In Vivo Thiophosphorylation of Chromosomal Proteins

RECOVERY AND ANALYSIS OF HeLa HISTONES AND DERIVATIVE PHOSPHOPEPTIDES

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HeLa cells are shown to incorporate \[^{35}S\]thiophosphate (added to the medium as sodium thiophosphate) into the \(\gamma\)-positions of ATP and GTP, and into other nucleotides. Under these conditions there is a transfer of radioactive thiophosphoryl groups to histones H1, H2A, H3, and H4. The newly thiophosphorylated chromosomal proteins can be recovered selectively by affinity chromatography on organomercurial-Sepharose columns. The thiophosphorylated histone H1 and its NH2-terminal and COOH-terminal fragments were subjected to tryptic digestion and the sulfur-derivatized phosphopeptides were isolated by Hg-affinity chromatography prior to electrophoretic separation of different sites of modification. Thiophosphate was found to be incorporated into both serine and threonine residues of histone H1 in HeLa cells during logarithmic growth. When \[^{3}H\]threonine and nonradioactive thiophosphate were employed simultaneously as precursors, the thiophosphorylated H1 molecules retained on the mercury column also showed the presence of the \[^{3}H\]threonine label. It follows that newly synthesized H1 molecules are subject to thiophosphorylation in the growing cell cultures.

The postsynthetic modification of nuclear proteins by phosphorylation involves both histones and nonhistone components of chromatin. Substrates for nuclear protein kinases include all of the major histones of the nucleosome "core," histone H1, and its variant forms (1-8), histone H5 (9, 10), the nuclear high mobility group proteins 14 and 17 (11-14), and a multitude of other DNA-binding proteins, including major subunits of the RNA polymerases (15).

The functional significance of phosphorylation of chromosomal proteins is largely unknown but clearly complex. In the case of histones, as well as some nonhistone proteins, phosphorylation influences the DNA-binding properties of the polypeptide chain and might be expected to affect both the structure and function of the DNA template (16, 17). But even with well defined substrates, such as histone H1, the functional significance of phosphorylation cannot be simply deduced by correlations with overall levels of phosphate incorporation, because there are multiple sites of modification in different domains of the polypeptide chain. Certain phosphorylations of histone H1 correlate with gene activation (e.g. by cyclic AMP-dependent mechanisms (18-20)), while other phosphorylations are related to cell cycle progression (21-26).

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and to the mechanisms of chromatin compaction and assembly of higher orders of chromatin structure (27-31).

In analyzing these phenomena, it would be very helpful to have a method for the selective isolation of the recently phosphorylated protein molecules, and for the unequivocal identification of those sites which were most recently modified. One such method for the labeling of nuclear proteins in vitro (32), as well as other substrates (33-36), employs the ATP analogue, adenosine \(5'-O\)-(3-thiotriphosphate) (37) as a thiophosphoryl group donor in the appropriate kinase reaction. We have shown that thiophosphorylated histones can be selectively recovered after labeling with ATP-\(\gamma\)-S and cyclic AMP-dependent protein kinase by affinity chromatography on organomercurial-Sepharose columns (32). However, because nucleoside triphosphates such as ATP-\(\gamma\)-S are not effective substrates for in vivo experiments, due to restricted permeation through the plasma membrane, what is needed is a sulfur-derivatized precursor that can readily enter living cells. We have employed a radioactive analogue of sodium orthophosphate, Na \[^{35}S\]thiophosphate, and tested for its uptake and utilization by intact HeLa cells. The \(^{35}S\)-labeling of specific histones is reported here, together with affinity methods for the isolation of the thiophosphorylated histone molecules, sulfur-derivatized peptides, and thiophosphorylated serine and threonine residues. The entry of \[^{35}S\]thiophosphate into intracellular nucleotide pools with the formation of radiolabeled ATP and GTP analogues is also demonstrated.

EXPERIMENTAL PROCEDURES

\[^{35}S\]Thiophosphate and \(^{32}P\)Orthophosphate Labeling of HeLa Cells—HeLa S-3 cells grown to log phase (3 \(\times\) 10\(^5\) cells/ml) in 100-ml spinner bottles were harvested by centrifugation at 50 \(\times\) g for 5 min and resuspended in 100 ml of phosphate-free medium A (Joklik-modified minimal essential medium without Na,PO\(_4\) (Grand Island Biological Co.). After 45 min at 37 \(^\circ\)C, the cells were collected by centrifugation and resuspended in 50 ml of medium A. Ten mCi of Na \[^{35}S\]thiophosphate of specific activity 989 mCi/mmol, or carrier-free \(^{32}P\)orthophosphate (New England Nuclear) were added and the cells were incubated for 3 h. More than 96% of the cells were viable at that time, as judged by trypan blue dye exclusion.

Labeling of ATP and GTP Pools by \[^{35}S\]Thiophosphate—HeLa S-3 cells (6 \(\times\) 10\(^4\) cells) at a concentration of 6 \(\times\) 10\(^3\) cells/ml were incubated in phosphate-free medium A containing 2 mCi of sodium \[^{35}S\]thiophosphate at a final concentration of 0.35 mCi. After 3 h at 37 \(^\circ\)C, the cells were harvested by centrifugation at 50 \(\times\) g for 5 min, and washed twice with ice-cold 50 mM Na phosphate buffer, pH 7.1. The nucleotide "pools" were extracted in 1 ml of 0.5 M KClO\(_4\), and the extract was washed by centrifugation before application to a column (1 \(\times\) 30 cm) of DEAE-Sephadex A-25 (Pharmacia). The nucleotides were eluted in a linear gradient of 0.1-0.4 M triethylamine.

The abbreviations used are: ATP-\(\gamma\)-S, adenosine \(5'-O\)-(3-thiotriphosphate); AMP-\(\alpha\)-S, adenosine \(5'-O\)-(1-thiononophosphate); ADP-\(\beta\)-S, adenosine \(5'-O\)-(2-thiodiphosphate); SDS, sodium dodecyl sulfate, PEI, polyethyleneimine.
bicarbonate as described by Eckstein (39). Fractions (3 ml) were monitored for ultraviolet absorption and radioactivity and those believed to contain [35S]ATP-γ-S and [35S]GTP-γ-S were pooled and concentrated in a rotary evaporator (to remove the buffer). Each residue was washed 3 times with methanol and further characterized by three different analytical methods: 1) chromatography on polyethyleneimine-impregnated cellulose (Polygram CIL 300 PEI-UV, Boehringer Mannheim). Unlabeled AMP-α-S, ADP-β-S, ATP-γ-S, and GTP-γ-S (50 μg each) were added as markers. The chromatograms were developed in 0.75 M potassium phosphate buffer, pH 3.5, for 3 h (39). The TLC plates were air-dried and the positions of the nucleotide markers were located by ultraviolet absorption. The positions of the [35S]-labeled nucleotides were then located by fluorography, after spraying the TLC plates with Enhance (New England Nuclear) and exposing to Kodak SB-5 preflashed x-ray film for 3-7 days.

2) High Voltage Electrophoresis—The nucleotide thiophosphates were analyzed by electrophoresis in a Eppendorf capillary electrophoresis system. The radioactive thiophosphate standards were pooled with unlabeled thiophosphate and either 5 mCi of [3H]threonine of specific activity 309 mCi/mmol, or 3 mCi of [3H]serine of specific activity 11 Ci/mmol (Amersham Corp.). After 3-h incubation at 37 °C, the histones were prepared as described below.

3) High Pressure Liquid Chromatography—The [35S]-labeled nucleotide fractions separated by DEAE-Sephadex chromatography were analyzed on a column (3.9 mm × 30 cm) of μBondpak NH2 (Waters Associates, Inc.). The nucleotides were eluted in 0.3 M potassium phosphate buffer, pH 3.5, at a flow rate of 3 ml/min. Unlabeled nucleotide thiophosphates were added as markers. Fractions (0.5 ml) were collected for measurement of radioactivity by scintillation spectrometry.

Thiophosphorylation of Newly Synthesized Histones—One hundred ml of HeLa cell suspensions (6 × 10⁶ cells/ml) were centrifuged and the cells were resuspended in synthetic phosphate-free medium B (minimal essential medium containing phosphate-free balanced salts, vitamins, and essential amino acids (Grand Island Biological). After incubation at 37 °C for 45 min, the cells were harvested and resuspended in 100 ml of medium B containing 1.0 mM Na thiophosphate (unlabeled) and either 5 μCi of [3H]threonine of specific activity 309 mCi/mmol, or 3 μCi of [3H]serine of specific activity 11 Ci/mmol (Amersham Corp.). After 3-h incubation at 37 °C, the histones were prepared as described below.

Isolation of Nuclei—All steps were carried out at 4-10 °C. The labeled cells were collected by centrifugation and washed twice in buffer C (8 mM NaH2PO4, 15 mM KH2PO4, 140 mM NaCl, 27 mM KCl). The washed cells were then lyzed by vortexing in buffer D (0.4 M NaCl, 50 mM MgCl2, 4 mM CaCl2, 0.01 M Tris-HCl, pH 7.4) containing 0.1% NP-40 (Particle Data Laboratories, Ltd., Elmhurst, IL). The nuclei were recovered by centrifugation at 1000 × g for 10 min and washed in buffer D without NP-40.

Extraction and Separation of Histones—Total histones were extracted from 107 nuclei in 0.25 N HCl and precipitated in 5 volumes of cold acetone. For preparation of histone H1, the isolated nuclei were extracted successively with 1.0 and 0.5% of 5% HClO4. The extracts were combined and histone H1 was precipitated in 18% (w/v) trichloroacetic acid (40). Further purification of histone H1 was achieved by preparative electrophoresis in 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (41), or by ion exchange chromatography on Bio-Rex 70 (42). Electrophoretically separated histone bands were identified in a thin slice of gel by staining with Coomassie blue dye, and the corresponding segments were excised for electrophoretic separation of the histones, using an ISCO Model 1750 Sample Concentrator.

Organometallic-Sepharose Column Chromatography—Thiophosphorylated histones were recovered by Hg-affinity chromatography (39). The histones were redissolved in N3-saturated 10 mM Tris-HCl buffer, pH 8.6, containing 2 mM EDTA, 10 mM NaCl, and 0.05% sodium dodecyl sulfate, and applied to Affi-Gel 501 columns (Bio-Rad). Adsorbed thiophosphorylated histone molecules were eluted in buffer containing 10 mM dithiothreitol (32). In [35S]-labeled experiments, the radioactive histones were dialyzed extensively against water containing 0.05 mM phenylmethylsulfonyl fluoride before lyophilization.

Preparation of Thiophosphorylated Peptides from Histone H1—Histone H1 (100 μg) which had been [35S]thiophosphorylated in vivo, was labeled with [3H]threonine and [3H]serine (43). The NH2-terminal and COOH-terminal fragments were separated by chromatography on Sephadex G-100, monitoring the elution by UV absorption at 218 nm (43) and by counting aliquots of the 0.6 ml fractions for [35S] activity. The products of the N-nucleosidetriphosphate cleavage of [35S]thiophosphorylated H1 were digested with trypsin according to Hohmann et al. (43) and the peptides in each digest were separated by high voltage electrophoresis on Whatman No. 1 paper, pH 7.9. The resulting thiophosphopeptide bands were located for [35S] activity by scintillation spectrometry of 0.5-ml strips. The presence of [3H] in bands 1 and 2, from the NH2-terminal region of H1 indicates the presence of serine [35S]thiophosphate, while [35S] activity in band IV from the COOH-terminal fragment indicates the presence of [3H]thiophosphothreonine (43).

Separation of Thiophosphorylated Threonine and Serine Residues—Histone H1 containing [35S]-labeled thiophosphate groups (5000 cpm) was subjected to partial acid hydrolysis in 10 μl of 6 N HCl at 98 °C for 60 min. After addition of 1 ml of H₂O, the solution was lyophilized, and the residue was redissolved and lyophilized repeatedly to ensure complete removal of HCl. Thio-phosphothreonine and thiophosphoserine were then separated by high voltage electrophoresis (1200 V, 1 h, pH 1.9) (44). The positions of the amino acids were located by reaction with ninhydrin and compared with those of the standards: phosphoserine and phosphothreonine, and thiophosphothreonine (45) and thiophosphoserine (46). The positions of the amino acids were determined by fluorography and by ultraviolet absorption and radioactivity by scintillation spectrometry.

RESULTS

Entry of [35S]Thiophosphate into Intracellular Nucleotide "Pools"—HeLa S-3 cells in log phase growth were incubated in a phosphate-free medium containing [35S]thiophosphate at a final concentration of 0.5 mM. The nucleotide "pools" were extracted in acid and analyzed by chromatography on DEAE-Sephadex A-25 (39), as shown in Fig. 1. Major peaks of radioactivity were consistently observed upon elution in a triethylamylamine gradient. The first (peak I) co-eluted with an AMP-α-S marker; the second (peak II) co-eluted with ATP-γ-S. The histones were resolved in ATP-γ-S and TATP-γ-S marker, and peak IV contained GTP-γ-S (inset, Fig. 1). Further analysis of these peaks by thin layer chromatography on polyethyleneimine-impregnated cellulose (39) confirmed that the [35S] activity in peak I co-migrated with the ADP-β-S standard (Fig. 2). The [35S] activity in peak III, while largely co-migrating with the ATP-γ-S standard, also showed some cross-contamination by ADP and thio-GTP (Fig. 2). Thiophosphorylated GTP was
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Fig. 1. Analysis of $^{35}$S-labeled nucleotides from HeLa cells incubated with Na $[^{35}$S]thiophosphate. The 0.5 M HClO$_4$ extract of the labeled cells was adjusted to pH 7.3, and aliquots containing 1.5 x 10$^6$ cpm were applied to columns of DEAE-Sephadex A-25 which had been equilibrated in 0.1 M triethylamine bicarbonate buffer, pH 7.3. The nucleotides were eluted in a 0.1 to 0.5 M buffer gradient, noting the positions of authentic nucleotide standards by UV spectrometry, and recording the positions of the $^{35}$S-labeled compounds by scintillation spectrometry. The inset shows a plot of the molarity of the eluting buffer, as determined by conductivity measurements, versus fraction number, and indicates the elution positions of AMP-$\alpha$-S, ADP-$\beta$-S, ATP-$\gamma$-S, and GTP-$\gamma$-S standards. Each of the peaks (I-IV) was recovered for further analysis of its nucleotide content.

Fig. 2. Analysis of $^{35}$S-labeled nucleotides by ascending thin layer chromatography on polyethyleneimine-impregnated cellulose. Fractions isolated by chromatography on DEAE-Sephadex were applied to PEI-thin layer plates with unlabeled nucleoside thiophosphates (50 µg each) added as markers. The positions of the markers were detected by UV absorption (A) while the positions of the $^{35}$S-labeled nucleotides in the various peaks were determined by fluorography (B). Note that peak III contains nucleotides which co-migrate with ATP-$\gamma$-S and GTP-$\gamma$-S, while peak IV contains more of the GTP analogue. The nucleotide spots corresponding to ATP-$\gamma$-S and GTP-$\gamma$-S were recovered from the TLC plate for further analysis.

Fig. 3. Analysis of $^{35}$S-labeled nucleotides by high voltage electrophoresis. The nucleotide spots separated by PEI-thin layer chromatography were separated by electrophoresis on paper, with unlabeled nucleoside thiophosphates added as markers. The distribution of $^{35}$S activity indicates that most of the radioactivity recovered from the ATP-$\gamma$-S spot in thin layer chromatography (Fig. 2) co-migrates with authentic ATP-$\gamma$-S on electrophoresis at pH 3.5.

Fig. 4. Analysis of $^{35}$S-labeled nucleotides by high pressure liquid chromatography. Nucleotide fractions separated by chromatography on DEAE-Sephadex A-25 (Fig. 1) were applied to a µBondapak-NH$_2$ column and eluted with 0.3 M potassium phosphate buffer, pH 3.5. The elution positions of nucleotide standards under these conditions, as monitored by UV spectrometry, are shown in A. B shows the $^{35}$S distribution in nucleotide fraction III, with a prominent peak co-eluting with ATP-$\gamma$-S. (The small peak emerging at 5 ml is inorganic $[^{35}$S]thiophosphate.) C shows the presence of ATP-$\gamma$-$^{35}$S and GTP-$\gamma$-$^{35}$S in peak IV, in agreement with the TLC results in Fig. 2.
peaks III and IV. It is clear that $^{35}$S-labeled nucleotides eluting in the positions of authentic ATP-$\gamma$S and GTP-$\gamma$S can be identified by high pressure liquid chromatography. Thus, by a variety of analytical criteria, $^{35}$Sthiophosphate enters the nucleotide pools of living cells in forms that should allow transfer of radioactive thiophosphoryl groups to proteins in ATP- or GTP-dependent reactions.

Thiophosphorylation of Histones in Cells Exposed to $^{35}$S Thiophosphate—Nuclei were isolated from HeLa cells incubated for 3 h in media containing $^{35}$Sthiophosphate or (32P)orthophosphate, and the histones were extracted for electrophoretic separation and analysis. The distribution of $^{32}$P phosphate in the various histone fractions is shown in Fig. 5A, which demonstrates extensive phosphate uptake into histones H1, H2A, and H3, and a slight $^{32}$P uptake into histone H4. The corresponding distribution of thiophosphorylated histones from cells incubated in the presence of $^{35}$Sthiophosphate is shown in Fig. 5B, which plots the $^{35}$S activity of the basic proteins at different regions of the gel. $^{35}$SThiophosphate incorporation into histones H1, H2A, H3, and H4 is clearly indicated. Note that the electrophoretic separation yields two prominent H1 bands, both of which are thiophosphorylated. However, the predominantly labeled band in $^{35}$S thiophosphate labeling experiments is the faster moving H1 band (Fig. 5B), whereas in $^{32}$Pphosphate labeling experiments, it is the slow moving H1 band that is more radioactive (Fig. 5A). It follows that the relative rates of $^{32}$Pphosphate uptake and turnover in individual nuclear proteins may not be accurately reflected when $^{38}$Sthiophosphate is employed as the precursor. However, the advantage of the thiophosphate label is the ability to recover selectively the recently thiophosphorylated molecules by Hg-affinity chromatography (32).

Affinity Purification of Thiophosphorylated Histone H1 Molecules—For proteins which lack sulfhydryl groups, such as histone H1, the chromatographic separation of newly thiophosphorylated molecules can be achieved directly by affinity chromatography on organomercurial-Sepharose columns (32, 45). Thiophosphorylated histones are effectively retained by such columns but can be readily eluted from them with dithiothreitol (32). This procedure was used to isolate the thiophosphorylated H1 molecules from HeLa cells incubated in the presence of $^{35}$Sthiophosphate. The histone H1 was extracted, purified electrophoretically, and then subjected to Hg-affinity chromatography on Affi-Gel 501. Fig. 6A shows the elution pattern of the $^{35}$S-labeled histone, with retention and subsequent displacement of the thiophosphorylated molecules by dithiothreitol. Fig. 6B shows the results of a different experiment in which $^{[3]}$Hthreonine and nonradioactive thiophosphate were employed simultaneously as precursors. In this case, the thiophosphorylated H1 molecules retained on the Hg column also show the presence of the $^{[3]}$Hthreonine label. A similar double labeling experiment, using $^{[3]}$Hserine and nonradioactive thiophosphate, is shown in Fig. 6C. The presence of the newly incorporated amino acids in histone molecules which are retained by the column establishes that recently synthesized molecules are subject to thiophosphorylation in the growing cell cultures.

Recovery of Thiophosphorylated Histone H1 Peptides—In a log phase HeLa culture, we would expect phosphorylation of histone H1 to take place at multiple growth-associated sites. Langan has identified four of those sites in mammalian cells as threonines at positions 16, 136, and 153, and serine at position 180 of the polypeptide chains (46). An additional site of phosphorylation at serine 37 is modified by a cyclic AMP-dependent protein kinase (47). In mitotic cells, phosphorylations take place on serine and threonine residues in the NH$_2$-terminal region, and on threonine residues in the COOH-terminal region (43). Affinity purifications of thiophosphorylated fragments of histone H1 should simplify the identification of which sites are subject to phosphorylation under different physiological conditions (or in different isolated enzyme systems).

We have tested the feasibility of this approach, as applied to histone H1 thiophosphorylation in log phase HeLa cells. Two methods were employed; in the first, $^{38}$S-labeled peptides were prepared from a limited tryptic digest of the entire H1 molecule; in the second, the H1 was first cleaved by treatment with N-bromosuccinimide, and peptides were prepared from the chromatographically separated NH$_2$-terminal and COOH-terminal fragments by tryptic digestion.

In the first procedure, we compared histone H1 molecules labeled in three separate cell cultures, using $^{35}$Sthiophosphate, $^{32}$Porthophosphate, or $^{[3]}$Hthreonine plus nonradio-
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Fig. 6. Recovery of thiophosphorylated histone H1 molecules by Hg-affinity chromatography on Affi-Gel 501. Histone H1 was purified electrophoretically, dissolved in 1 ml of 10 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA, 0.1% SDS, and 0.5 mM 2-mercaptoethanol, and applied to a column (0.5 × 8 cm) of Affi-Gel 501 which had been equilibrated in the buffer. After washing to remove the unbound histone molecules, 10 mM dithiothreitol was added to elute the thiophosphorylated histone from the column. Radioactivities were determined by scintillation spectrometry. A, affinity purification of 35S-labeled histone H1 after incubating HeLa cells for 3 h in the presence of [3H]threonine and nonradioactive thiophosphate. B, separation of thiophosphorylated H1 molecules containing [3H]threonine after incubating HeLa cells in the presence of [3H]threonine and nonradioactive thiophosphate. C, separation of thiophosphorylated H1 molecules containing [3H]serine after incubating HeLa cells in the presence of [3H]serine and nonradioactive thiophosphate. The radioactive peaks not retained by the Hg-column (B and C) represent newly synthesized H1 molecules which were not thiophosphorylated (although they may have been phosphorylated) under these conditions. The retained [3H]threonine- or [3H]serine-labeled peaks displaced by dithiothreitol contain newly synthesized H1 molecules which were thiophosphorylated during the 3-h labeling period.

active thiophosphate, respectively, as precursors. In each case, histone H1 was purified electrophoretically, and the 35S-labeled and 3H-labeled histones were further fractionated by Hg-affinity chromatography to select the thiophosphorylated forms of the protein molecules. Each histone preparation was subjected to limited trypsin digestion and the resulting peptides were separated by electrophoresis in SDS-polyacrylamide gels. The separation of peptides derived from the affinity purified 35S-labeled histone is shown in Fig. 7A. There are four prominent peaks of 35S activity separated by this procedure. The same peptide peaks are observed in the tryptic digest of the 32P-labeled histone (Fig. 7B), supporting the view that the thiophosphate modification can be used to probe the multiple sites of protein kinase action in vivo.

The separation of thiophosphorylated peptides from newly synthesized H1 molecules is illustrated in Fig. 7C. In these experiments, HeLa cells were incubated in the presence of [3H]threonine and nonradioactive thiophosphate. The histones were extracted from the isolated nuclei and separated by electrophoresis. Histone H1 bands were eluted from the gel and the thiophosphorylated molecules were recovered by Hg-affinity chromatography. They were subjected to limited trypsin digestion and the resulting peptide mixture was rechromatographed on the Hg-affinity column for selective recovery of the thiophosphorylated peptides. Subsequent separation of the 3H-labeled thiophosphopeptides by electrophoresis in SDS-polyacrylamide gels gave the pattern shown in Fig. 7C, with several peaks of [3H]threonine activity corresponding to the 35S-labeled peptides (Fig. 7A) and the 32P-labeled peptides in Fig. 7B.

It is known that histone H1 has multiple phosphorylation sites involving serine residues (4, 6, 7, 46). The incorporation of 32P-phosphate by HeLa cell cultures confirms the serine modification. The thiophosphorylated histone H1 was subjected to limited acid hydrolysis and the free amino acids were separated by high voltage electrophoresis. Both serine and threonine residues were found to be thiophosphorylated. As judged by their respective 3S activities (62% of the total recovered counts in thiophosphothreonine and 38% of the
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total in thiophosphoserine), threonine residues represent the major sites of H1 phosphorylation under these conditions.

Chemical and enzymatic cleavages of thiophosphorylated histone H1 were combined to demonstrate that \[^{35}\text{S}\] thiophosphate is incorporated into different amino acids in different regions of the molecule. The thiophosphorylated H1 molecules were purified by Hg-affinity chromatography and treated with N-bromosuccinimide. The resulting NH\(_2\)-terminal and COOH-terminal fragments were separated chromatographically, and both were found to be labeled with \[^{35}\text{S}\] thiophosphate (Fig. 8A). Each fragment was then digested with trypsin and the peptides separated by high voltage electrophoresis, as described by Hohmann et al. (43). The distribution of \(^{35}\text{S}\) activity in different peptide bands is plotted in Fig. 8E. Particular attention is drawn to the \(^{35}\text{S}\)-thiophosphorylation of band I from the NH\(_2\)-terminal fragment (indicated by the arrow in the top of Fig. 8B). This peptide band has been shown to contain only phosphoserine after \(^{32}\text{P}\)-labeling of Chinese hamster ovary cells (43); its \(^{35}\text{S}\)-activity in these experiments is presumed to correspond to the thiophosphorylation of serine in the NH\(_2\)-terminal region. Similar separations of the \(^{35}\text{S}\)-labeled tryptic peptides from the COOH-terminal fragment of histone H1 show the presence of thiophosphate groups in band IV (indicated by the arrow in the bottom of Fig. 8B) in which threonine is the only site of modification (43). It follows that thiophosphate can be used to study the modification of different amino acids in different regions of the polypeptide chain. The presence of the thiophosphate group also permits re-isolation of the peptides or modified amino acids by Hg-affinity chromatography (Fig. 7C).

![Fig. 8: Separation of \(^{35}\text{S}\)-thiophosphorylated peptides from NH\(_2\)-terminal and COOH-terminal fragments of histone H1.](image)

**DISCUSSION**

To our knowledge, this is the first indication that inorganic thiophosphate can be used to label the nucleotide "pools" within living cells. It is known that ATP-\(\gamma\)-S can be formed in *vitro* in reactions involving glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase (48). Isolated liver mitochondria have been reported to form a little ATP-\(\gamma\)-S from thiophosphate, but this is strongly inhibited by F, (48). Isolated mitochondria incorporate \(^{35}\text{S}\) thiophosphate in an acid-precipitable fraction which would have included proteins but the sites of modification were not identified, and the uptake was found to be insensitive to inhibitors of oxidative phosphorylation such as aurovertin and oligomycin (49).

We have shown that formation of the thiophosphorylated analogues of ATP and GTP, as well as other nucleotides, takes place in intact cells. The nature of the products has been confirmed by DEAE-Sephadex chromatography, PEI-thin layer chromatography, high voltage electrophoresis, and high pressure liquid chromatography. (Compounds identified as ATP-\(\gamma\)-S and ADP-\(\beta\)-S could, of course, have more than one thiophosphoryl group, depending upon the balance between multiple nucleotide kinase reactions in the cell and their preference for phosphorylated, as compared to thiophosphorylated substrates.)

One important consequence of the formation of ATP-\(\gamma\)-S and GTP-\(\gamma\)-S is the subsequent transfer of thiophosphoryl groups to phosphorylatable sites on histones and other phosphoproteins *in vivo*. Although other mechanisms of protein modification by thiophosphate might be proposed, it has been clearly established that histones and other proteins are thiophosphorylated by ATP-\(\gamma\)-S in kinase-mediated reactions (32-36), and we presume that this is the major route of protein thiophosphorylation in the living cell.

Because the thiophosphorylation of proteins permits their purification by affinity chromatography on organomercurial-
Sepharose columns (32), the use of \(^{35}\)S thiophosphate (or nonradioactive thiophosphate) allows the selective recovery of histones and other protein molecules that have just been modified in vivo. This is an important extension of the previous method for the study of kinase-mediated protein phosphorylations in vitro (32). In both cases, the modification permits recovery of a variety of thiophosphorylated proteins.

The present study extends the affinity purification method to the separation of thiophosphorylated peptides and thiophosphoamino acids produced by proteolytic or chemical treatment with iodoacetate before purification of the thiophosphopeptides from similar peptides containing previously incorporated phosphate groups. Since only the sites of most divalent cations, and factors controlling progression through the cell cycle, the feasibility of analyzing the thiophosphorylations of different amino acids in different regions of the histone H1 molecule is indicated by the experiments summarized in Fig. 8. However, it is clear from the differences in rates of labeling of histone variants with \(^{35}\)S thiophosphate and \(^{32}\)P orthophosphate (Fig. 5), that caution must be exercised in the interpretation of labeling differences in different phosphoproteins and in different regions of the same protein.

While this paper has emphasized the thiophosphorylation of histones in cultured HeLa cells using \(^{35}\)S thiophosphate as a precursor, the procedure is not limited to cell cultures nor to components of the nucleus. Other experiments have shown that \(^{35}\)S thiophosphate is utilized for protein phosphorylation in intact animals; e.g., specific protein thiophosphorylations permit the recovery of a subset of membrane proteins from the "smooth" endoplasmic reticulum of rat liver.

Finally, the entry of thiophosphate into the nucleotide "pools" of living cells has other important consequences. Among them is the ability to label newly synthesized RNA and DNA and to recover them selectively, excluding previously synthesized nucleotides by Hz-affinity chromatography. Some applications of this technique will be described elsewhere.

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