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Biochemistry

Phosphorylation of hydroxylysine residues in collagen synthesized by cultured aortic smooth muscle cells

(phosphohydroxylysine/collagen/protein phosphorylation)

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ABSTRACT **O^5-Phosphohydroxylysine was chemically synthesized and techniques were established for its identification by combined use of cation-exchange chromatography, thin-layer electrophoresis at pH 1.9 and 3.5, and thin-layer chromatography. Clean separation of phosphohydroxylysine from the other phospho amino acids, phosphoethanolamine, and phosphocholine was achieved. Conditions were also determined to permit hydrolysis of proteins in 2 M HCl without loss of the phosphono group of phosphohydroxylysine residues. Experiments were then performed showing that ^{32}P was incorporated into the hydroxylysine residues of cell-associated collagens when cultured calf aorta medial smooth muscle cells were incubated with [^{32}P]orthophosphate. In other experiments, the cells incorporated [^{3}H]lysine into hydroxylysine residues of cell-associated collagen and then ^{32}P into phosphohydroxylysine residues. The doubly labeled phosphohydroxylysine subsequently isolated showed nearly 1:1 stoichiometry with respect to incorporation of precursor lysine and phosphorus. Finally, in preliminary experiments done with a cell-free extract of the smooth muscle cells, ^{32}P was transferred from [γ-^{32}P]ATP to hydroxylysine residues in several kinds of collagenous substrates. Thus, this work shows that smooth muscle cells have the capacity to phosphorylate hydroxylysine residues in their cell-associated collagens and provides preliminary evidence that a protein kinase is involved.**

With the discovery of proteins containing phosphotyrosine residues (1, 2), all of the coded hydroxy amino acids inserted into polypeptide chains have been shown to undergo post-translational phosphorylation by action of protein kinases and ATP. Two other hydroxy amino acids are in themselves formed by post-translational hydroxylation reactions; these are hydroxyproline in collagens and elastins, formed from proline residues, and 5-hydroxylysine in collagens, formed from lysine residues. We have begun studies of the phosphorylation of hydroxylysine and hydroxyproline residues in proteins.

In its free form, O^5-phosphohydroxylysine was identified tentatively in the extracellular fluids of bovine embryos in the early screening applications of paper chromatography (3). More recently, a system has been found in liver for phosphorylation of free hydroxylysine by a hydroxylysine kinase that utilizes GTP as the phosphono donor (4, 5). That reaction is the first step in the complete degradative metabolism of hydroxylysine liberated by digestion of collagenous proteins as, for instance, shown by our laboratory, by the combined actions of trypsin and carboxypeptidase B (6).

For many years, we have been concerned with possible functions for hydroxylysine residues. It is known that such residues, like those of lysine, can undergo oxidation at C-6 to form corresponding aldehyde groups, which can participate in crosslink formation (7). The hydroxyl groups of hydroxylysine residues can undergo glycosylation by action of a transferase and UDP-galactose, and the substituted galactose residue can be glycosylated further by action of a second transferase and UDP-glucose (8). In some collagens or collagenous proteins (basement membranes, C1q of complement) most of the hydroxylysine residues are glycosylated (9, 10). Of the 14 hydroxylysine residues in C1q, 11 are glycosylated. In some collagens, such as type I, the percentage of hydroxylysine residues glycosylated is much less (11).

A question that persists is what is the function of hydroxylysine residues that do not become glycosylated? Indeed, they appear too few in number to have major significance in hydrogen bonding of the structure as do the hydroxypyroline residues that occur in much greater number. The possibility that hydroxylysine residues in protein undergo phosphorylation like other hydroxy amino acid residues requires exploration. Accordingly, in this report we present the logical first steps in such studies, addressing several points: First, the chemical synthesis of phosphohydroxylysine as a standard and the establishment of means of its identification in biological systems and second, the capacity of cultured cells making collagens to incorporate inorganic [^{32}P]phosphate into hydroxylysine residues of those proteins. In addition, in a preliminary way, we report the capacity of a cell-free system to incorporate ^{32}P from [γ-^{32}P]ATP into hydroxylysine residues of collagens, thus implicating a hydroxylysine protein kinase. Pursuit of the last two points is facilitated considerably by the fact that hydroxylysine residues occur only in collagens and the ability to follow incorporation of ^{32}P into an already uniquely labeled hydroxylysine residue formed from a lysine residue previously labeled with tritium.

MATERIALS AND METHODS

Reagents. 5-Hydroxylysine, lysine, *O*-phosphoserine, phosphocholine, *O*-phosphothreonine, *O*-phosphotyrosine, *O*-phosphoethanolamine, N^G-phosphoarginine, and inositol 2-monophosphate were purchased from Sigma. [^{32}P]Orthophosphate (carrier-free), L-[4,5-^{3}H]lysine (97 Ci/mmol, 3.59 TBq/mmol), and [γ-^{32}P]ATP (19 Ci/mmol, 703 GBq/mmol) were obtained from Amersham. Type I collagen was obtained from Sigma, as was calf skin gelatin. All other reagents, including amino acids, were of high purity.

Basal minimum essential medium for tissue culture was obtained from Flow Laboratories; fetal calf serum and L-ascorbic acid were obtained from GIBCO.

Chemical Synthesis of Phosphohydroxylysine. Phosphohydroxylysine was synthesized by a method patterned after that for phosphotyrosine as described by Eckhart *et al.* (12). Phosphorus oxychloride (10 mmol) was added to 5 mmol of hydroxylysine in 25 mmol of trimethylphosphate. The mixture was stirred overnight at room temperature. The resulting yellow syrup was dissolved in water to a volume of 200 ml. The strongly acidic solution was applied directly to a column of Dowex 50X8 (20 × 50 mm, hydrogen form), and the resin was washed with water. Elution was effected with 1 M

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ammonium hydroxide. The effluent (about 200 ml) was dried by flash evaporation and dissolved in water. The solution was adjusted to pH 7.0 and applied to a column of Dowex 1X8 (20 × 50 mm, acetate form); the column was then washed with water. Elution was effected with 1 M acetic acid. The eluate was dried by flash evaporation and then dissolved in water. The phosphohydroxylysine in this solution was about 90% pure as judged by automatic amino acid analysis. Final purification was accomplished by collection of the peak material from the column of the amino acid analyzer. Determination of the phosphate and hydroxylysine contents after hydrolysis showed it to be nearly 100% pure. Mass spectrometric analysis of suitable derivatives, as will be reported elsewhere, also established the structure to be phosphohydroxylysine.

Cell Cultures. Calf aorta medial smooth muscle cells were used; starter cultures were obtained from A. Adamany and O. O. Blumenfeld of the Department of Biochemistry of this institution. These cells are diploid cells of finite life span in culture and are capable of considerable synthesis of collagens in media supplemented with L-ascorbate (13). Cultures were obtained at a population doubling level of 18 and grown in Corning culture flasks of 150 cm^2. The culture medium was basal minimal essential medium supplemented with 10% fetal calf serum and L-ascorbic acid (50 μg/ml). For further incubations, as described in *Results*, confluent monolayers from a number of flasks (2.0×10^7 cells per flask) were washed with Krebs–Ringer bicarbonate buffer without phosphate but with 20 mM Hepes, pH 7.4.

Acid Hydrolysis of Proteins. Preliminary experiments with authentic phosphohydroxylysine showed that the compound was stable when heated at 95°C for 4 hr in presence of 2 M HCl. Accordingly, proteins to be analyzed for phosphohydroxylysine were hydrolyzed under those conditions. After hydrolysis, the sample was dried by flash evaporation, dissolved in 0.5 ml of water, and applied to a column of Sephadex G-50 (8 × 200 mm). Elution was with water, and the effluent of amino acids was collected and dried by flash evaporation.

Amino Acid Analysis. Amino acid analysis of hydrolysates was performed by using the 2.5-hr single-column system of a Model 119 BL analyzer (Beckman) equipped with a column of W-2 resin (9 × 320 mm) and employing the buffer systems suggested in the instrument manual. Effluents were examined at 570 nm after reaction with ninhydrin. In the case of samples containing radioactive components, effluent was collected directly with a fraction collector and the radioactivity of each fraction was measured in a liquid scintillation counter.

Analysis by Thin-Layer Electrophoresis and Chromatography. Acid hydrolysates were analyzed on cellulose thin-layer plates (Kodak) by electrophoresis at pH 3.5 for 2.5 hr at 300 V in a medium of glacial acetic acid/pyridine/water, 10:1:170 (vol/vol), and at pH 1.9 for 2 hr at 300 V in glacial acetic acid/formic acid (88% by vol)/water, 87:25:888 (vol/vol). Ascending chromatography was for 5 hr with a developer of isobutyric acid/0.5 M NH_4OH, 5:3 (vol/vol). The markers were stained for amino groups by reaction with a ninhydrin reagent and for phosphate groups by reaction with an acid molybdate reagent (Sigma). Autoradiography was performed by exposure to Kodak X-Omat AR film for 3 days.

RESULTS

Elution Profiles of Phosphohydroxylysine and Other Phospho Amino Compounds on the Amino Acid Analyzer. Table 1 shows the characteristic times of elution of the various phospho compounds from a column of W-2 cation-exchange resin. The phospho compounds eluted rapidly, before aspartic acid, with the first buffer system. Because of its ε-amino group, phosphohydroxylysine was eluted more slowly than the other phospho amino acids. Phosphohydroxylysine emerged as a doublet corresponding to the *threo* and *allo* isomers. The phosphohydroxylysine peak position and doublet character are shown in Fig. 1.

Table 1. Elution times in amino acid analysis of phosphohydroxylysine and relevant compounds

Compound	Elution time, min
Cysteic acid	
Phosphoserine	11.0
Phosphothreonine	
Phosphotyrosine	11.3
Phosphoethanolamine	12.8
Phosphohydroxylysine	16.7
Aspartic acid	19.9
Hydroxylysine	70.4
Lysine	78.5

Analysis of Phosphohydroxylysine and Other Phospho Compounds by Thin-Layer Electrophoresis and Chromatography. With the pH 3.5 electrophoresis system, phosphoserine, phosphothreonine, and phosphotyrosine moved toward the anode with relative electrophoretic mobilities of −0.77, −0.66, and −0.51 when the mobility of inorganic orthophosphate was considered to be −1.0. Phosphohydroxylysine moved slightly toward the cathode with a mobility of +0.04, quite similar to the mobilities of phosphoethanolamine and phosphocholine. Of course, hydroxylysine and lysine moved more rapidly toward the cathode. At pH 1.9, phosphoserine, phosphothreonine, and phosphotyrosine moved toward the anode with mobilities shown in Table 2, whereas phosphohydroxylysine moved toward the cathode with a mobility different from those of phosphoethanolamine and phosphocholine, which remained near the origin. Thin-layer chromatography allowed separation of phosphohydroxylysine from all the other compounds tested except phosphotyrosine; phosphohydroxylysine and phosphotyrosine could be separated easily by electrophoresis. Thus by a combination of these methods, one can readily separate and distinguish phosphohydroxylysine. Table 2 summarizes the electrophoretic and chromatographic characteristics of the various phospho compounds examined.

Stability of Phosphohydroxylysine to Acid. As determined by use of the amino acid analyzer, only 18% of a sample of phosphohydroxylysine remained unhydrolyzed after heating at 110°C for 24 hr in presence of 6 M HCl. On the other hand, about 85% of another sample survived treatment with 2 M HCl at 95°C for 4 hr; accordingly, the latter conditions were used routinely. In some experiments a rough estimate from ninhydrin reaction values showed that these conditions per-

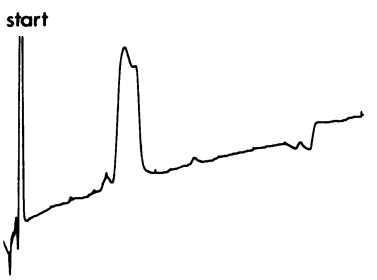

FIG. 1. Elution profile of phosphohydroxylysine obtained on the automatic amino acid analyzer. Phosphohydroxylysine, synthesized from hydroxylysine (mixed DL-*threo* and DL-*allo* forms), was observed as a doublet after ninhydrin reaction. Tracing shows arbitrary A_{570}.

Table 2. Characteristics of phosphohydroxylysine and relevant compounds

	Electrophoretic mobility*		
	pH 3.5	pH 1.9	R_f in TLC†
Orthophosphate	−1.0	−1.0	0.33
Phosphoserine	−0.77	−0.39	0.24
Phosphothreonine	−0.66	−0.26	0.31
Phosphotyrosine	−0.51	−0.26	0.38
Phosphohydroxylysine	+0.04	+0.58	0.39
Phosphoethanolamine	+0.02	+0.14	0.49
Phosphocholine	+0.02	+0.14	0.62
Phosphoarginine	−0.11	+0.38	0.47
Phosphoinositol	−0.86	−0.64	0.18

*Relative to orthophosphate.
†Cellulose TLC developed with isobutyric acid/0.5 M NH_4OH (5:3, vol/vol).

mitted about 95% complete proteolysis of collagenous proteins.

Formation of Phosphohydroxylysine Residues in Calf Aorta Smooth Muscle Cells Incubated with Inorganic [^{32}P]Phosphate. For these studies, the endogenous collagens produced by the cell were examined for phosphorylation. These cells fabricate mostly type I collagen and export it to the medium. The cells also manufacture collagens that remain associated with the cells in an insoluble form. The cell-associated collagens are relatively rich in hydroxylysine residues, and many but not all of these are glycosylated (unpublished data). In preliminary experiments in which the smooth muscle cells were incubated with inorganic [^{32}P]phosphate, we found that very little label was incorporated into the collagens exported to the medium, but significant amounts were incorporated into the cell-associated proteins. Accordingly, most of the experiments reported here deal with phosphorylation of the collagens associated with the cells.

In the first group of experiments, repeated several times with similar results, 10 flasks of confluent cells were washed with Krebs–Ringer bicarbonate buffer (no phosphate) containing 20 mM Hepes (pH 7.4), L-ascorbic acid (100 μg/ml), and amino acids (same concentration as in basal minimal essential medium). To each flask, 1.2 mCi of [^{32}P]orthophosphate and 11 nmol of carrier orthophosphate were added in 15 ml of the same fortified buffer. The cells were incubated for 6 hr at 37°C in a humid 5% CO_2 atmosphere and then washed twice with cold phosphate-buffered saline, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride and 0.01% sodium azide. The cells were scraped from the flasks with a rubber policeman, centrifuged, transferred to a dialysis sack, and dialyzed overnight against water at 4°C. The cells were then lyophilized. The sample was hydrolyzed in 2 M HCl, and the hydrolysate was analyzed for phosphohydroxylysine as outlined above.

Fig. 2*A* shows the pattern obtained by electrophoresis at pH 3.5 of the cell hydrolysate; radioactivity due to ^{32}P was visualized by autoradiography. Comparison was made with standards of several phospho compounds that could possibly occur. In the lane obtained with the cell hydrolysate, the arrow points to a component at the position corresponding to phosphohydroxylysine, phosphoethanolamine, and phosphocholine standards. Fig. 2*B* compares the pattern obtained when the hydrolysate was electrophoresed at pH 1.9 with patterns of standards under the same conditions. The component in the hydrolysate indicated by the arrow is clearly identified with standard phosphohydroxylysine and distinguished from phosphoethanolamine and phosphocholine. Fig. 2*C* shows the pattern obtained when the hydrolysate was subjected to two-dimensional electrophoresis at pH 3.5

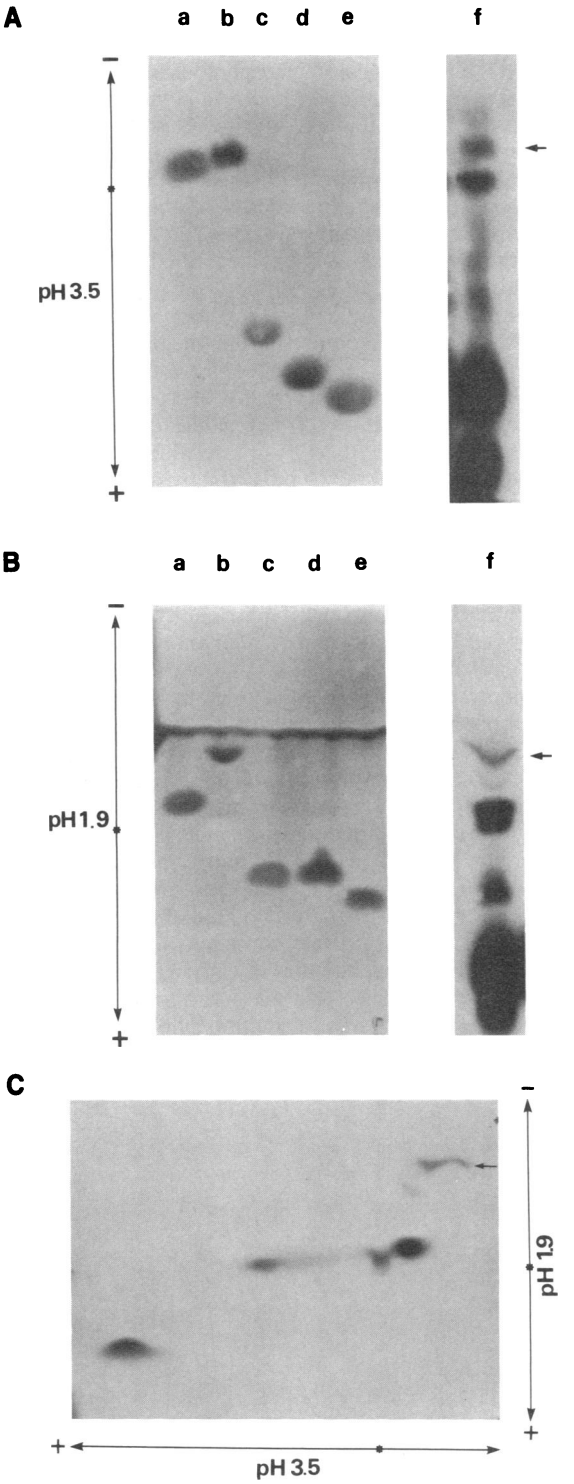

FIG. 2. Analysis by thin-layer electrophoresis of phospho amino acids and an acid hydrolysate of ^{32}P-labeled cells. Lanes: a, phosphoethanolamine; b, phosphohydroxylysine; c, phosphotyrosine; d, phosphothreonine; e, phosphoserine; f, acid hydrolysate of ^{32}P-labeled cells. The details of procedure are described in *Materials and Methods*. Each phospho amino acid and the hydrolysate were analyzed on cellulose thin-layer plates by electrophoresis at pH 3.5 (*A*) and again at pH 1.9 (*B*). The hydrolysate was further analyzed by two-dimensional thin-layer electrophoresis (*C*). The position of origin (*) and position of phosphohydroxylysine (arrow) are indicated. In lanes f, the acidic region shows considerable radioactivity due to free orthophosphate and unhydrolyzed peptides.

and 1.9, respectively. The component indicated by the arrow can be identified clearly as phosphohydroxylysine. The less intense spot below phosphohydroxylysine may be due to an-

other form of the compound or to a peptide containing phosphohydroxylysine.

Further Identification of Phosphohydroxylysine Formation by a Double-Labeling Experiment. As mentioned in the Introduction, the fact that hydroxylysine residues *per se* arise by post-translational modification of certain lysine residues provides a unique opportunity to double-label phosphohydroxylysine. Several experiments of this kind were done with cells from confluent cultures. Ten flasks of confluent cells were washed in buffer as described above. However, the incubation medium contained 1 mCi of L-[4,5-^{3}H]lysine in addition to the 1.2 mCi of [^{32}P]orthophosphate, and nonradioactive lysine was omitted from the medium. The cells were then incubated in this medium for 6 hr. [^{3}H]Lysine would be incorporated into many of the cell proteins but would appear as hydroxylysine residues only in collagenous molecules. If those hydroxylysine residues were then phosphorylated metabolically, only the resulting residues of phosphohydroxylysine would contain both ^{3}H and ^{32}P. (This assumes that one can rule out, as we did subsequently, that residues of *N*-phospholysine form through an -N—P- bond involving the ε-amino groups of lysine residues. In all of the analytical systems presented phosphohydroxylysine could be distinguished easily from *N*-phospholysine.)

Analyses of hydrolysates of cell layers obtained in these experiments are represented in Fig. 3. Fig. 3*A* shows the electrophoretic pattern obtained at pH 3.5; a peak containing ^{3}H and ^{32}P is observed. However, electrophoresis at this pH does not separate phosphoethanolamine and phosphocholine sharply from phosphohydroxylysine, so that the peak could contain, in addition to doubly labeled phosphohydroxylysine, singly labeled (with ^{32}P) phosphoethanolamine and phosphocholine. Accordingly, another sample of the hydrolysate was subjected to analysis on the amino acid analyzer. Because the analyzer buffers contained sufficient salt to diminish counting efficiency for ^{3}H, only ^{32}P radioactivity was measured; nevertheless, this method allows the separation of phosphohydroxylysine from phosphoethanolamine and phosphocholine. Results are shown in Fig. 3*B*. Each of the peaks obtained in this procedure was analyzed separately by electrophoresis at pH 3.5, in which case both ^{3}H and ^{32}P could be measured (Fig. 3*C*). Only peak 2, known to be at the position of authentic phosphohydroxylysine, was doubly labeled. In fact, peak 1, containing only singly-labeled material contained phosphoethanolamine, phosphocholine, and other phospho amino acids. Thus, the combination of methods of separation and analysis showed clearly the presence of phosphohydroxylysine. Furthermore, by correcting the cpm for efficiencies, and from the specific activities of ^{32}P and ^{3}H, we were able to calculate that these occurred almost in the expected stoichiometric ratio of 1 (data not shown).

Preliminary Experiments To Detect the Presence of a Protein Kinase for Hydroxylysine Residues. In the several kinds of experiments described above, metabolically active cells were incubated with [^{32}P]orthophosphate, resulting in the incorporation of radioactivity into a number of compounds, including serine, threonine, and tyrosine residues in several or many proteins, and specifically into residues of hydroxylysine in collagenous proteins. The assumption is, of course, that the cells incorporate orthophosphate into ATP which then, through the agency of a kinase, phosphorylates the amino acid residues. For hydroxylysine residues, preliminary evidence to that effect has been obtained in experiments using cell-free systems and [γ-^{32}P]ATP.

In one such experiment, homogenates were made from confluent aorta smooth muscle cells, and a cell-free extract was prepared in which acid-labile ATPase activity was eliminated by treatment at pH 3.2. The fraction was centrifuged at 900 × *g* and the resulting supernatant, at 23,000 × *g*. When the 23,000 × *g* supernatant was incubated for 10 min at 20°C with [γ-^{32}P]ATP and a collagenous substrate such as gelatin prepared from calf skin collagen, labeled phosphohydroxylysine was isolated and identified unequivocally (data not shown). Further work is required for selection of the best collagenous substrate and most favorable conditions of incubation.

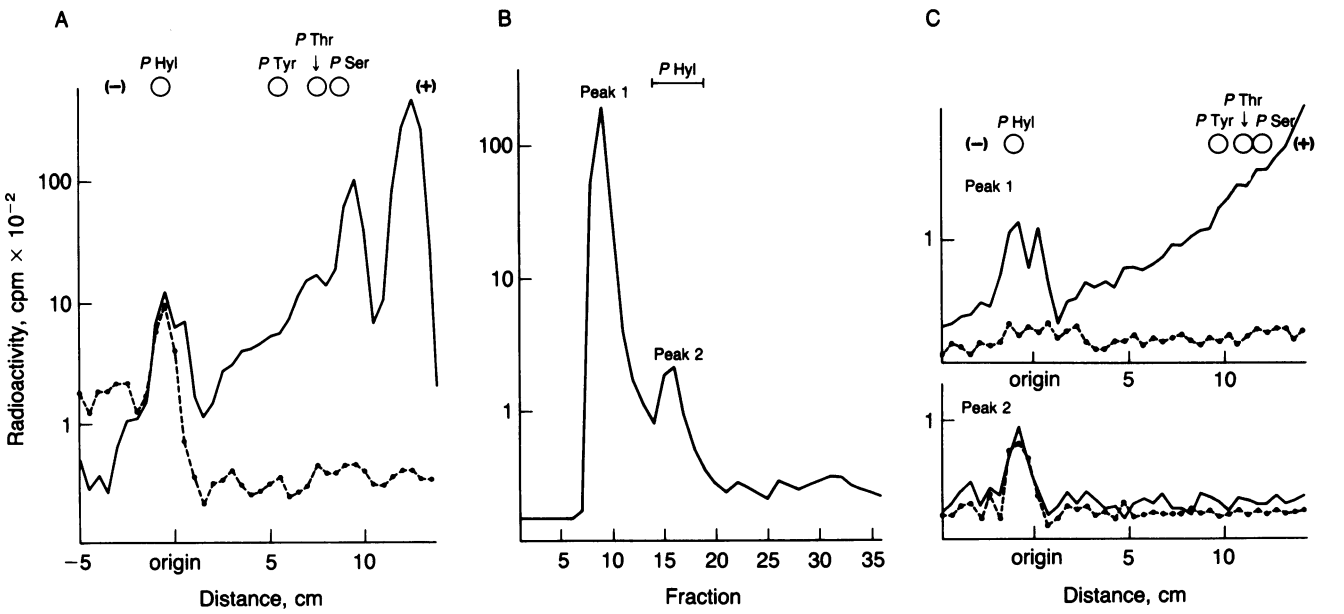

FIG. 3. Analysis of acid hydrolysate of cells labeled with [^{32}P]orthophosphate and [^{3}H]lysine (see text). (*A*) Acid hydrolysate of double-labeled cells was analyzed by thin-layer electrophoresis at pH 3.5. Thin-layer plates were sliced into 5-mm pieces and the ^{32}P (solid line) and ^{3}H (broken line) in each fraction were measured. The ordinate scale (radioactivity) is logarithmic. (*B*) ^{32}P profile of the hydrolysate obtained on the amino acid analyzer. Fraction volume, 0.9 ml. The ordinate scale is logarithmic. (*C*) Peaks 1 and 2, resolved on the amino acid analyzer (see *B*) were collected separately and applied to a Dowex 1 column (acetate form) at pH 7.0. Effluents obtained by elution with 1 M acetic acid were evaporated and subjected to thin-layer electrophoresis at pH 3.5. Radioactivity in successive 5-mm slices (^{32}P, solid line; ^{3}H, broken line) was measured; the radioactivity scale is logarithmic. *P*Hyl, phosphohydroxylysine.

DISCUSSION

Serine and threonine residues in collagens or in denatured collagens (gelatins) are known to be phosphorylated by several protein kinases. To our knowledge, the results presented here provide the first evidence that residues of hydroxylysine, an amino acid unique to collagens, can be phosphorylated. Phosphorylation of endogenous collagen was demonstrated in cultured calf aorta medial smooth muscle cells incubated with [^{32}P]orthophosphate; and in preliminary experiments, phosphorylation of exogenous collagenous substrates occurred when they were incubated with [γ-^{32}P]ATP and cell-free extracts from the smooth muscle cells. For this work, we developed a protocol for analyzing phosphohydroxylysine, based on cation-exchange chromatography, two-dimensional thin-layer electrophoresis, and thin layer chromatography. Although the experiments with intact cells would appear to provide an adequate demonstration of phosphorylation of hydroxylysine residues, the studies implicating the participation of a protein kinase are positive but preliminary. We stress that the kinase activity observed in these experiments is that of a protein kinase distinct from the GTP-dependent liver kinase for free hydroxylysine; the latter represents part of a system for the complete degradation of free hydroxylysine released by digestion of collagens (4, 5).

At this point, one can only speculate concerning the function(s) of phosphorylation of hydroxylysine residues in collagenous proteins. As judged from several kinds of double-label experiments, not all of which are described in this communication, the phosphorylation appeared to occur shortly after labeled lysine was incorporated into collagen chains and post-translationally hydroxylated to form labeled hydroxylysine residues. This suggests that phosphorylation may be important in the processing and transport of collagen chains and assemblies.

Some insight into possible functions of this kind of phosphorylation may be gained from studies with two proteins that have covalently attached collagenous chains: namely, C1q of complement and acetylcholine esterase. C1q has a biological action linking antigen–antibody associations to the activation of the C1 esterase (protease) function of complement. Interestingly, acetylcholine esterase exerts its enzymatic function in association with cell membranes. If these proteins are found to undergo phosphorylation of some of their hydroxylysine and hydroxyproline residues, investigation could be conducted to determine whether that phosphorylation is important in regulation of their activities. Such systems would be expected to be coordinated with dephosphorylation systems represented by specific phosphatases.

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