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Protein histidine phosphorylation: Increased stability of thiophosphohistidine

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Abstract

Posttranslational phosphorylation of proteins is an important event in many cellular processes. Whereas phosphoesters of serine, threonine and tyrosine have been extensively studied, only limited information is available for other amino acids modified by a phosphate group. The formation of phosphohistidine residues in proteins has been discovered in prokaryotic organisms as well as in eukaryotic cells. The ability to biochemically analyze phosphohistidine residues in proteins, however, is severely hampered by its extreme lability under acidic conditions. In our studies we have found that by replacing the phosphate linked to the histidine residue with a thiophosphate, a phosphohistidine derivative with increased stability is formed. This allows the analysis of phosphohistidine-containing proteins by established biochemical techniques and will greatly aid in the investigation of the role of this posttranslational modification in cellular processes.

Keywords: histidine phosphorylation; posttranslational modification; signal transduction; thiophosphate

Regulation by transient phosphorylation of proteins is a common process in a wide variety of biological processes, including signal transduction events (Cohen, 1982). The phosphorylation of serine, threonine, and tyrosine residues in proteins of eukaryotic organisms has been well established in many steps of signal transduction. Phosphorylation of these residues can lead to the activation of enzymatic activities or the association of other proteins with the newly phosphorylated residue in a sequence-specific context (Ullrich & Schlessinger, 1990).

It is estimated that phosphohistidine (pH) may account for approximately 6% of phosphorylated proteins in prokaryotes as well as eukaryotes (Matthews, 1995). Although not as abundant as serine and threonine phosphorylation, histidine phosphorylation apparently is more common than phosphorylation of tyrosine residues. Known cases of histidine phosphorylation, however, are less numerous than the ones for hydroxy amino acids, presumably because of the widespread use of experimental techniques that fail to preserve this acid-labile modification (Wei & Matthews, 1991).

In prokaryotes, several signal transduction events are governed by so-called two-component systems that involve the formation of phosphorylated histidine residues (Alex & Simon, 1994). These systems typically consist of a sensor protein and a response regulator protein. Sensor proteins autophosphorylate in response to specific inputs. The site of phosphorylation in most cases is a conserved histidine residue. The sensor protein transduces signals by transferring the phosphate group to an aspartic acid residue in a second protein, the response regulator. Phosphorylation of the response regulator leads to an output signal such as transcriptional activation or protein–protein interaction (Alex & Simon, 1994).

The phosphorylation of histidine residues has been observed in eukaryotic cells. The *Sln1* protein in *Saccharomyces cerevisiae* that is involved in osmosensing shares high amino acid homology with the bacterial two-component protein systems, including a presumed histidine-autophosphorylation site (Ota & Varshavsky, 1993; Posas et al., 1996). The *Arabidopsis thaliana* gene, *ETR1*, also encodes a histidine kinase, which mediates the ethylene response (Chang et al., 1993; Gamble et al., 1998). In *Dictyostelium discoideum*, the sensor histidine kinase *dhkC* plays an important role in development (Singelton et al., 1998). Phosphohistidine was originally detected in mammalian cells in mitochondria derived from bovine liver (Boyer et al., 1962) and nuclei from rat tissue (Smith et al., 1973). It was also found that phosphorylation of a histidine on the cytoplasmic tail of P-selectin takes place following platelet activation with thrombin or collagen (Crovello et al., 1995). Thus, it appears that this posttranslational modification is also increasingly recognized in mammalian systems that are related to signal transduction. The difficulties in studying histidine phosphorylation events have their origin in the high energy state of the

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phosphate-imidazole bond, which resembles that of a phosphoramidate (Wei & Matthews, 1991). Phosphohistidine is therefore not as stable as its phosphoester relatives and hydrolyzes readily under acidic conditions, which are employed commonly in biochemical techniques (Boyle et al., 1991; Wei & Matthews, 1991).

Methods such as radioactive peptide mapping and Edman degradation have greatly facilitated the analysis of serine, threonine, and tyrosine phosphorylations in proteins (Boyle et al., 1991). However, due to their acidic reaction conditions, they cannot be applied to the analysis of phosphohistidine. Both methods aid in mapping the sites of phosphorylation in a protein, which is a prerequisite for subsequent functional studies.

In this paper, we describe a method that allows for the stabilization of the imidazole-phosphate bond in phosphohistidine through the incorporation of thiophosphate instead of regular phosphate. This method will facilitate studies on the role of histidine-phosphorylation in signal transduction and other cellular processes.

Results

In our efforts to develop and improve methods for the analysis of histidine kinases and histidine-phosphorylated proteins (Medzihradszky et al., 1997; Senderowicz et al., 1997), we sought a way to increase the stability of the phosphohistidine residue. At the same time we wanted to preserve the overall structure of phosphohistidine as much as possible. The latter aspect is important since we wanted to maintain the ability of a histidine kinase to incorporate the phosphate derivative into a substrate protein during a kinase reaction. One way of increasing the stability of the N-P bond in a phosphohistidine is to replace the double-bonded oxygen with a sulfur atom. Due to its lower electronegativity, the sulfur atom should stabilize the N-P bond and thereby decrease the hydrolysis sensitivity of the phosphohistidine.

In the studies described below, we used synthetic peptides with either a phosphohistidine or thiophosphohistidine (tpH) and compared their acid sensitivities. In addition, we have analyzed the acid sensitivities of an autophosphorylated or autothiophosphorylated histidine kinase. For this purpose we used the catalytic domain of the yeast histidine kinase *Sln1* in a glutathione S-transferase (GST) fusion protein (Posas et al., 1996).

The synthetic peptide with the sequence Ac-HGGGGAAAL-NH_2 was either phosphorylated, Ac-(pH)GGGGAAAL-NH_2, or thiophosphorylated, Ac-(tpH)GGGGAAAL-NH_2, on the histidine. This peptide sequence was chosen because it exhibits favorable elution characteristics on reversed phase–high performance liquid chromatography (RP-HPLC) columns and except for the imidazole side chain of histidine it has no other functional groups that are susceptible to modification by the reagents employed during the phosphorylation reactions. The unphosphorylated and phosphorylated/thiophosphorylated peptides could be resolved using standard RP-HPLC solvents and gradients (Fig. 1). The histidine phosphorylation of the peptide was carried out with potassium phosphoramidate as previously described (Wei & Matthews, 1991; Medzihradszky et al., 1997; Senderowicz et al., 1997). For the preparation of the histidine-thiophosphorylated peptide, we developed a procedure that employs $PSCl_3$ as a phosphorylation reagent. We had to resort to this rather reactive compound because all attempts to prepare potassium thiophosphoramidate for use as the thiophosphorylating reagent failed. All peptides were purified by RP-HPLC and characterized by mass spectrometry. Although the phosphopeptides slowly degrade during the mass spectrometry analysis, monoprotonated peptide ions with the correct molecular masses were detected for the histidine-phosphorylated and -thio-phosphorylated peptides, which are characterized by a molecular mass shift of 80 and 96 Da, respectively (Fig. 2). No other side products were observed indicating that $PSCl_3$ treatment of the peptide only results in the desired thiophosphorylation of the imidazole side chain of histidine. Figures 1 and 2 show the RP-HPLC chromatograms and mass spectra of the peptide Ac-HGGGGAAAL-NH_2 before (Figs. 1A, 2A) and after the treatment with potassium phosphoramidate (Figs. 1B, 2B) and $PSCl_3$ (Figs. 1C, 2C), respectively. The earlier eluting peaks on RP-HPLC labeled with an asterisks correspond to the phosphorylated (Fig. 1B) and thiophosphorylated (Fig. 1C) peptides, respectively.

To examine which nitrogen on the imidazole ring gets thiophosphorylated after overnight treatment with $PSCl_3$, we carried out phosphorylation reactions with the histidine monomer. Earlier studies had shown that after 12 h treatment of histidine with potassium phosphoramidate 3-phosphohistidine is the predominant isomer present in the reaction mixture (Wei & Matthews, 1991). Previous NMR analyses that we carried out with a histidine-containing synthetic peptide indicated that the major isomer that is formed after 12 h treatment with potassium phosphoramidate also is the 3-phosphohistidine derivative (Medzihradszky et al., 1997). In Figure 3 we show the results of a thin layer chromatography (TLC) analysis of histidine phosphorylation/thiophosphorylation using potassium phosphoramidate and $PSCl_3$, respectively (Besant et al., 1998). A 15 min reaction of histidine with potassium phosphoramidate results in the expected mixture of the 1- and 3-phosphohistidine isomers (Fig. 3, lane 2). After 12 h treatment with potassium phosphoramidate only 3-phosphohistidine is left (Fig. 3, lane 4). The overnight reaction of histidine with $PSCl_3$ also results in a product that comigrates with 3-phosphohistidine, and no spot that comigrates with 1-phosphohistidine is detectable (Fig. 3, lane 3). We therefore assume that the major reaction product after 12 h treatment with $PSCl_3$ is the 3-thiophosphohistidine isomer. In future studies, we will confirm this finding by NMR analyses of synthetic thiophosphohistidine-containing peptides.

To analyze the acid sensitivities of the phosphopeptides, they were treated at pH 0–7 for 180 min and at pH 1 for 1–180 min at RT. Subsequently the mixtures were injected onto the RP-HPLC column and the areas under the phosphorylated/thiophosphorylated and unphosphorylated peaks integrated. Figure 4 shows the results of these studies after subtraction of the background values of the untreated peptides. To determine the background values, the phospho/thiophospho peptides were dissolved in water and subjected to RP-HPLC under the same gradient conditions. Both analyses demonstrate that the thiophosphohistidine is significantly more stable than the phosphohistidine under acidic conditions. Both histidine derivatives are stable down to pH 3 when treated at room temperature for 3 h. However, whereas the thiophosphohistidine continues to be stable at pH 1, the phosphohistidine shows greater than 50% hydrolysis. At pH 0 all of the phosphohistidine is hydrolyzed whereas only 29.1% of the thiophosphohistidine was converted to histidine (Fig. 4A). When treated for increasing times at pH 1 and room temperature, the phosphohistidine showed significant hydrolysis at 60 min whereas the thiophosphohistidine remained essentially intact at least up until 180 min of reaction time (Fig. 4B). Acid hydrolysis studies of 1-phosphohistidine, 3-phosphohistidine, phosphoimidazole, and 1,3-diphosphoimidazole have been reported (Hultquist, 1968) and are consistent with the data presented here.

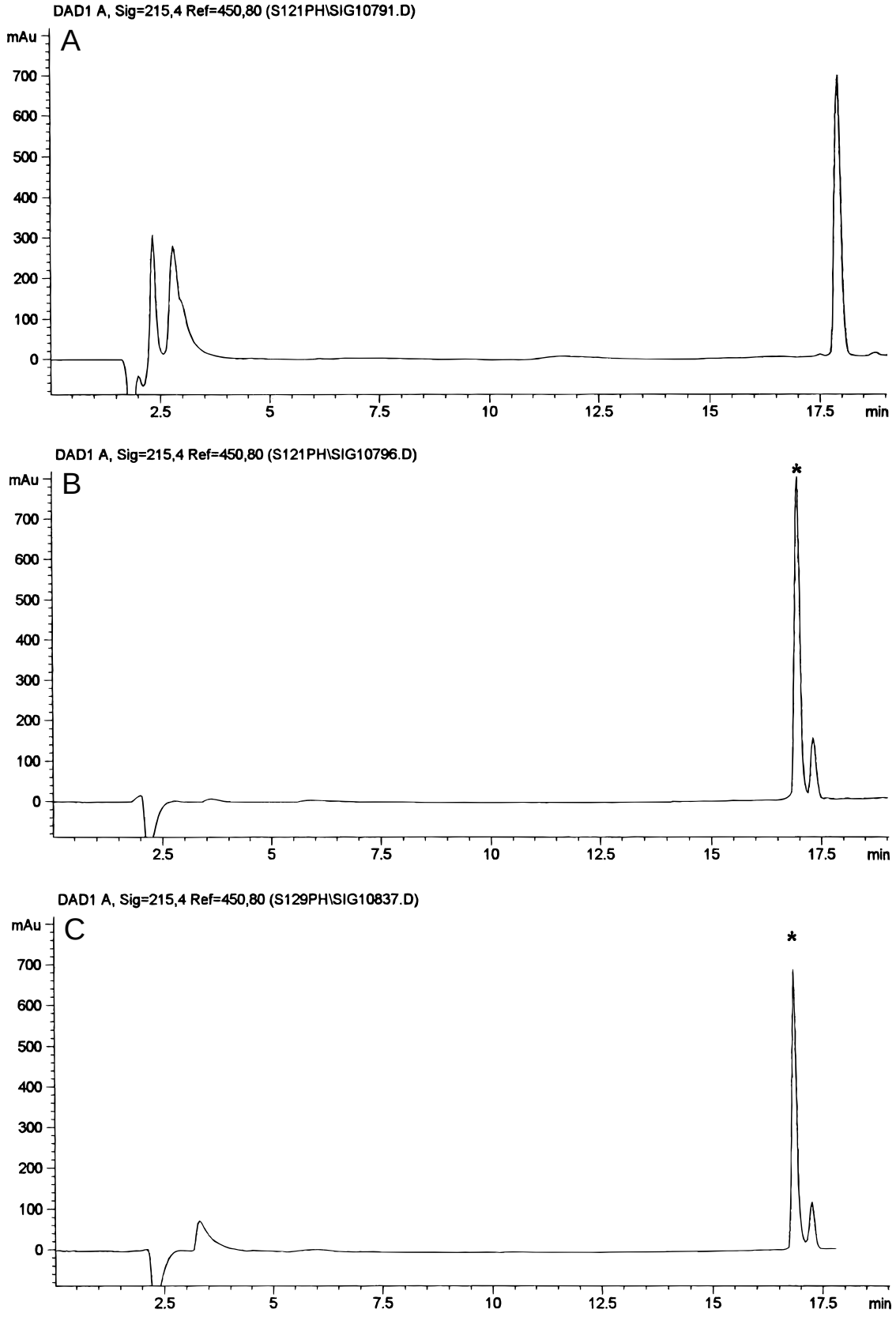

Fig. 1. RP-HPLC of synthetic peptides Ac-HGGGGAAAL-NH_2; (**A**) unphosphorylated; (**B**) after treatment with potassium phosphoramidate; (**C**) after treatment with $PSCl_3$. Peptides were chromatographed as described in Materials and methods. The earlier eluting peaks in **B** and **C** labeled with an asterisks correspond to the phosphorylated Ac-(pH)GGGGAAAL-NH_2 and thiophosphorylated Ac-(tpH)GGGGAAAL-NH_2 peptides, respectively.

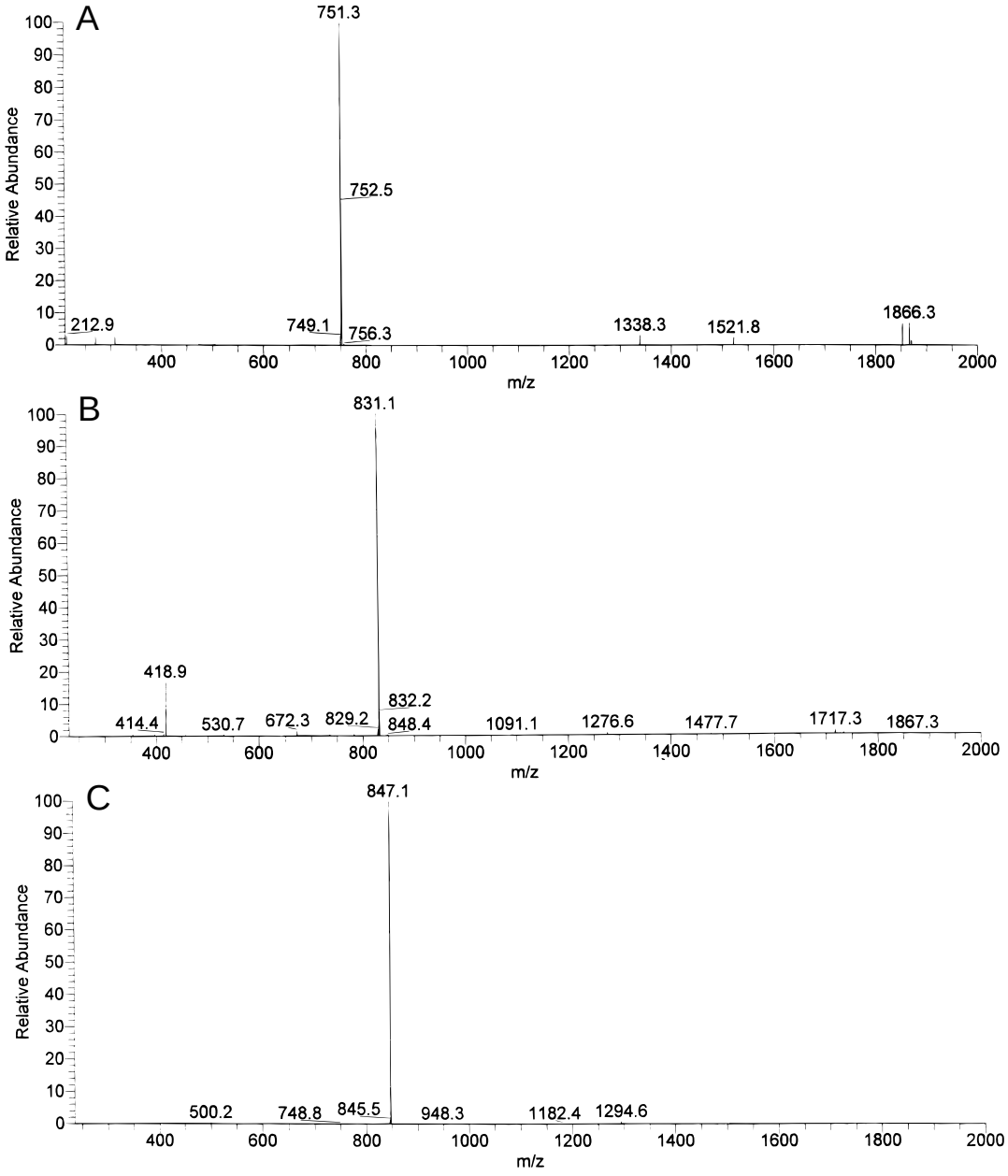

Fig. 2. Electrospray mass spectrometry analyses of synthetic peptides purified by RP-HPLC; (**A**) unphosphorylated; (**B**) after treatment with potassium phosphoramidate; (**C**) after treatment with $PSCl_3$; shown are the monoprotonated molecular ions of the peptides (**A**) Ac-HGGGGAAAL-NH_2, (**B**) Ac-(pH)GGGGAAAL-NH_2, and (**C**) Ac-(tpH)GGGGAAAL-NH_2.

However, due to differences in the hydrolysis conditions and the compounds that were studied, we cannot directly compare our results to the ones of Hultquist.

In the next step, we wished to confirm these results in a protein that contains a phosphorylated histidine residue. For this purpose, we used the yeast histidine kinase *Sln1* catalytic domain (amino acids 450–1070) as a GST fusion protein (Posas et al., 1996). To use the *Sln1* histidine kinase for our studies, we had to make certain that the enzyme can utilize γ-thio-ATP as a substrate. Since the double-bonded sulfur also results in an increased stability of the γ-phosphate bond in ATP, there was the possibility that the kinase would be unable to cleave the γ-phosphate during the enzyme reaction and hence prevent the autophosphorylation of histidine from taking place. The following results demonstrate that the *Sln1* kinase fusion protein can incorporate phosphate and thiophosphate from the ATP and thio-ATP substrates (Figs. 5, 6). At this point we have not performed any kinetic analyses on the phosphate and thiophosphate uptakes. In the studies described below, we used the autophosphorylated and autothiophosphorylated *Sln1* proteins to compare the acid stabilities of phosphohistidine and thiophosphohistidine residues in a protein.

To analyze the hydrolysis of phosphohistidine and thiophosphohistidine, we prepared autophosphorylated GST-*Sln1* fusion proteins in the following manner. Glutathione resin-immobilized

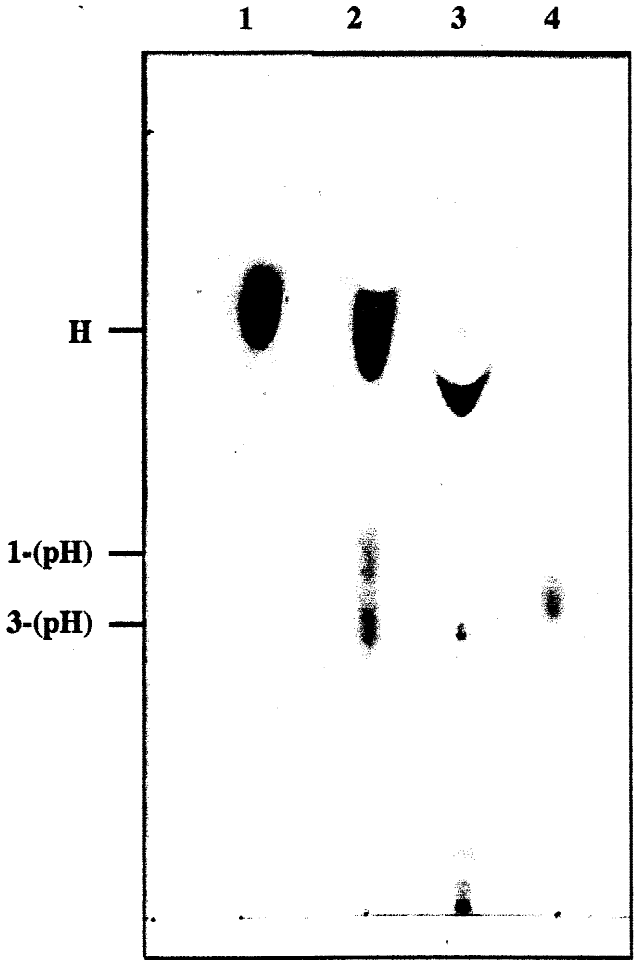

Fig. 3. Thin layer chromatography of phosphorylation reactions of histidine with potassium phosphoramidate and $PSCl_3$, respectively. Unphosphorylated histidine was run in lane 1. Histidine was phosphorylated with potassium phosphoramidate for 15 min (lane 2) and 12 h (lane 4) or $PSCl_3$ for 12 h (lane 3). The positions of histidine, 1-phosphohistidine, and 3-phosphohistidine are indicated on the left. A reaction product that comigrates with 3-phosphohistidine is detectable for the 12 h thiophosphorylation reaction.

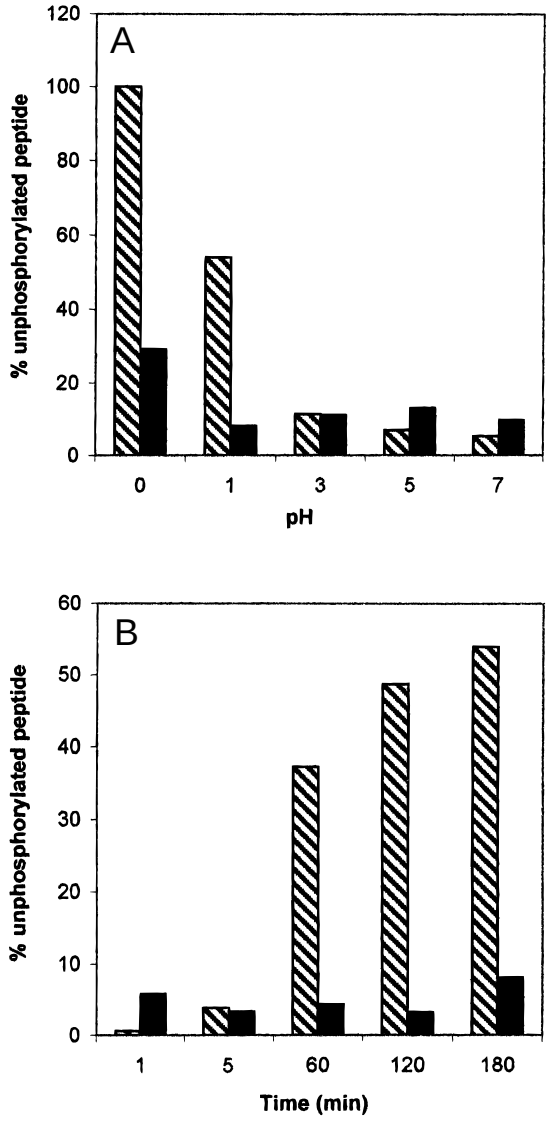

Fig. 4. Treatment of peptides Ac-(pH)GGGGAAAL-NH_2 (▨) and Ac-(tpH)GGGGAAAL-NH_2 (■) with aqueous HCl; (**A**) pH dependence, 3 h, RT; (**B**) time course, pH 1, RT. Phosphopeptides were treated in fluid phase and the degree of phosphate hydrolysis determined by RP-HPLC.

GST-*Sln1* fusion protein was subjected to in vitro kinase reactions using either γ-^{32}P-ATP or γ-^{35}S-thio-ATP and the histidine-autophosphorylated and -thiophosphorylated proteins eluted. The proteins were then run on a sodium dodecyl sulfate (SDS) gel and subsequently transferred to a polyvinylidenedifluoride (PVDF) membrane. After exposure of the PVDF-immobilized proteins to X-ray film and the phosphorimager for quantitation individual, lanes were cut out and treated as described in Figures 5 and 6. After treatment, the individual PVDF strips were reassembled and exposed again to X-ray film and the phosphorimager. Subsequent to radioactivity quantitation the PVDF membranes were stained with Amidoblack for protein comparison. Evaluation of the results revealed that the thiophosphohistidine shows increased acid stability over the phosphohistidine in the *Sln1* fusion proteins. Figure 5 shows that treatment of phosphohistidine-containing GST-*Sln1* leads to complete hydrolysis of the phosphate at pH 0

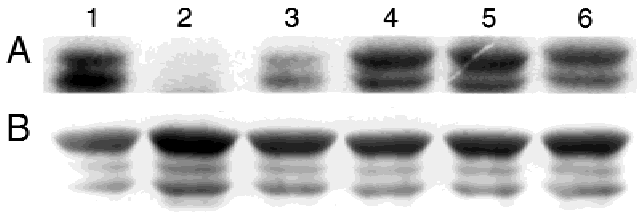

Fig. 5. Treatment of autophosphorylated (lanes 1–3) and autothiophosphorylated (lanes 4–6) *Sln1*-GST fusion protein with 10 mM Tris-HCl, pH 8 (lanes 1 and 4), 1 N HCl (lanes 2 and 5), 3 N KOH (lanes 3 and 6) for 3 h at RT. **A:** Phosphorylated/thiophosphorylated proteins were treated on PVDF membranes and exposed to X-ray film. **B:** After exposure the PVDF membranes were stained with Amidoblack.

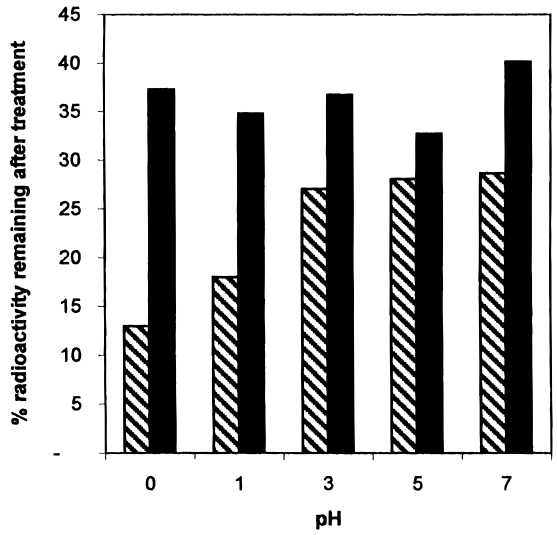

Fig. 6. Treatment of autophosphorylated (▨) and autothiophosphorylated (■) *Sln1*-GST fusion proteins with aqueous HCl at various pH for 3 h at RT. Phosphorylated/thiophosphorylated proteins were treated on PVDF membranes and exposed to a phosphorimager before and after treatment.

(Fig. 5A, lane 2), whereas the radioactive signal for the thiophosphorylated protein is no different at pH 0 than it is at pH 7 (Fig. 5A, lane 5). No differences in the amounts of GST-*Sln1* protein present on the PVDF membrane after the various treatments are detectable as judged by the Amidoblack staining profile (Fig. 5B). Figure 6 shows the pH dependence of the histidine-phosphorylated/thiophosphorylated GST-*Sln1* fusion proteins. The obtained results are consistent with the data obtained for the synthetic peptides (Fig. 4A). Whereas the thiophosphorylated GST-*Sln1* protein shows similar radioactive signals at all pH values, the phosphorylated protein undergoes significant phosphohistidine hydrolysis at lower pH values. However, we were unable to precisely quantitate the differences in phosphate/thiophosphate hydrolysis. This is presumably due to inconsistent protein washout from the PVDF membranes during the acid treatments at various pH values. Assuming that the protein washout from the PVDF membrane is the same for a given pH value, the presented data can be used for stability comparisons only. The results presented in Figure 6 are therefore not absolute numbers but only show relative stabilities of phosphohistidine and thiophosphohistidine in the *Sln1* fusion proteins.

To apply the increased stability of thiophosphohistidine over phosphohistidine to a commonly used biochemical analysis, we prepared a phosphohistidine-containing protein that has a high stoichiometry of phosphorylation. We then subjected the autophosphorylated protein to peptide mapping and Edman degradation. Both procedures employ acidic reagents that are known to lead to phosphohistidine hydrolysis. The protein we chose is nucleoside diphosphate kinase (NDPK) from bovine liver. This enzyme is not a histidine kinase but uses phosphohistidine as an intermediate in the phosphorylation of nucleoside diphosphates (Lascu et al., 1983; Moréra et al., 1995). The intermediate histidine-phosphorylated NDPK can be isolated and was used for the peptide mapping experiments. Figure 7 shows the autoradiogram of equal amounts of the autophosphorylated (Fig. 7,

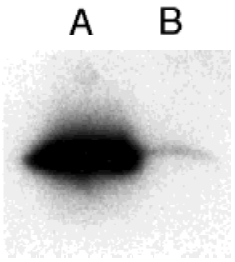

Fig. 7. NDPK kinase reactions carried out with γ-^{32}P-ATP and γ-^{35}S-thio-ATP as substrates, respectively. Equal amounts of the (**A**) autophosphorylated and (**B**) autothiophosphorylated NDPK proteins were subjected to SDS gel electrophoresis, stained with Coomassie Blue and the dried gel exposed to X-ray film.

lane A) and autothiophosphorylated (Fig. 7, lane B) NDPK proteins run on an SDS gel. Although we did not assess the specific activities of the two radiolabeled proteins, it is apparent that the ^{32}P-labeled band corresponding to the autophosphorylated NDPK results in a signal that is by orders of magnitude stronger than the corresponding ^{35}S-labeled band that represents the autothiophosphorylated NDPK. Equal amounts of the phosphorylated/thiophosphorylated enzymes derived from the SDS gel shown in Figure 7 were subjected to an in-gel tryptic digestion and the extracted peptides fractionated on a RP-HPLC column using a 0.1% trifluoroacetic acid (TFA)/acetonitrile buffer system. Aliquots of each fraction were subjected to scintillation counting resulting in higher apparent numbers for the ^{35}S-labeled vs. the ^{32}P-labeled NDPK-derived peptides. As can be seen from Figure 8, a major radioactive peak in fraction 30 was obtained for both the phosphohistidine- and thiophosphohistidine-containing proteins. The other minor peaks of radioactivity presumably represent peptide fragments that are due to incomplete digestion of the protein. Prolonged storage of the RP-HPLC frac-

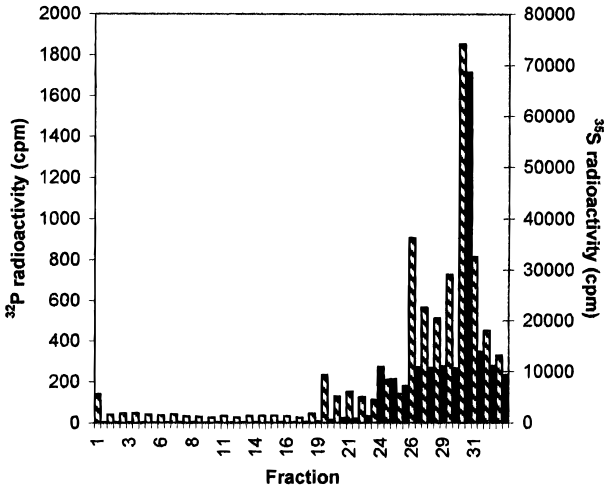

Fig. 8. Peptide mapping analyses by RP-HPLC of autophosphorylated (▨) and autothiophosphorylated (■) NDPK tryptic digests. In-gel tryptic digests from equal amounts of NDPK were fractionated on a RP column and 10% aliquots of each fraction were scintillation counted.

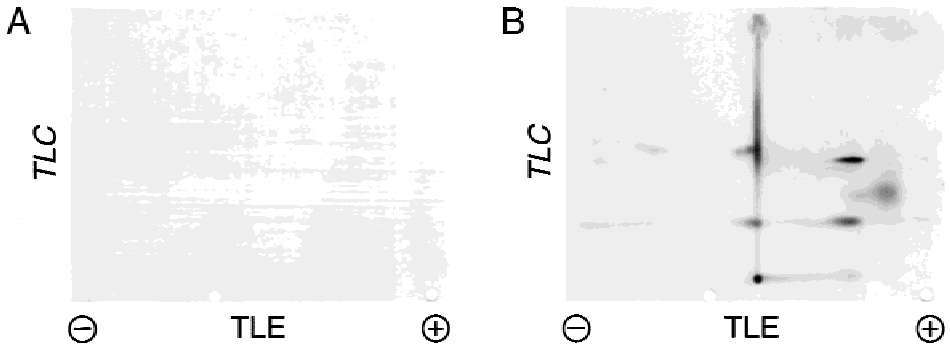

Fig. 9. Autoradiogram of peptide mapping analyses by TLE/TLC of (**A**) autophosphorylated and (**B**) autothiophosphorylated NDPK tryptic digests. In-gel tryptic protein digests from equal amounts of NDPK were spotted onto a cellulose plate and subjected to high-voltage TLE in the first dimension and TLC in the second dimension. Plates were exposed to X-ray film.

tions at 4 °C resulted in significant phosphate hydrolysis whereas the thiophosphorylated peptide stayed intact (data not shown). Both digest mixtures derived from the same protein amount of ^{32}P-labeled and ^{35}S-labeled NDPK were also run on a two-dimensional peptide map, including thin layer electrophoresis (TLE) and TLC. Whereas the thiophosphorylated protein digest resulted in several signals (Fig. 9B), no radioactive signals were obtained from the phosphorylated protein digest (Fig. 9A). Based on the results of Figure 7 the ^{32}P-labeled peptides should have resulted in significantly stronger signals than the ^{35}S-labeled peptides. We therefore conclude that under the conditions used, the phosphohistidine is not sufficiently stable. The radiolabeled peptides derived from the major peaks of the RP-HPLC runs were then subjected to radioactive Edman degradation. Whereas the phosphohistidine-containing peptide resulted in spurious sequence data with variable amounts of radioactivity released in the Edman degradation cycles, the thiophosphohistidine-containing peptide showed a clear signal above background for a released radioactive anilinothiazolinone (ATZ)-amino acid in position 4 (Fig. 10). The only tryptic peptide derived from bovine liver NDPK with a histidine in position 4 has the sequence ^{115}NII HGSDSVESAEK^{128}. This peptide contains the predicted phosphorylated histidine residue in position 118 of NDPK from bovine liver. The homologous histidine residue 122 has been shown to be the site of phosphorylation in NDPK from *D. discoideum* (Moréra et al., 1995).

Discussion

Histidine-phosphorylation plays an important role in prokaryotic and eukaryotic signal transduction events in two-component systems (Chang et al., 1993; Ota & Varshavsky, 1993; Alex & Simon, 1994; Singelton et al., 1998). The function of histidine-phosphorylation in mammalian cells and its involvement in signal transduction remain elusive. A major reason for this situation is the extreme lability of the phosphohistidine bond. Biochemical analysis using established methods has been hampered by the phosphohistidine lability. This prompted us to look for a phosphohistidine analog with improved stability toward acidic conditions commonly used in many biochemical methods. Our findings suggest that replacing the double-bonded oxygen of the phosphate with a sulfur leads to a more stable N-P bond due to the lower electronegativity of the sulfur. This is exemplified by an increased stability of thiophosphohistidine in acidic solvents. Our data using synthetic peptides and an autophosphorylated histidine kinase suggest that thiophosphohistidine-containing proteins can be subjected to solvents of pH 1 without any significant loss of the thiophosphate group. This opens up the possibility of analyzing these phosphoproteins by methods like peptide mapping and Edman degradation, which are important techniques for determining the sites of histidine phosphorylation in a protein (Boyle et al., 1991). We have demonstrated that the histidine-thiophosphorylated protein NDPK can be analyzed in this manner, and we have been able to confirm the site of histidine-phosphorylation in this enzyme. The procedures used can be employed in the identification of histidine-phosphorylation sites in proteins and the functional analysis of these residues in signal transduction and other cellular events. Similar approaches have been very successful with tyrosine-phosphorylated peptides and have resulted in the delineation of sites of interaction of phosphotyrosines and phosphotyrosine-

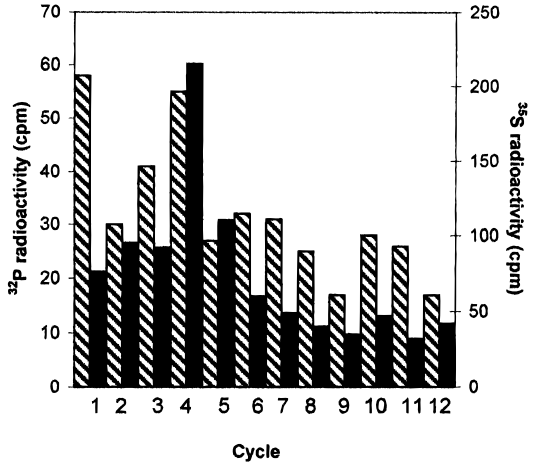

Fig. 10. Radioactive Edman degradation of the peptides eluting in fraction 30 of RP-HPLC runs of the autophosphorylated (▨) and autothiophosphorylated (■) NDPK tryptic digest, respectively. Fraction 30 containing the major radioactive peaks from equal amounts of the autophosphorylated and autothiophosphorylated protein digests were concentrated and spotted onto a covalent sequencing membrane. Extracts from each sequencing cycle were subjected to scintillation counting.

specific protein modules such as *src* homology 2 (SH2) and phosphotyrosine binding (PTB) domains (Peters et al., 1992; Kavanaugh et al., 1995).

The increased stability of thiophosphohistidine over phosphohistidine greatly simplifies the handling of histidine-phosphorylated proteins and peptides. We have found that even at −20 °C regular phosphohistidine slowly decomposes whereas thiophosphohistidine remains unhydrolyzed for several months (data not shown). This method will therefore be of great value in the purification of histidine-phosphorylated proteins. Furthermore, the ability to determine phosphorylated histidine residues by peptide mapping and radioactive Edman degradation will aid in the identification and functional analysis of these residues in proteins derived from prokaryotic and eukaryotic cells.

Materials and methods

Peptides

Synthesis of the peptide Ac-HGGGGAAAL-NH_2 was carried out using a PE Biosystems (Perkin Elmer Biosystems, Foster City, California) instrument, Model 433 with standard fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids and resin (Anaspec, San Jose, California). After cleavage and purification, the peptide was lyophilized to dryness. For histidine phosphorylation, 5 mg of peptide was dissolved in 200 μL 10 mM ammonium bicarbonate and the pH adjusted to 8 with 6 N KOH. To this peptide solution, 50 mg of potassium phosphoramidate (Wei & Matthews, 1995; Medzihradszky et al., 1997) was added and the mixture reacted overnight at RT (20 °C). For histidine thiophosphorylation, 5 mg of peptide was dissolved in 200 μL water and 5 μL triethylamine. Five 2.5 μL-aliquots of $PSCl_3$ (Sigma Aldrich, St. Louis, Missouri) were then added to the peptide solution maintaining pH 8 with 6 N KOH. The mixture was reacted overnight. Both phosphorylation and thiophosphorylation mixtures were subjected to RP-HPLC in 0.1% TFA with an acetonitrile gradient on a Vydac C_{18} RP column, 1 × 25 cm (The Separations Group, Hesperia, California) using a Gilson HPLC system (Gilson, Middleton, Wisconsin). The eluted phosphorylated/thiophosphorylated peptides were immediately neutralized with triethylamine, aliquoted and dried in a speedvac. Peptide identity was confirmed by mass spectrometry with an LCQ instrument (FinniganMat, San Jose, California).

Phosphohistidine analysis

Phosphorylation and thiophosphorylation of monomeric histidine were carried out as described above for the synthetic peptide. An aliquot of the phosphorylation reaction using potassium phosphoramidate was stopped after 15 min to preserve 1-phosphohistidine in the reaction mixture and immediately spotted onto a silica TLC plate (EM Science, Gibbstown, New Jersey). The TLC plates were developed in ethanol/20% ammonium hydroxide/water (65:12:18), dried at room temperature, and stained with a 0.2% ninhydrin (Sigma Aldrich) solution in ethanol.

Kinase reaction

The GST-*Sln1* expression plasmid, pGEX-4T-1, was obtained from Dr. Haruo Saito (Harvard Medical School) (Posas et al., 1996). *Escherichia coli* DH5α cells were initially grown in LB medium at 37 °C. For protein expression the cells were expanded in LB medium and expression induced with 100 μM isopropyl β-D-thiogalactopyranoside (IPTG) at RT. After 5 h the cells were spun down and the cell pellet immediately transferred into liquid nitrogen. Cells were lysed with phosphate buffered saline (PBS), 0.5% Tween-20, 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM dithiothreitol (DTT), and sonication for 2 min at 4 °C. The lysate was centrifuged at 14,000 RPM, and the supernatant applied to a 0.5 mL glutathione Sepharose (Amersham Pharmacia Biotech, Piscataway, New Jersey) column. After loading, the column was washed with 20 mL PBS/0.1% NP-40 and 10 mL 10 mM Tris-HCl, pH 8. The Sepharose beads containing the GST-*Sln1* fusion protein were then washed twice with kinase buffer (25 mM Tris-HCl, pH 8, 10 mM $MgCl_2$). For the kinase reaction 5 μCi of γ-^{32}P-ATP or γ-^{35}S-ATP (Amersham Pharmacia Biotech) were added to 10 μL Sepharose beads in kinase buffer and the reaction carried out for 3 h at room temperature. The resin beads were then washed with 10 mM Tris-HCl, pH 7.5 and the autophosphorylated fusion proteins eluted into 25 μL SDS sample buffer. Electrophoresis was carried out on a 10% SDS gel at 50 V for 12 h. Proteins in the gel were transferred to Immobilon membrane (Millipore, Bedford, Massachusetts) at 250 mA for 3 h. The membrane was dried and exposed to X-ray film at −80 °C and subsequently to a phosphorimager (Molecular Dynamics, Sunnyvale, California).

Peptide treatments

Aliquots (10 μg) of histidine-phosphorylated or -thiophosphorylated peptides were dissolved in aqueous HCl at various pH and treated for the times indicated. The mixtures were then injected onto a Vydac C_{18} RP column, 1 mm × 25 cm (The Separations Group) using an HP1090 HPLC system (Hewlett Packard, Palo Alto, California). A 30 min gradient of acetonitrile in 0.1% TFA was applied and peptides detected at 215 nm. Peak areas were integrated and used for the calculations.

Protein treatments

Individual Immobilon strips (Millipore) containing the histidine kinase fusion protein were treated under the conditions shown in the figure legends in 15 mL polypropylene tubes using 10 mL of the respective reagent. After the treatment the membrane strips were reassembled and exposed to X-ray film at −80 °C and subsequently to the phosphorimager (Molecular Dynamics) for quantitation. Finally, the membranes were stained with Amidoblack (Sigma Aldrich, St. Louis, Missouri) for protein comparison. All experiments were performed in duplicates.

Peptide mapping

Twenty micrograms of bovine liver NDPK (Sigma Aldrich) was subjected to an in vitro kinase reaction using 100 μL of kinase buffer and 5 μCi of γ-^{32}P-ATP or γ-^{35}S-ATP (Amersham Pharmacia Biotech), respectively, for 1 h at RT. Excess labeling reagent was then removed by Centricon (Amicon, Beverly, Massachusetts) centrifugation and washing the protein with 10 mM Tris-HCl, pH 7.5. The remaining labeled proteins were dissolved in 25 μL SDS sample buffer. The proteins were run on a 15% SDS gel and stained with Coomassie Blue (BioRad, Richmond, California). The gel was then destained with water/methanol/acetic acid (60:35:5),

air-dried and exposed overnight to X-ray film at −80 °C. The stained NDPK-containing gel slices were cut into small pieces and washed twice with 20 mM ammonium bicarbonate/acetonitrile (50:50). The gel pieces were then dried under a stream of nitrogen. For digestion 1 μg of trypsin (Promega, Madison, Wisconsin) in 50 μL of 20 mM ammonium bicarbonate was added and incubated overnight at 37 °C. Peptides were extracted twice with 0.1% TFA/acetonitrile (40:60) for 45 min at 37 °C. Aliquots of the resultant peptide mixtures were then either subjected to a Vydac 1 mm × 25 cm C_{18} RP-HPLC column (The Separations Group) or spotted onto a 20 × 20 cm cellulose plate (Baker, Phillipsburg, New Jersey). For HPLC the peptides were eluted with a linear gradient of acetonitrile in 0.1% TFA over 1 h at a flow rate of 100 μL/min. Peptide fractions were collected at 1 min, and 10% of each fraction was subjected to scintillation counting (Beckman, Fullerton, California). For TLE the cellulose plate was run for 2 h at 900 V in pyridine/acetic acid/acetone/water (1:2:8:40) using a high-voltage electrophoresis chamber (Savant Instruments, Holbrook, New York). After drying overnight the plate was subjected to TLC and developed in pyridine/n-butanol/acetic acid/water (50:75:15:60). After drying the cellulose plate was exposed overnight to X-ray film at −80 °C.

Edman degradation

Radiolabeled phosphopeptides in 5 μL of 0.1% TFA were spotted onto a Sequelon AA membrane and the peptides coupled to the membrane according to the manufacturer's instructions (PE Biosystems, Foster City, California). The membranes were then placed into the cartridge of a Procise Protein Sequencer, Model 492 (PE Biosystems) and subjected to automated Edman degradation. The released ATZ-amino acids were collected and scintillation counted.

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