Protein Science (1997), 6:1405–1411. Cambridge University Press. Printed in the USA.

Synthesis and characterization of histidine-phosphorylated peptides

KATALIN F. MEDZIHRADSZKY,[1] NANCY J. PHILLIPPS,[2] LIONEL SENDEROWICZ,[3] PING WANG,[4] AND CHRISTOPH W. TURCK[3,4,5]

[1]Department of Pharmaceutical Chemistry, Mass Spectrometry Facility, University of California San Francisco, School of Pharmacy, Parnassus, U-6, San Francisco, California 94143-0446
[2]Department of Pharmaceutical Chemistry, University of California San Francisco, School of Pharmacy, Parnassus, S-926, San Francisco, California 94143-0446
[3]Howard Hughes Medical Institute, University of California San Francisco, School of Medicine, Parnassus, U-426, San Francisco, California 94143-0724
[4]Department of Medicine, University of California San Francisco, School of Medicine, Parnassus, U-426, San Francisco, California 94143-0724
[5]Cardiovascular Research Institute, University of California San Francisco, School of Medicine, Parnassus, U-426, San Francisco, California 94143-0724

(RECEIVED November 13, 1996; ACCEPTED April 14, 1997)

Abstract

Posttranslational phosphorylation of proteins is an important event in many cellular processes. Whereas phosphoesters of serine, threonine, and tyrosine have been studied extensively, only limited information is available for other amino acids modified by a phosphate group. The formation of phosphohistidine residues in proteins was discovered originally in prokaryotic organisms, but also has been found recently in eukaryotic cells. We describe methods for the synthesis and analysis of phosphohistidine-containing peptides, a prerequisite for the investigation of the role of this posttranslational modification in cellular processes.

Keywords: histidine phosphorylation; mass spectrometry; peptide synthesis; posttranslational modification

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 in 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). In prokaryotes, several signal transduction events are governed by so called two-component systems that involve the formation of phosphohistidine (H(p)) 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 component, the response regulator. Phosphorylation of the response regulator leads to an output signal such as transcriptional activation or protein–protein interaction (Alex & Simon, 1994).

It is estimated that phosphohistidine may account for approximately 6% of total protein phosphorylation in prokaryotes as well as eukaryotes (Matthews, 1995). Although not as abundant as serine and threonine phosphorylation, it apparently is much 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).

The phosphorylation of histidine residues has also been observed in eukaryotic cells. The Sln1 protein in yeast that is involved in osmosensing shares high amino acid homology with the bacterial two-component protein systems, including a histidine that has been shown to undergo autophosphorylation in vivo (Ota & Varshavsky, 1993; Posas et al., 1996). Sln1 is part of a "two-component" osmosensor system that is regulated by a multistep phosphorelay mechanism involving three proteins. It has been shown that, in this system, the phosphate group is first transferred from a phosphohistidine in Sln1 to an aspartic acid residue of the same protein. Subsequently, the phosphate is transferred from the aspartic acid residue of Sln1 to a histidine of Ypd1 and finally again to

Reprint requests to: Christoph W. Turck, University of California San Francisco, School of Medicine, Howard Hughes Medical Institute, Parnassus, U-426, San Francisco, California 94143-0724; e-mail: turck@itsa.ucsf.edu.

an aspartic acid residue within Ypd1. This phosphorelay mechanism was found in vivo and the nature of the phosphorylated residue, i.e., histidine or aspartic acid, was confirmed by its lability toward acid and base, respectively (Posas et al., 1996). The *Arabidopsis thaliana* gene, ETR1, also encodes a histidine kinase, which mediates the ethylene response (Chang et al., 1993). Recently, phosphohistidine was even detected in mammalian cells. Phosphorylation of a histidine on the cytoplasmic tail of P-selectin takes place following platelet activation with thrombin or collagen (Sweeney Crovello et al., 1995). Thus, it appears that this post-translational modification is becoming increasingly apparent in many systems that are related to signal transduction. Methods to study histidine phosphorylation in proteins are therefore needed. The difficulties in developing these methods have their origin in the high-energy state of the phosphate imidazole bond, which resembles that of a phosphoramidate. Phosphohistidine is therefore not as stable as its phosphoester relatives and hydrolyzes readily under acidic conditions (Wei & Matthews, 1991).

One method that has facilitated greatly the analysis of serine, threonine, and tyrosine phosphorylations in proteins has been the generation of synthetic phosphopeptides. These peptides provide powerful probes not only in mapping the sites of phosphorylation, but also in delineating phosphorylated residues that are involved in protein–protein interactions (Escobedo et al., 1991; Fantl et al., 1992; Kavanaugh et al., 1995).

In this report, we describe a method that allows the synthesis and purification of histidine-phosphorylated peptides. In addition, we outline strategies for the characterization of these peptides by mass spectrometry. Both methods will facilitate studies on the role of histidine-phosphorylation in signal transduction and other cellular processes.

Results

For the synthesis of histidine-phosphorylated peptides, we used the potassium phosphoramidate method, which had been developed for the synthesis of the phosphohistidine monomer (Wei & Matthews, 1991; Hultquist et al., 1966). Potassium phosphoramidate is a relatively mild phosphorylating reagent and does not lead to modification of any of the hydroxyamino acids, serine, threonine, and tyrosine (Wei & Matthews, 1991; Hultquist et al., 1966). Yields for peptide histidine phosphorylation were 60–80%.

For the current study, we synthesized four model peptides that are all derived from the human tyrosine phosphatase, PTP-PEST. The reason for selecting PTP-PEST-derived peptide sequences is a recent report that suggests the involvement of a phosphohistidine residue of PTP-PEST in the protein's association with the adaptor protein *shc* in *NIH 3T3* cells (Charest et al., 1996). The *shc* protein has been shown to play important roles in the formation of protein complexes in signal transduction events of tyrosine kinase receptors (Bonfini et al., 1996). These associations are mediated by *src* homology 2 (SH2) and phosphotyrosine-binding (PTB) domains within *shc* that have high affinities for phosphotyrosine residues. The report by Charest et al. suggests that PTP-PEST does not associate with the PTB domain of *shc* via a phosphotyrosine, but presumably a phosphorylated histidine residue at sequence position 602. This residue is located in a PTB domain-specific consensus sequence motif that normally includes phosphotyrosine (Kavanaugh et al., 1995) instead of phosphohistidine. This leads the authors to speculate that phosphohistidine can replace phosphotyrosine in the binding of PTP-PEST to the *shc* PTB domain (Charest et al., 1996). The synthetic phosphohistidine-containing peptides will provide us with probes to investigate this association in greater detail.

The success of the postsynthesis histidine-phosphorylation reaction with potassium phosphoramidate was assessed initially by reversed-phase HPLC (RP-HPLC). Due to the acid lability of phosphohistidine, we performed the chromatography on a pH-stable RP-HPLC column using an ammonium bicarbonate solvent system. In all cases, we found excellent resolution of phosphorylated from unphosphorylated peptide under these conditions. Figure 1B shows a typical chromatogram of the phosphorylation reaction mixture of peptide Ac-PLSFTNPLHSDDWH-NH_2. The histidine-phosphorylated peptide always eluted at lower acetonitrile concentrations than its unphosphorylated counterpart. In some cases, a third peak eluted before the mono-phosphorylated peptide and possibly represents a N1,N3-diphosphorylated histidine peptide (data not shown). This earlier eluting peptide was unstable. Rechromatography showed that it converted completely into the mono-phosphorylated histidine peptide after 24 h at 4 °C (data not shown). The observation is consistent with data obtained from the synthesis of the phosphohistidine monomer, which also results in the generation of unstable diphosphorylated histidine (Wei & Matthews, 1991). The peak eluting at 5 min in Figure 1B corresponds to potassium phosphoramidate, which was used in the phosphorylation reaction. As mentioned above, the reaction with potassium phosphoramidate does not lead to phosphorylation of hydroxyamino acids. This was confirmed by mass spectrometry analyses described below, where, in all cases, the only site of phosphorylation that was found was the imidazole ring of histidine. Due to the use of the mild phosphorylating reagent potassium phosphoramidate, no selective protection of the hydroxyamino acids during the phosphorylation reaction is necessary.

Phosphohistidine can be identified by peptide base hydrolysis and subsequent amino acid analysis (Wei & Matthews, 1991). However, this procedure only leads to partial hydrolysis of peptide bonds and the destruction of certain amino acids. We therefore tried to develop alternative ways to examine these peptides by several mass spectrometry techniques. Peptides were analyzed by electrospray ionization mass spectrometry (ESI-MS) and by matrix-assisted laser desorption ionization (MALDI) mass spectrometry.

Figure 2 shows the ESI-MS mass spectra of the phosphorylated peptide Ac-PLSFTNPLHSDDWH-NH_2 in positive- (Fig. 2A) and negative-ion modes (Fig. 2B). In positive-ion mode, doubly charged ions at *m/z* 916.3, 927.3, 938.2, 949.3, 960.7 represent the phosphopeptide coordinating 2–6 Na ions, respectively. Similarly, a series of doubly charged ions were detected for the dephosphorylated peptide, with 1–4 Na ions: 865.3, 876.3, 887.7, 898.8. Some of the components exhibited triply charged ions as well (*m/z* 592.1, 633.6). In negative-ion mode, the most abundant ions for both the phosphopeptide and its dephosphorylated counterpart are the doubly charged ions, not containing any metal ions at *m/z* 892.5 and 852.3, respectively.

Because MALDI usually induces fragmentation, the peptides were analyzed both in linear and reflectron modes. In-flight metastable decomposition does not affect the masses detected in linear experiments, whereas the products of this decomposition appear as wider, nonfocused peaks in the reflectron spectra. Figure 3 shows the positive-ion MALDI spectrum of the histidine-phosphorylated peptide Ac-SFTNPLH(p)AAA-NH_2 acquired in linear mode. The matrix in this experiment was α-cyano-4-hydroxycinnamic acid (CHCA). All the abundant ions in the spectrum represent the de-

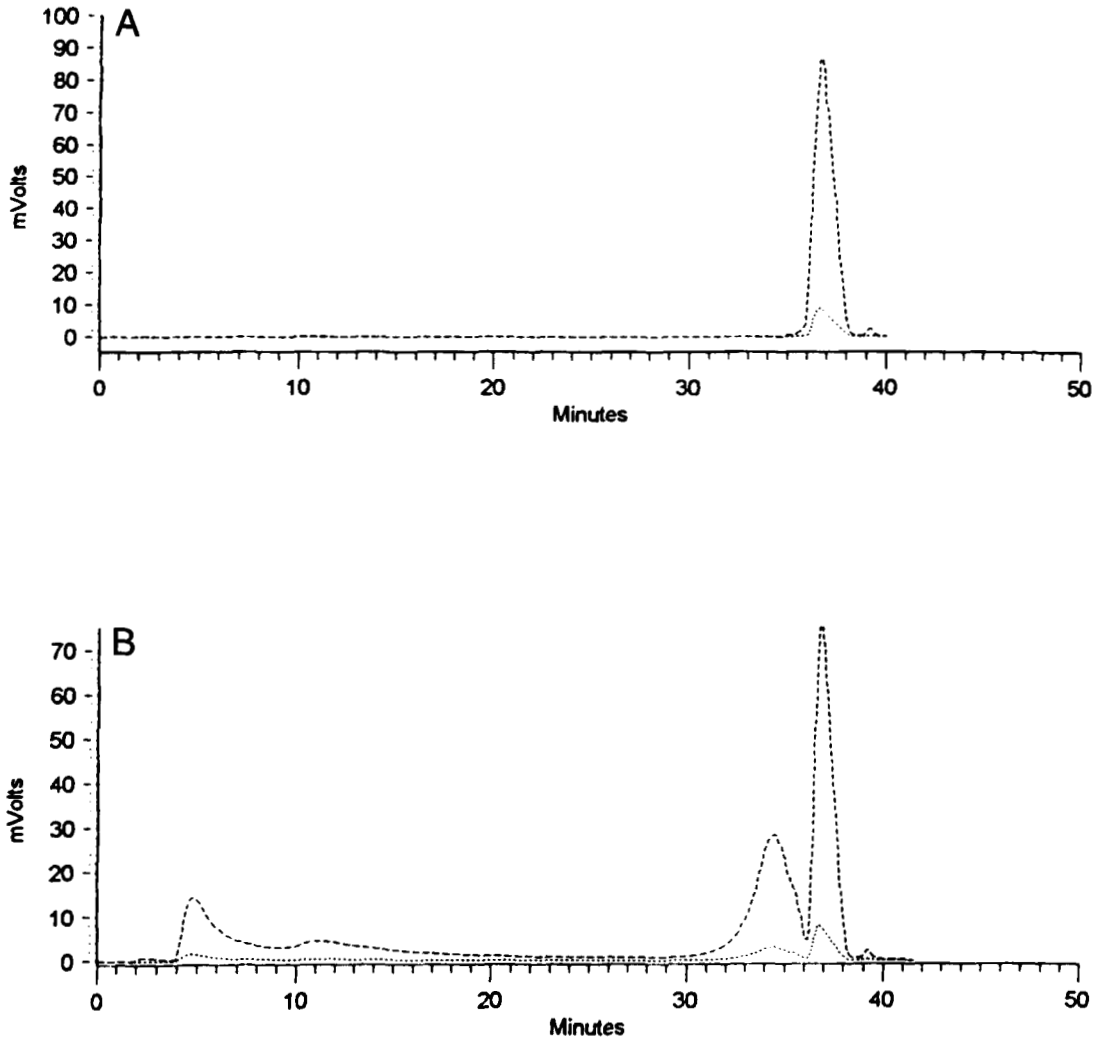

Fig. 1. RP-HPLC profile of peptide Ac-PLSFTNPLHSDDWH-NH_2 (**A**) before and (**B**) after phosphorylation with potassium phosphoramidate. Peptides were chromatographed in an ammonium bicarbonate, pH 7 solvent system on a pH-stable RP-HPLC column with an ascending linear gradient of acetonitrile. Peptides were detected at 215 nm (coarse dashed line) and 280 nm (fine dashed line).

phosphorylated peptide: 1,070.2 is MH^+, whereas the ions at *m/z* 1,094.3 and 1,110.3 are the Na- and K-adducts, respectively. Figure 4 shows the negative-ion MALDI spectrum of the same sample, acquired in reflectron mode. The ion at *m/z* 1,147.9 represents the deprotonated molecular ion of the phosphopeptide.

In order to establish the amino acid sequence of a peptide and to determine the site of phosphorylation, fragmentation of the singly or multiply charged molecular ions has to be induced. In collision-induced dissociation (CID) analysis, the ion to be activated is collided with chemically inert gas atoms, leading to fragmentation along the peptide backbone and in the amino acid side chains. Low-energy CID experiments were performed on some of the electrospray-generated protonated molecular ions of the phosphohistidine-containing peptides. However, the low-energy CID spectra did not yield sufficient information on the peptide's structure and the site of phosphorylation. While electrospray-generated ions are not suitable for high-energy CID experiments that could yield more complete sequence information, MALDI–high-energy CID has been used successfully for the analysis of peptides. However, no MH^+ was detected for the phosphohistidine-containing peptides in positive-ion MALDI analysis, when CHCA or 2,5-dihydroxy-benzoic acid (DHB) were used as matrices. It has been reported that a mixture comprising of 2,6-dihydroxy-acetophenone (DHAP) and di-ammonium hydrogen citrate (DAHC) is a suitable matrix for the MALDI analysis of a series of chemically labile compounds (Gorman et al., 1996). Using this "cooler" matrix, we eventually succeeded in obtaining molecular ions of the phosphorylated histidine-containing peptides in reflectron mode, although abundant ions were still detected due to dephosphorylation (Fig. 5). Using the MALDI-generated molecular ion as precursor, the metastable decomposition of this phosphohistidine-containing peptide was studied by recording its "post source decay" (PSD) spectrum. Similarly, its high energy-CID spectrum was also recorded. However, the PSD did not yield sufficient information on the peptide's structure. Figure 6 shows the MALDI–high-energy CID spectrum of the peptide Ac-SFTNPLH(p)SAAW-NH_2. The low-mass region of such a spectrum usually contains a wealth of information on the amino acid composition of the peptide in the form of immonium ions with a structure of $^+NH_2{=}CH{-}R$. The immonium ion for the modified histidine indeed was detected at

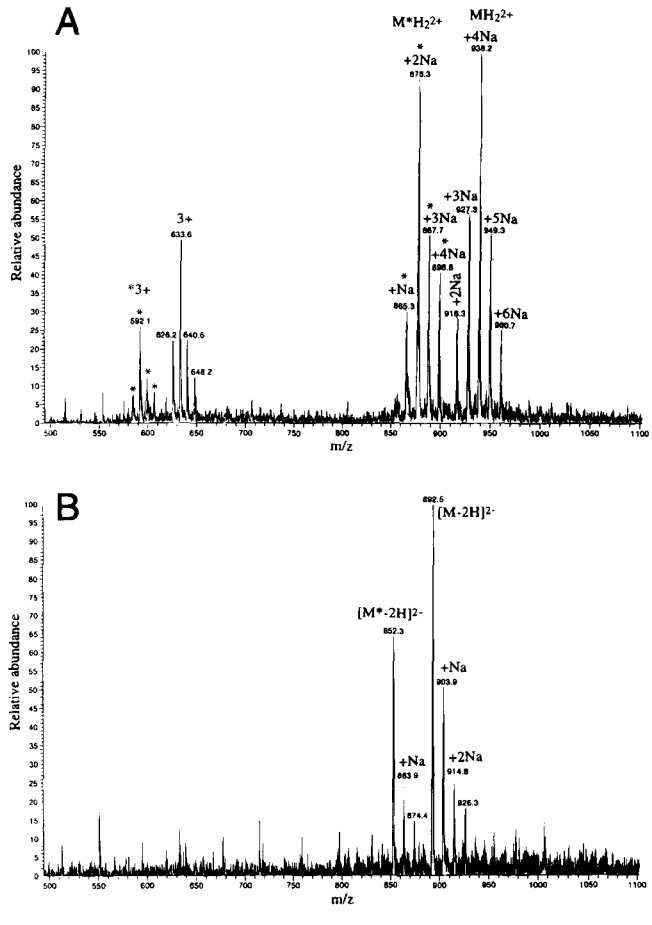

Fig. 2. Electrospray ionization mass spectrum of the phosphorylated peptide Ac-PLSFTNPLHSDDWH-NH_2 in (**A**) positive-ion and (**B**) negative-ion modes. Asterisks designate signals derived from the dephosphorylated peptide.

m/z 190, although gas-phase dephosphorylation seems to be a preferred fragmentation step, and the unphosphorylated histidine ion was detected as well (*m/z* 110). Immonium ions at *m/z* 60, 70, 74, 86, 120, and 159 indicate the presence of the other amino acids

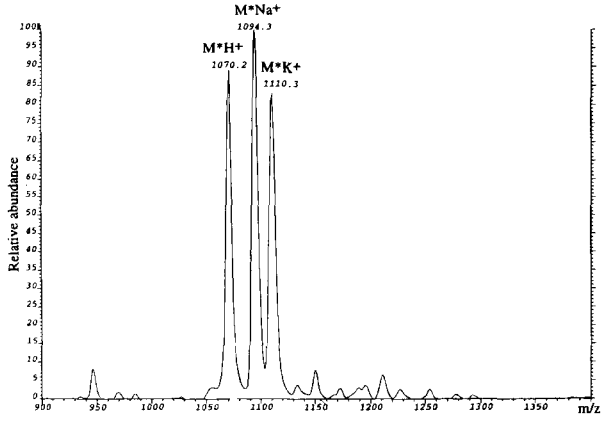

Fig. 3. Positive-ion MALDI spectrum of peptide Ac-SFTNPLH(p)AAA-NH_2 acquired in linear mode. CHCA was used as matrix.

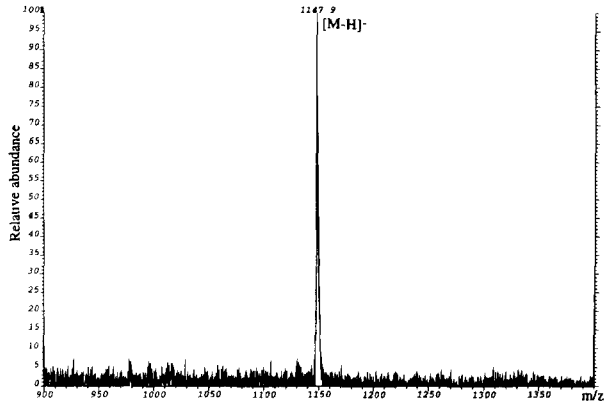

Fig. 4. Negative-ion MALDI spectrum peptide Ac-SFTNPLH(p)AAA-NH_2 acquired in reflectron mode. CHCA was used as matrix. Only the phosphorylated species is detected in this mode.

in the peptide (S, P, T, L/I, F, and W, respectively). Larger fragments formed via cleavages along the peptide backbone can yield sequence information. In the peptide studied, this fragmentation occurred mostly at the peptide bounds, yielding **b**- and **y**-type ions with charge retention at the amino- and the carboxy-termini, respectively. Carboxy-terminal sequence ions (numbered from the carboxy-terminus) $\mathbf{y_7}$ (at *m/z* 860), $\mathbf{y_8}$ (974), $\mathbf{y_9}$ (1,075), and $\mathbf{y_{10}}$ (1,222) showed the presence of the phosphate group (80-Da shift in comparison to the unmodified peptide). The same was true for the amino-terminal sequence ions $\mathbf{b_8}$ (1,006) and $\mathbf{b_{10}}$ (1,148). Altogether the presence of these phosphorylated fragments indicate that the modification must have occurred on the LHS sequon (Fig. 6). Some of the same sequence ions were also detected gas-phase dephosphorylated, i.e., at an 80 Da lower *m/z* value (for example, $\mathbf{y_7^*}$ at *m/z* 780). However, no satellite ions were observed at an 98 Da lower *m/z* value than the phosphorylated fragment ions. These fragments that are formed by β-elimination of the phosphate ester are characteristic of serine- and threonine-phosphorylated peptides. A further indication that the hydroxy-amino acids are, in fact, unmodified is the presence of abundant satellite ions formed by water loss from amino-terminal sequence ions at *m/z* 259 ($\mathbf{b_2}$-18), 360 ($\mathbf{b_3}$-18), and 474 ($\mathbf{b_4}$-18). Gas-phase dephosphorylation of the molecular ion was also ob-

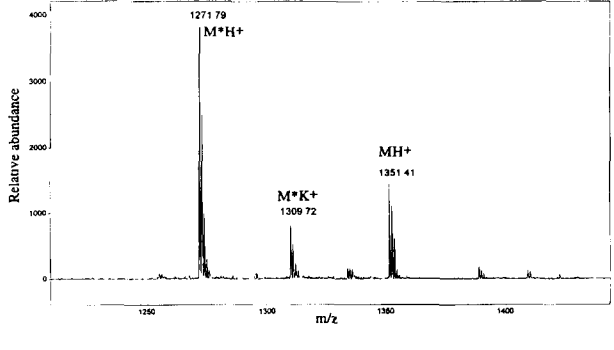

Fig. 5. Positive-ion MALDI spectrum of peptide Ac-SFTNPLH(p)SAAW-NH_2 in reflectron mode, with delayed extraction. DHAP/DAHC was used as matrix. Asterisks designate signals derived from the dephosphorylated peptide.

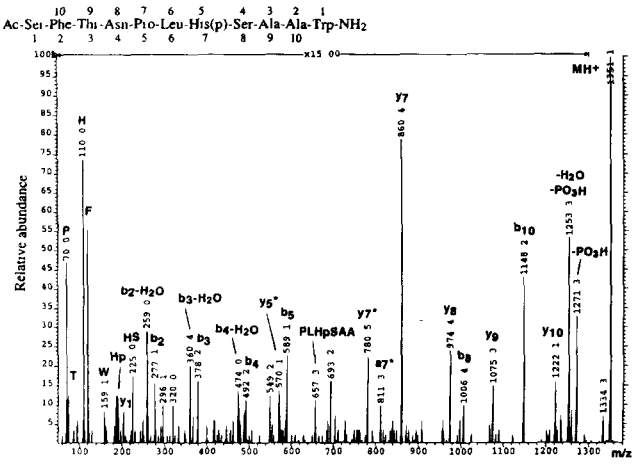

Fig. 6. MALDI-high energy CID spectrum of peptide Ac-SFTNPLH (p)SAAW-NH_2. DHAP/DAHC was used as matrix. Signals labeled with an asterisk represent gas-phase dephosphorylated ions.

served (*m/z* 1,271 = MH^+-80 Da), whereas another abundant ion at *m/z* 1,253 (MH^+-98 Da) corresponds to the cleavage of the phosphate group and water loss from one of the hydroxy-amino acids, respectively.

Previous studies by others have shown that, during phosphohistidine synthesis using potassium phosphoramidate, the N1-phosphohistidine is generated first, followed by N1,N3-diphosphohistidine and then N3-phosphohistidine (Wei & Matthews, 1991; Hultquist et al., 1966). In order to investigate the site of phosphorylation after treatment of the peptides with potassium phosphoramidate, we subjected the peptide Ac-SFTNPLHAAA-NH_2 to proton NMR. Previous NMR studies on histidine-phosphorylated proteins had revealed that the C2 and C4 protons of the imidazole ring show characteristic differences in their chemical shifts, depending on which of the two nitrogens is phosphorylated (Gassner et al., 1977). Figure 7 shows the proton NMR spectra of the peptide Ac-SFTNPLHAAA-NH_2 before (Fig. 7A) and after 24-h treatment (Fig. 7B) with potassium phosphoramidate. Two additional signals are apparent for the imidazole C2 (δ 7.738) and C4 (δ 7.091) protons after phosphorylation. The downfield shifts of those signals at pH 10.2 are consistent with an N3-phosphorylated histidine (Gassner et al., 1977). These results agree with studies by others, which showed that, after 23 h, the major product of histidine phosphorylation is the N3 isomer (Wei & Matthews, 1991; Hultquist et al., 1966).

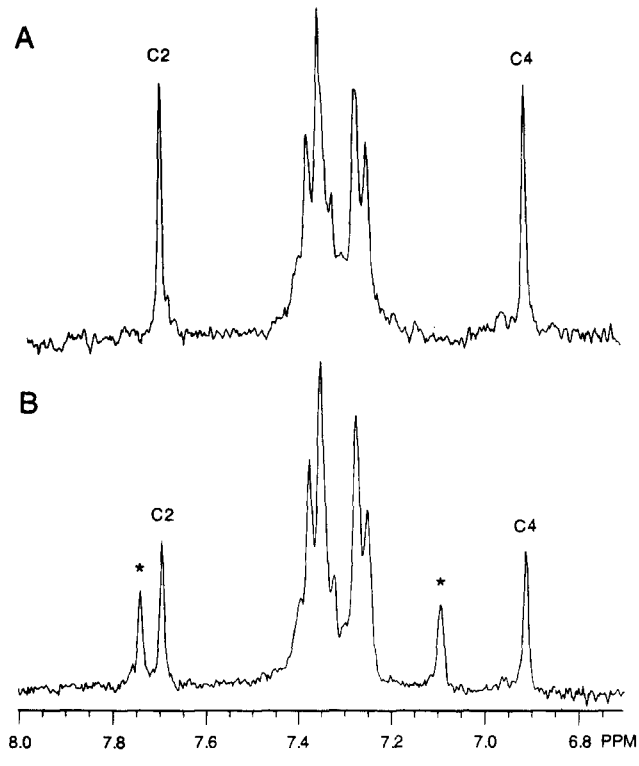

Fig. 7. Sections of the 300-MHz proton NMR spectra of the peptide Ac-SFTNPLHAAA-NH_2 (**A**) before and (**B**) after treatment with potassium phosphoramidate, recorded at pH 10.2. The signals labeled C2 and C4 correspond to the C2 and C4 protons of the imidazole ring of unphosphorylated histidine, respectively. The signals labeled with an asterisks are derived from the C2 and C4 protons of the N3-phosphorylated histidine.

Discussion

Histidine-phosphorylation plays an important role in prokaryotic signal transduction events in two-component systems (Alex & Simon, 1994). The recent identification of phosphohistidine in eukaryotic cells and its involvement in signal transduction cascades (Chang et al., 1993; Ota & Varshavsky, 1993; Sweeney Crovello et al., 1995; Posas et al., 1996) prompted us to develop methods for the synthesis and analysis of phosphohistidine-containing peptides. These procedures 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-specific protein modules, such as SH2 and PTB domains (Escobedo et al., 1991; Fantl et al., 1992; Kavanaugh et al., 1995). Tyrosine-phosphorylated peptides are able to compete for the association of SH2 or PTB domains with a phosphotyrosine-containing protein, such as a growth factor receptor.

A recent report describes the association of the phosphotyrosine-specific PTB domain of *shc* with the tyrosine phosphatase PTP-PEST in *NIH 3T3* cells (Charest et al., 1996). The association is mediated by the sequence ^{599}NPLH^{602} of PTP-PEST, which resembles the consensus binding sequence for the PTB domain, NPXY(p) (Y(p) = phosphotyrosine). Although not proven, the authors of the paper speculate that the histidine residue in PTP-PEST is phosphorylated. This modification may lead to an enhanced association with the PTB domain of *shc*. Our ability to synthesize and analyze phosphohistidine-containing peptides will allow us to investigate this association in more detail and address the question of whether phosphohistidine can replace phosphotyrosine in the NPXY(p) motif that confers high binding affinity to the PTB domain of *shc* (Kavanaugh et al., 1995).

The synthesis and analysis of phosphohistidine-containing peptides is difficult due to the lability of the phosphate on the imidazole ring. The phosphoramidate approach, in combination with RP-HPLC at neutral conditions, enabled us to synthesize phosphohistidine-containing peptides chemically. Proton NMR analysis of one peptide revealed that the phosphorylation reaction resulted in the N3-histidine-phosphorylated species. Mass spectrometry

revealed that, although the phosphate gets cleaved to different extents during the analysis depending on the conditions used, the molecular ion of the histidine-phosphorylated peptide species can be detected.

RP-HPLC-purified histidine-phosphorylated peptides were analyzed by ESI and MALDI mass spectrometry in positive- and negative-ion modes. ESI-MS is usually the method of choice for the analysis of labile structures because it normally does not induce fragmentation. While analyzing the phosphopeptides, both positive- and negative-ion ESI-MS spectra exhibited ions due to dephosphorylation. The dephosphorylation may be the result of decomposition upon storage, as well as fragmentation in the electrospray source. Furthermore, the salt content of the samples leads to multiple metal-adduct formation in positive-ion ESI-MS analysis, that may prevent unambiguous molecular weight determination. In contrast to the soft ESI, it has been reported that MALDI may induce extensive fragmentation. Phosphorylated serine- and threonine-containing peptides usually do not show decomposition in CHCA or DHB when analyzed in positive-ion, linear mode, whereas an abundant metastable peak is detected for most of them besides the molecular ion in reflectron mode. Under these conditions, phosphohistidine-containing peptides displayed only ions present due to dephosphorylation either chemically or promptly upon ionization. We found that the molecular weight determination of these peptides can be performed most successfully by MALDI in negative-ion mode. It is somewhat surprising that, although the MALDI technique usually induces extensive fragmentation, the peptides were detected even in negative-ion reflectron mode without significant dephosphorylation. Similar findings have been reported for labile acidic glycoconjugates, which also show a lower metal-adduct formation tendency in negative-ion MALDI analysis (Gibson et al., 1996).

CID analysis can be utilized for amino acid sequence and phosphorylation site determinations. It has been reported that low-energy CID spectra obtained from electrospray-generated ions usually yield only partial sequence information and extensive gas-phase dephosphorylation for phosphorylated serine- or threonine-containing peptides (Busman et al., 1996). Unfortunately, not all the phosphorylated histidine-containing peptides exhibited abundant enough MH^+ ions by MALDI suitable for high-energy CID analysis when the DHAP/DAHC matrix was used. Interestingly, high-energy CID spectra of the phospho-histidine-containing peptides featured an immonium ion for the modified histidine at *m/z* 190. Phosphoserine or -threonine residues do not exhibit the posttranslationally-modified immonium ions. In addition, phosphorylated serine- or threonine-containing peptides show abundant ions due to the β-elimination of the phosphate ester (i.e., at an *m/z* value 98 Da lower than the corresponding phosphorylated sequence ions), whereas, for phosphorylated histidine, similar satellite ions were observed 80 Da lower than the phosphorylated fragments.

The ability to determine phosphorylated histidine residues by mass spectrometry will greatly aid in the identification of these residues in proteins derived from prokaryotic and eukaryotic cells. Furthermore, the synthesis of peptides containing phosphohistidine residues will allow us to identify phosphohistidine-specific protein domains involved in protein–protein interactions. Similar associations of phosphotyrosine-specific protein domains (SH2 and PTB) have been shown to play important roles in tyrosine kinase receptor signal transduction events (Ullrich & Schlessinger, 1990; Kavanaugh et al., 1995).

Materials and methods

Peptide synthesis

Peptides were synthesized using the Fmoc chemistry. After cleavage and deprotection, the peptides were purified by RP-HPLC and lyophilized. Peptide identity was confirmed by mass spectrometry. For phosphorylation, 0.5 mg of the deprotected and lyophilized peptide was dissolved in 10 mM ammonium bicarbonate and the pH was adjusted to 8 with 0.1 M NaOH. Ten milligrams of potassium phosphoramidate were added and the mixture was tumbled overnight at room temperature (Wei & Matthews, 1991; Hultquist et al., 1966). Purification of the peptides was performed on a Vydac pH-stable reversed-phase column (The Separations Group, Hesperia, California) using 10 mM ammonium bicarbonate as solvent A and acetonitrile/10 mM ammonium bicarbonate (9:1) as solvent B. A linear gradient was applied, increasing solvent B concentration from 0% to 35% over 60 min at a flow rate of 0.5 mL/min. Peptides were detected at 215 nm and 280 nm. The eluted peptides were stored in solution at 4 °C.

Mass spectrometry

Electrospray experiments were conducted on a Micromass Quattro triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a nanoflow electrospray source or on a LCQ iontrap mass spectrometer (FinniganMat, San Jose, California). The low-energy CID experiments were performed using the triple quadrupole mass spectrometer. The collision gas was Ar, the collision energy was approximately 50 eV. MALDI experiments were performed either on a Micromass TofSpec SE (Micromass) or on a PerSeptive BioSystems Voyager Elite time-of-flight mass spectrometer (Perseptive Biosystems, Framingham, Massachusetts). Samples were analyzed both in linear and reflectron modes. The CZE-standard peptide mixture (Biorad, Richmond, California) was used for external calibration. Matrices used were CHCA and DHB (Hewlett-Packard, Palo Alto, California) or a mixture comprising of DHAP and DAHC (Aldrich, St. Louis, Missouri). An aliquot of the 1 M DAHC solution in water was mixed with a 10 mg/mL solution of DHAP in ethanol-acetonitrile (1:1, v/v) at a ratio of 1:10 (v/v) (Gorman et al., 1996). Usually 1 μL of the sample solution was mixed with 1 μL of matrix solution and this mixture was deposited onto the target surface. MALDI–high-energy CID experiments were performed on a Micromass AutoSpec SE-orthogonal acceleration time-of-flight tandem mass spectrometer equipped with a MALDI source (Micromass). The collision gas was Xe, the collision energy was 800 eV (Medzihradszky et al., 1996). For these experiments, the DHAP/DAHC mixture was used as the matrix.

NMR spectroscopy

^{1}H-NMR spectra were recorded on a General Electric QE-300 MHz spectrometer at 22 °C. Peptide samples were first lyophilized twice from 99.96% D_2O (Aldrich, St. Louis, Missouri), and then dissolved in 0.5 mL of 99.996% D_2O (Cambridge Isotope Laboratories, Andover, Massachusetts) and adjusted to pH 10.2 with NaOD. A trace of sodium 3-(trimethylsilyl) $^2H_4$ propionate (Sigma, St. Louis, Missouri) was added as an internal reference.

Acknowledgments

This work was supported by the Howard Hughes Medical Institute (C.T.) and grants NIH NCRR BRTP RR01614 and NSF DIR 8700766 (A.L. Burlingame, P.I.). We thank David Maltby and Jenny Kong for technical assistance and Micromass for the loan of the nanoLC probe.

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