cooled in ice, mixed vigorously with a vortex mixer, and centrifuged at 4 °C for 10 min at 15,000 rpm in a Tomy model MTX-150 centrifuge. The supernatant fluid was transferred to a 15-ml polypropylene tube, brought to 4 ml with cold filter-sterilized water, and extracted with phenol as described below. It is important to note that prothymosin α in its entirety leaks out of the nuclei when digitonin-treated cells, as well as electroporated or normal cells, are lysed with nonionic detergents (2) and that prothymosin α yields in all cases are virtually identical.

Pulse-Chase Experiments with Electroporated Cells—HeLa S3 Cells were electrophorized in the presence of [32P]ATP in the medium to determine the degree to which molecules outside the cell had equilibrated with the internal environment. An aliquot consisting of 1 ml of cells and 1 ml of disposable electroporation chamber with a 0.4-cm gap (catalog number 11601-028, Life Technologies, Inc.); the chamber was cooled in ice for 10 min and shocked with an electrical discharge of 875 V/cm and 330 microfarads in a Life Technologies, Inc. Cell-Porator at room temperature with the low resistance setting. The amount of a known concentration of [32P]ATP taken up by the cells, 70–80% of which survived, was measured and found to represent 70% equilibration.

For pulse-chase experiments, HeLa S3 cells were labeled in 175 cm² flasks under the conditions noted above, trypsinized, washed in Puck’s saline, and resuspended at 5 × 10⁶ cells/ml in warm cytoxin to which 20 mM ATP and 20 mM MgCl₂ at pH 7.0 had been added. The chase was initiated by electroporating as noted above. These conditions resulted in a 14–15-fold instantaneous decrease in the specific activity of intracellular ATP. Following electroporation, the chamber was again cooled to 4 °C for 10 min and brought to room temperature for 10 min. Cells recovered from each chamber were washed thoroughly with Puck’s saline, centrifuged free of the wash solution, resuspended in 5 ml of complete DMEM (DMEM containing serum and additives), seeded into a 60-mm dish, and maintained in an incubator as described earlier. The unphysiological concentration of ATP achieved inside the cells after electroporation did not significantly affect the viability of the cells for the duration of the chase.

For the harvest, the cells in each dish were scraped free with a cell lifter, transferred to a centrifuge tube, cooled to 4 °C, washed with PBS, recovered as a pellet, and disrupted in 1 ml of cold lysis buffer. After removing the nuclei by centrifugation, the supernatant fluids were transferred to disposable electroporation chambers with a 0.4-cm gap above noted for digitonin-treated cells, and subjected to a phenol extraction (see below).

Pulse-Chase Experiments with Sodium Phosphate—HeLa S3 cells were labeled in flasks in phosphate-free complete DMEM for 4 h as indicated for the electroporated cells. To initiate the chase, the cells were washed free of the labeling solution, trypsinized, washed again, resuspended in complete DMEM containing 40 mM sodium phosphate at pH 7.0, and seeded into dishes at a concentration of 6 × 10⁶ cells/dish. At the end of the chase, the cells were recovered and lysed using the methods for electroporated cells.

Purification and Analysis of Prothymosin α—Supernatant fluids from lysed cells containing virtually all of the prothymosin α in the cell in 4 ml were made 0.5% in SDS and extracted at 65 °C with 2 ml of phenol saturated with 2 × ACHE buffer (20 mM sodium acetate at pH 5.1, 100 mM NaCl, and 6 mM disodium EDTA). The aqueous phase was recovered by centrifugation and extracted twice with phenol using the same methods. The final aqueous phase was precipitated with 4 volumes of acetone in dry ice for 1 h or overnight at −20 °C, and the sample was washed free of the labeling solution, trypsinized, washed again, resuspended in complete DMEM containing 40 mM sodium phosphate at pH 7.0, and dissolved into 20 μl of acetone. The sample was recovered by precipitation in acetone, and the pellet was dissolved in water and analyzed in an 18% polyacrylamide gel (catalog number EC6506, Novex). A general description of our methods for purification of the protein and for electrophoresis has appeared (2, 12). Prothymosin α was visualized by staining with Coomassie Brilliant Blue, and, when labeled with [32P] or [35S], radioactivity was detected by exposing the dried gel to XAR-x-ray film. Stained protein and radioactive protein were quantified by scanning wet gels and films, respectively, with an ImageQuant scanning densitometer (Molecular Dynamics). To ensure that half-lives were determined, were fit using linear least squares analysis.
The required enzymes may be absent from COS cells. The acetylserine is not readily achieved by cellular kinases and that these data strongly suggest that direct phosphorylation on cellular proteins occurred (data not shown). As shown in Fig. 1, when the lysates were fractionated by nickel chromatography to recover the specific peptides, no co-extraction of the control peptide evaluated at pH 7 in the absence of Mg²⁺, more, the peptides did not become radioactive when added to the buffer in which cells labeled [³²P]orthophosphate were lysed (Fig. 1, top, [³²P]Labeled Lysates, and bottom). These data strongly suggest that direct phosphorylation on acetylserine is not readily achieved by cellular kinases and that the required enzymes may be absent from COS cells. The failure to phosphorylate serine or threonine residues directly on either prothymosin α or related peptides under a variety of conditions or to transfer phosphate intermolecularly from [³²P]-labeled molecules of all types to prothymosin α during cell disruption (20) seriously weakened the case for the biosynthesis of phosphoserine or phosphothreonine. Having already demonstrated the intramolecular transfer of prothymosin α’s labile phosphates to stable positions and having accounted for 90% of the stable phosphate in this manner (20), we concluded that virtually all of the stable phosphate arises from glutamyl phosphate.

Use of Stable Serine Phosphate as an Assay for Unstable Glutamyl Phosphate—Quantitative correlation between glutamyl phosphate and serine (or threonine) phosphate on prothymosin α was established in two steps. In the first step, determinations were made of the amount of stable [³²P]prothymosin α recovered from cells labeled in vivo and the amount of prothymosin α protein recovered from COS cells that were transfected with increasing amounts of the gene. The data, displayed in Fig. 2A, show that, in each experiment consisting of eight measurements with the protein resolved in the same gel, the relative amounts of stained prothymosin α and of stable radioactive phosphate were proportional. (See open squares, triangles, or circles.) To consider three independent experiments at once, both the total intensity from the stained gel and the total intensity on the autoradiogram were obtained, averaged, and set equal to 100 arbitrary units for each data set. Data points are percentages of that value for their respective experiments. In this way, the axes in Fig. 2A are arbitrary, but the fit of the points to a line is not arbitrary. The data show that the specific activity of prothymosin α, measured as stable [³²P]prothymosin α divided by total prothymosin α in each experiment, is constant regardless of how much prothymosin α the cell contained.

In the second step, both the amount of tritium incorporated into prothymosin α at sites of acyl phosphorylation as a consequence of reduction with [³H]NaBH₄ and the total amount of prothymosin α were directly determined. Fig. 2B compares the amount of nonexchangeable tritium found in proline after amino acid analysis and the absolute amount of prothymosin α from which it came using two reduction protocols. The data, which reflect 20 completely independent determinations, show that the amount of tritium, a measure of glutamyl phosphate, and the amount of prothymosin α were directly proportional. Since stable [³²P]prothymosin α is proportional to prothymosin α and since labile phosphate, measured as tritium incorporated into proline upon reductive cleavage, is also proportional to prothymosin α protein, stable serine phosphate must be proportional to labile glutamyl phosphate. This relationship provides the basis for quantifying the relative amounts of [³²P]phosphogluate in vivo.

Half-life of [³²P]Prothymosin α in Permeabilized HeLa Cells—Digitonin, which removes cholesterol from plasma membranes, leaving the cytoplasm of treated cells in direct communication with the external medium (39), was the agent chosen for permeabilization. Cells were pulse-labeled, permeabilized, and chased in the presence of 10 mM ATP, a large concentration relative to the measured intracellular concentration of 0.9 mM ATP (see “Experimental Procedures”). Under these conditions, the specific activity of [³²P]ATP decreased rapidly, and a pronounced decline in the radioactivity incorporated into prothymosin α was observed. As shown in Fig. 3A, which displays an autoradiogram of electrophoretically purified prothymosin α, the chase appeared to be effective within 0.5 h, leaving less than 10% of the initial radioactivity associated with the protein after 8 h. These data, corrected for the recovery of prothymosin
a in each sample, are shown in Fig. 3. Because the decay followed first order kinetics, we were able to determine a half-life of 80 min for phosphoprothymosin α from the curve.

During the interval (0–8 h), [32P]inorganic phosphate continued to accumulate in cytoplasmic mRNA, the most prevalent polynucleotide in our preparations (data not shown). The observation suggests that these permeabilized cells, which are no longer viable, retain the ability to synthesize RNA and export it from the nucleus.

Half-life of [32P]Prothymosin α in Electroporated HeLa Cells—To measure the half-life of the phosphate covalently attached to prothymosin α in living cells, we made use of electroporation as a means of diluting intracellular labeled ATP with an excess of its nonradioactive counterpart. When HeLa cells were pulse-labeled with [32P]orthophosphoric acid and chased by electroporation in cytomix containing 20 mM ATP, the half-life of phosphoprothymosin α was 90 min (Fig. 4), a value in good agreement with the half-life measured in digitonin-treated cells. However, unlike the experiment with digitonin, in which vanishingly small quantities of label remained in prothymosin α after 18–24 h, the cells chased by electroporation always retained 10–20% of the radioactivity incorporated during the pulse (data not shown). This observation is consistent with an experimental design in which the

![Graph showing Radioactive Phosphate vs Prothymosin α (Arbitrary Units)](#)

![Graph showing % [32P] Prothymosin α Remaining vs Hours)](#)
specific activity of ATP is reduced by an instantaneous 14–15-fold increase in the intracellular concentration of ATP rather than by continuous exchange of labeled and unlabeled precursors across the plasma membrane throughout the chase. The residual label might also reflect the existence of a subpopulation that failed to be perforated by electroporation or dead cells that ceased normal phosphate turnover on prothymosin α. Although all of the label was not fully eliminated during the chase, the results are in accord with stable prothymosin α molecules undergoing phosphorylation and rapid dephosphorylation.

**Half-life of [32P]Prothymosin α and RNA in HeLa Cells Chased with Sodium Phosphate**—An experimental design in which cells are labeled with inorganic phosphate and chased with ATP requires the use of permeabilized cells. Neither method, however, is ideal; digitonin kills the cells, and intracellular ATP concentrations, which are >10-fold higher than normal, may affect the very parameters one seeks to measure. To minimize both problems, we again labeled cells with orthophosphate and chased with a high concentration of sodium phosphate (Fig. 5, A and B). The technique makes use of living cells and avoids puncturing the cell membrane. Instead, a chase is initiated with a molecule (inorganic phosphate) that may not be an immediate precursor of phosphoprothymosin α. With the revised conditions, the half-life of phosphate on prothymosin α was 75 min, and greater than 90% of the label turned over (Fig. 5B). Even after 24 h of chase, however, ~5% of the radioactivity initially incorporated into prothymosin α was retained (Fig. 5A).

The pulse-chase kinetics of RNA were measured in the same cytosolic samples. From the point of view of RNA, the chase with sodium phosphate was not effective, since equivalent amounts of labeled mRNA were observed immediately following the pulse and for 18 h thereafter (data not shown). By 24 h of chase, a diminution in radioactivity in RNA was observed but, on the average, about 60% of the label incorporated into RNA during the 4-h pulse remained. These values serve to illustrate the difficulties usually encountered in chasing large nucleoside triphosphate pools with inorganic phosphate.

**Effect of Inhibition of Translation on the Phosphorylation of Prothymosin α**—Our experiments indicate that the half-life of the phosphate on prothymosin α (<1.5 h) is very much shorter than the half-life of the protein moiety, itself (~24 h) (12). However, the experimental design does not allow one to distinguish transient phosphorylation of newly synthesized molecules from rapid reversible phosphorylation of preexisting protein. Toward this end, HeLa cells were either treated with cycloheximide to prevent the accumulation of newly synthesized molecules or incubated in the absence of the inhibitor as a control and pulse-labeled with [32P]inorganic phosphate. Phosphorylation of prothymosin α was not affected by cycloheximide (Fig. 6); the amount of [32P]prothymosin α and total prothymosin α remained unchanged regardless of whether new synthesis of prothymosin α was curtailed. From these data, it can be inferred that both new and old prothymosin α molecules can be phosphorylated. Furthermore, there is no evidence to suggest that newly synthesized prothymosin α molecules undergo preferential phosphorylation. The data are also consistent with the known stability of prothymosin α, because the total amount of prothymosin α (stained protein) was essentially unaffected by a short hiatus in protein synthesis (data not shown).

**Turnover of Prothymosin α’s Phosphates in Resting and Growing NIH3T3 Cells**—The stability of the phosphates was determined in NIH3T3 cells. Cells, either quiescent or growing, were pulse-labeled and chased in the presence of phosphate. As shown in Fig. 7, the values differed. The stability of prothymosin α’s glutamyl phosphate in growing murine cells was very similar to that measured in growing HeLa cells; a value of 70 min in NIH3T3 cells is not significantly different from 75 min measured in HeLa cells using the same technique. In resting NIH3T3 cells, however, there was a marked change. The half-life was 30–35 min, approximately half the value found in...
Discussion

In this paper, we have examined the kinetics of the loss of phosphate from prothymosin \(\alpha\) under a variety of conditions. Our assay depends on a proportionality between the amount of glutamyl phosphate on prothymosin \(\alpha\) and the amount of stable phosphate retained by the purified protein. We have shown that the amount of stable radioactive phosphate covalently attached to homogeneous prothymosin \(\alpha\) is proportional to the amount of prothymosin \(\alpha\) protein, despite manipulation of the amount of prothymosin \(\alpha\) in the cell by transfection of the gene. We have also shown that the amount of acyl phosphate in prothymosin \(\alpha\), measured as \([3H]\)proline after the reaction with \([3H]\)borohydride, is proportional to the amount of prothymosin \(\alpha\) protein. It follows that prothymosin \(\alpha\)'s abundant phosphoglutamated in vivo is proportional to its stable phosphate in vitro; a fixed fraction of the phosphate on glutamate residues in vivo serendipitously transfers preferentially to serine and to threonine at the time of cell lysis, while the remainder is subject to hydrolysis. There is no evidence to suggest that phosphorylation directly on serine or threonine occurs in vivo (Fig. 1 and Ref. 20).

The assay does have limitations. Many acyl phosphates must compete for few positions (perhaps only one position) near the amino terminus of each protein molecule. It is possible that our determinations distinguish only phosphorylated from unphosphorylated molecules, without consideration for the number of phosphates possessed by each protein molecule. Alternatively, it is possible that the probability of finding a stable phosphate depends on the total number of glutamyl phosphates available on each molecule of prothymosin \(\alpha\). According to the latter view, a molecule with few glutamyl phosphates would be unlikely to retain one as a phosphate ester, whereas a richly endowed molecule would have a heightened probability. In neither case is the assay ideal, because the fate of a specific glutamyl phosphate cannot be determined. Nevertheless, like the standard assay for ribonuclease, which responds to loss of trichloroacetic acid precipitable RNA rather than marking each catalytic event, an insensitive assay of either type noted above can still be used to obtain useful kinetic information.

The stability of the glutamyl phosphates initially present on prothymosin \(\alpha\) was measured in vivo using cells that were pulse-labeled, and chased using three independent techniques: cells were electroporated in the presence of a high concentration of ATP with the goal of diluting the intracellular \([32P]\)ATP pool; cells were permeabilized with digitonin to allow extracellular ATP in the bathing fluids to equilibrate with the intracellular pool; and cells were incubated in the presence of inorganic phosphate. In all cases, the half-life of the glutamyl phosphate on prothymosin \(\alpha\) was ~80 min, considerably shorter than the 24-h half-life of the protein. In these experiments, the rate of disappearance of radioactive phosphate on prothymosin \(\alpha\) was determined by noting the change in the specific activity \(([32P]/\text{total prothymosin }\alpha)\) of the isolated protein. This approach is valid because the decay of prothymosin \(\alpha\) itself is negligible, and new synthesis of the protein does not make a significant contribution to the denominator during the time frame required for the chase (12). It should also be noted that stable phosphate is apparently not subject to phosphatases in the lysates; the addition of phosphatase inhibitors had no effect on the recovery of prothymosin \(\alpha\)’s phosphoserine/phosphothreonine (20).

A measured half-life depends not only on the inherent stability of the labeled molecule in question but also on the rate of loss of label from the precursor pools. When \([32P]\)prothymosin \(\alpha\) was chased using two of the conditions noted above (digitonin and electroporation), instantaneous dilution of the label at the commencement of the chase was achieved by violating the cells. However, with inorganic phosphate as the chase vehicle in physiologically maintained cells, the chase also became effective virtually immediately, and the half-life obtained was equivalent. Stated in different words, the values obtained at very short times of phosphate chase, including the zero time point, fell on the same line generated by the data obtained after several hours of chase. There was no indication that the data required correcting for the kinetics of isotope dilution in the labeling pool. Such corrections, when necessary, make calculated half-lives shorter than measured ones by mathematically eliminating a subpopulation that continues to become labeled during an inadequate chase.

The effectiveness of the phosphate chase indicates that the source of prothymosin \(\alpha\)'s label is a small, rapidly equilibrating pool. This pool, however heretical the idea, does not seem to be the 0.9 mM ATP pool unless a small, isolated compartment of ATP, which readily equilibrates with the extracellular milieu, can be identified in the nucleus. Although ATP is the most common source of phosphate for protein kinases, and casein kinase II has been reported to phosphorylate prothymosin \(\alpha\) in vitro with ATP or GTP (28), it is not at all clear how prothy-
mosin α is phosphorylated in vivo. We have not identified the immediate precursor pool for acyl phosphorylation and, obviously, have not yet determined the mechanism in vivo by which the phosphate is removed; nor do we understand how the energy stored in glutamyl phosphate bonds is utilized. However, it should be evident that phosphorylation in vitro directly on serine and threonine in defined solutions will not be an aid to understanding acyl phosphorylation in vivo.

The kinetics of the acquisition and loss of 32P in prothymosin α provide additional insight. We have previously shown that, in synchronized cells, prothymosin α acquires equivalent amounts of label during each interval, suggesting that prothymosin α molecules do not become phosphorylated at specific stages in the cell cycle (12). We show here that when unsynchronized cells are pulse-labeled and chased, virtually all of the label disappeared with the same kinetics, suggesting a single class of molecules. Furthermore, the ability to label prothymosin α was not affected by the presence or absence of cycloheximide, data that imply that both newly synthesized and aged prothymosin α molecules form a single pool, members of which bear phosphate at any instant in time. Taken together, these observations argue for the equivalence of all prothymosin α molecules. Our findings are not consistent with a model in which prothymosin α discharges its phosphate as cells pass a strategic cell cycle check point. Such a model predicts that radioactivity acquired by pulse-labeled, unsynchronized cells would persist in significant amounts until one complete cell cycle had been traversed, a projection at odds with a half-life of ~80 min. As a consequence, we believe it is necessary to abandon the idea that prothymosin α plays a role at a specific stage of the cell cycle.

Prothymosin α is required for cell growth and is found in increasing amounts in highly proliferative cells and tissues (12–16). Here we show that the glutamyl phosphate borne by the protein is unstable and surmise that the continuous turnover of these phosphates is synonymous with prothymosin α’s activity. Since prothymosin α has been closely identified with cell growth, we performed a simple experiment to determine whether noncycling cells, also, make use of the phosphoprotein. We compared the half-life of prothymosin α’s phosphates in quiescent and growing NIH3T3 cells and found the values to be 30–35 and 70 min, respectively. Because the chase in both metabolic states appeared to be effective almost immediately, there is no reason to suspect that the difference is an artifact caused by an alteration in the rate of loss of label from the precursor pools; the disparity in stability seems to be genuine. We do not yet know the significance of the increase in phosphate turnover in quiescent cells (whether it reflects greater or lesser use of a meager amount of prothymosin α), but we recognize that the half-life in both cases is considerably shorter than that expected from the known chemical stability of acyl phosphate-bearing model compounds (40) and considerably longer than the in vitro half-lives of the aspartyl phosphates found in two component systems (23–25). All told, it is difficult to escape the view that prothymosin α functions in quiescent cells, that its activity can be regulated (a subject we examine further), and that the phosphoprotein is required not just for cell growth, but for cell survival as well.

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