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Mammalian Histidine Kinases: Do They REALLY Exist?†

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I. Introduction

Protein phosphorylation is one of the most extensively investigated forms of post-translational modification. Its significance has been demonstrated by the critical role that kinases and phosphatases play in the regulation of most aspects of eukaryotic cellular function. To date, most research on protein kinases has focused on the hydroxyamino acid protein kinases, namely, tyrosine and serine/threonine kinases, with the number of known kinases of these types having increased enormously over the last three decades. However, early reports in the literature indicated that the proportion of total phosphoamino acids present as phosphohydroxyamino acids in mammalian cells was only ~50% (*1*−*3*). This finding indicates the presence of cellular proteins that are phosphorylated on residues other than serine, threonine, and tyrosine and that these types of phosphorylation are abundant. A more recent analysis of basic nuclear proteins from *Physarum polycephalum* showed that 6% of the phosphoamino acids present in these proteins was phosphohistidine (ref *4*, as quoted in ref *5*). Thus, at least in this subset of proteins, the proportion of phosphoamino acids present as phosphohistidine is ~2 orders of magnitude greater than the cellular abundance of phosphotyrosine (*6*). The known importance of protein phosphorylation to cellular function and the relative abundance in cellular proteins of phosphoamino acids other than the phosphohydroxyamino acids suggests that kinases that catalyze these phosphorylations, including histidine kinases, may have an impact on cellular function similar to those of the better known tyrosine and serine/threonine kinases.

In phosphohydroxyamino acids, the phosphate is covalently bound to the amino acid via an O−P phosphoester bond (see Figure 1) which is stable under the acidic conditions that are used in many research techniques used to study protein phosphorylation, such as partial acid hydrolysis of phosphoproteins, TCA[1] precipitation of phosphoproteins, SDS−PAGE gel staining and fixation procedures, etc. In phosphohistidine, however, the phosphate group is bound to the amino acid via an N−P phosphoramidate bond which is acid-labile (see Figure 1). Thus, many of the techniques used to study the hydroxyamino acid protein kinases cannot be used to study histidine kinases. Figure 1 also shows that there are three possible forms of phosphohistidine; both the 1-phospho and 3-phospho forms have been reported to occur in mammalian cellular phosphoproteins (*7*−*12*) and *P. polycephalum* (*13*), but there have been no reports of the 1,3-diphospho form.

Although protein histidine phosphorylation in mammalian cells has been known to exist for more than 40 years, little research has been performed to investigate mammalian histidine kinases, in comparison to that focused on serine/threonine and tyrosine kinases. One of the reasons for this

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[1] Abbreviations: TCA, trichloroacetic acid; PCNA, proliferating cell nuclear antigen; DEPC, diethyl pyrocarbonate; BCKDHK, branched-chain α-ketoacid dehydrogenase kinase; PDHK, pyruvate dehydrogenase kinase.

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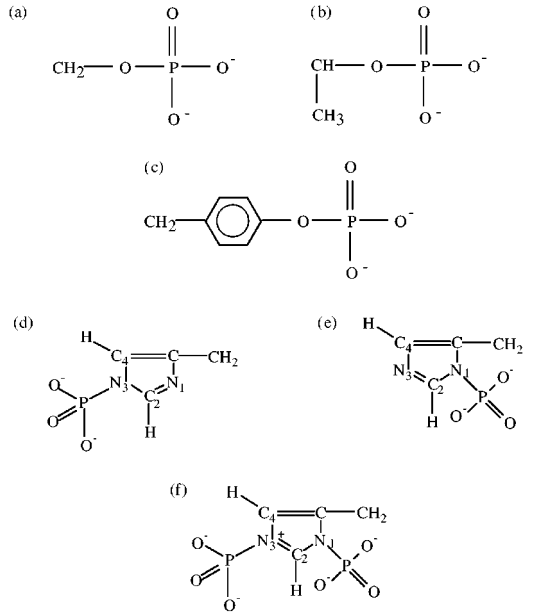

FIGURE 1: Structures of the phosphoamino acid functional groups: (a) phosphoserine, (b) phosphothreonine, (c) phosphotyrosine, (d) 1-phosphohistidine, (e) 3-phosphohistidine, and (f) 1,3-diphosphohistidine.

lack of research activity stems from the fact that we still know very little about the cellular biology of mammalian protein histidine phosphorylation. In addition, the acid-labile nature of the N−P bond in phosphohistidine has meant that new techniques have had to be developed to study this type of phosphorylation.

Readers of this article will probably be aware that there is a well-known group of histidine kinases, namely, the so-called two-component histidine kinases (for recent reviews, see refs *14* and *15*). These kinases were discovered in prokaryotes, but in more recent years, similar enzymes have been found in lower eukaryotic organisms such as yeast, *Dictyostelium discoideum*, and plants. The majority of these histidine kinases are environment-sensing plasma membrane receptors that are somewhat akin to the well-known tyrosine kinase receptors in vertebrate cells in that in response to fluctuations in the extracellular environment, e.g., changes in osmolarity, ethylene concentration, and heat shock, they autophosphorylate on a histidine residue. This phosphoryl group is then transferred to an aspartate residue either on another domain of the receptor protein or on a separate response regulator protein. In the simplest systems, the phosphorylated response regulator protein induces the cellular response, usually by regulating gene transcription. More complex systems exist in which there is further transfer of the phosphoryl group from aspartate to histidine on another protein and back to aspartate on yet another protein.

To date, only two vertebrate proteins, branched-chain α-ketoacid dehydrogenase kinase and pyruvate dehydrogenase kinase, have been found to share sequence homology with that of the two-component histidine kinases (*16*), and the evidence that these proteins autophosphorylate on a histidine residue will be briefly discussed later in this paper. This review is primarily focused on histidine kinases that are similar to the better-known mammalian protein kinases where a separate, different substrate protein is phosphorylated. Studies which report the evidence of this type of enzyme will be presented, along with methodological developments in light of the renewed interest in this area of research. In addition, evidence for histidine phosphatases will be discussed as well as the possible biological roles of histidine kinases.

2. Histone H4 Phosphorylation

The covalent modification of histone proteins has been shown to occur in response to various physiological cues. Serine phosphorylation of histones H1 and H3, in particular, has undergone intense investigation with reference to cell division (*17*−*19*). However, reports over the past decade have also documented the existence of histone H4 kinases in various cells such as neutrophils (*20*−*22*), reticulocytes (*23*), and lymphoid cells (*24*) as well as the placenta (*25*−*27*). These are all histone H4 kinases that share some remarkable similarities. For example, all have a requirement for proteolytic cleavage in vitro (*22*, *25*, *28*) and in vivo (*24*) for activation. Cellular signaling pathways that lead to histone H4 kinase activation are not clear, although in neutrophils this occurs in response to chemotactic stimuli (*20*) downstream of phosphatidylinositol-3-kinase activation (*26*). The histone H4 kinases described above are all serine kinases; however, the existence of inducible histone H4 kinases suggests that this type of covalent modification plays a role in the regulation of some cellular processes, even though these processes are as yet unclear.

3. Histidine Phosphorylation on Histone H4

Early reports of histone H4 phosphorylation on histidine residues originated from Smith et al. (*2*). Studies analyzing the phosphorylation of histones by nuclear proteins from a variety of rat tissues revealed two histone kinases, which were distinct on the basis of substrate specificity and pH optima for maximal activity. One of them phosphorylated histone H4 with a pH optimum of 9.5 (*2*). Partial purification and characterization of this enzyme from a Walker 256 carcinosarcoma cell line revealed other distinguishing qualities such as its susceptibility to inhibition by GTP and CTP and an absolute requirement for Mg^{2+} (*29*). Phosphoamino acid analysis and the acid lability of the phosphorylated histone H4 led to the conclusion that the site of phosphorylation was on a histidine residue (*2*, *11*, *12*). The isoform of phosphohistidine found in phosphorylated histone H4 varied with respect to the tissue source of the kinase. ^{31}P NMR data demonstrated that the enzyme obtained from regenerating rat liver catalyzes the formation of 1-phosphohistidine, while the carcinosarcoma histidine kinase catalyzes the formation of 3-phosphohistidine (*12*). The significance of these differences with reference to possible function is currently unresolved. However, the histidine kinase from both tissue sources appears to phosphorylate both of the histidine residues in histone H4 (H18 and H75) (*12*, *29*). A common substrate recognition sequence adjacent to the target histidines is not obvious, and the similarity of the amino acid sequence around each histidine is limited to a single Lys residue, two amino acids C-terminal to these residues (see Figure 2). A histone H4 histidine kinase in a nuclear extract from *P. polycephalum* phosphorylates histone H4 on H75 alone to give 1-phosphohistidine (*13*), and this type of phosphorylation of histone H4 is also catalyzed by the fairly well-characterized enzyme from yeast (*30*).

1 MSGRGKGGKG LGKGGAKRHR KVLRDNIQGI TKPAIRRLAR RGGVKRISGL 50
51 IYEETRGVLK VFLENVIRDA VTYTEHAKRK TVTAMDVVYA LKRQGRTLYG 100
FGG 102

FIGURE 2: Amino acid sequence of histone H4 with the putative phosphorylation sites highlighted in bold.

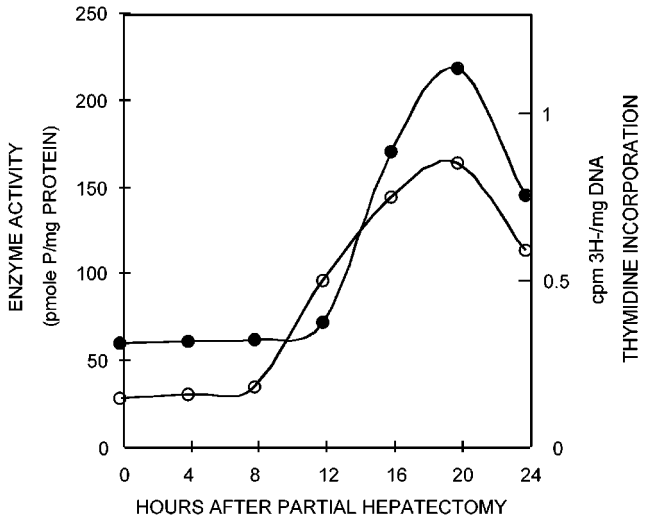

FIGURE 3: Reproduction of data from ref *11* illustrating the correlation of histone kinase activity and extent of DNA synthesis after partial hepatectomy: (○) enzyme activity and (●) level of [^{3}H]thymidine incorporation.

Mammalian histone H4 histidine kinase activity has been observed mainly in proliferative tissues, such as regenerating rat liver and a Walker 256 carcinosarcoma cell line (*2*). In vivo studies have demonstrated that acid-labile phosphates are formed on histone H4 only in regenerating, but not sham-operated, rat liver. Furthermore, upregulation of this pH 9.5 kinase correlates with the period that precedes DNA synthesis (*11*). This finding has been reproduced in work currently undertaken in this laboratory. In addition, enhanced histidine kinase activity has been observed in regenerating liver resulting from acute and chronic liver damage (unpublished data) compared to normal liver. In the first instance, regeneration arises from pre-existing hepatocytes, whereas liver stem cells account for regeneration in chronically damaged liver, as determined by PCNA staining of liver sections. Interestingly, we have evidence that histidine kinase activity is present in developing fetal liver (unpublished data) but not in proliferating hepatocytes during postnatal development, which suggests that its upregulation is specifically involved with either liver regeneration or development, and not general hepatocyte proliferation. It is not clear at this stage whether the increase in histone H4 histidine kinase activity following partial hepatectomy or liver damage results from increased enzyme expression or an increase in the specific activity of the pre-existing enzyme.

In the original report of a histone H4 kinase activity in regenerating rat liver (*2*), evidence was also presented that there were histone histidine kinases present in the nuclei of cells from other rat tissues. Screening of rat tissues revealed that thymus was a good source of histone H4 histidine kinase (*31*), and Besant and Attwood (*32*) carried out a partial purification of the enzyme from porcine thymus. This preparation was shown to contain up to four putative histone H4 histidine kinases with apparent molecular masses in the range of 34−41 kDa (*32*). Recent work by P. V. Attwood in collaboration with C. W. Turck at the Department of Medicine (University of California at San Francisco, San Francisco, CA) has shown that there are in fact multiple kinases in both nuclear and cytoplasmic thymus preparations that phosphorylate histone H4 on histidine (unpublished data). To date, it has not been determined which of the two histidines in histone H4 are phosphorylated by any of these thymic histidine kinases.

Currently, the biological function of histone H4 histidine kinases remains to be determined. The reports of the presence of such kinases in proliferating tissues and the induction of their activity in regenerating liver suggest that they may play a role in cell proliferation. Although this is speculative, the correlation between histone phosphorylation and cell division, as well as chromatin condensation associated with entry into mitosis, has been well-documented (*33*−*38*; for reviews, see refs *17* and *39*). In addition, H75 of histone H4 has been determined to be located one amino acid residue away from the DNA binding site within the nucleosome core in calf thymus (*40*). The phosphorylation of H75 could possibly facilitate the displacement of histone H4 from the nucleosome core to allow for the initiation of DNA duplication (*41*). However, the imidazole ring of H75 has also been shown to hydrogen bond with E90 of histone 2B within the intact nucleosome core, thus helping to stabilize the histone octamer (*42*). Phosphorylation of H75 could thus result in the destabilization of the nucleosome structure. H18 has been shown to reside in the highly basic N-terminal tail of histone H4, which strongly interacts with the acidic region of the histone H2A−2B dimer of an adjacent nucleosome particle (*42*). However, Wei et al. (*41*) showed that histone H4 in nucleosome core particles is not a substrate for the histidine kinase from *P. polycephalum*, but Chen et al. (*3*) presented evidence that it was not newly synthesized histone H4 that was phosphorylated. Thus, phosphorylation of pre-existing histone H4 at the time the histones are displaced from DNA during replication may disrupt particle−particle interactions and binding to DNA to prevent pre-existing histones from prematurely forming nucleosome complexes during DNA synthesis (*3*, *41*). Thus far, no conclusive evidence in support of these possible consequences of histone H4 histidine phosphorylation has been forthcoming.

4. Evidence for Other Mammalian Histidine Kinases

Some mammalian cellular proteins that contain phosphohistidine have turned out to be enzyme reaction intermediates resulting from autophosphorylation of an enzymic histidine residue. Examples of such enzymes are phosphoglycerate mutase (*43*, *44*), ATP citrate lyase (*45*, *46*), human prostatic acid phosphatase (*47*), 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (*48*), and nucleoside diphosphate kinase (*10*, *49*). However, other phosphohistidine-containing mammalian proteins reported below have provided strong evidence for the existence of mammalian histidine kinases.

Protein histidine phosphorylation was reported to occur in synaptic plasma membranes (*50*). It was found that incubation of synaptic plasma membranes with ATP in the presence of Mg^{2+} resulted in phosphoryl transfer to not only serine but also histidine residues on membrane proteins. Unlike phosphoserine formation, phosphorylation of the histidine was unaffected by cAMP. The phosphorylation of

histidine also did not conform to Michaelis−Menten kinetics which suggested the possibility that two or more kinases were involved with different K_m values for ATP. The entire process of synaptic membrane protein phosphorylation was thought to play a role in modulating synaptic transmission. Although the kinetics of phosphorylation and phosphoamino acid analysis of the synaptic membrane proteins were determined, further investigations into the identity of the histidine-phosphorylated proteins, the stoichiometry of the phosphorylations, or the identity of the putative histidine kinase(s) were never attempted.

Another membrane protein reported to be phosphorylated on histidine was isolated from human leukemia (HL-60) cells (*51*). This protein was phosphorylated with [γ-^{32}P]GTP and comigrated with β-subunits of heterotrimeric GTP-binding proteins (G proteins) on SDS−PAGE. In addition, the phosphoprotein produced by incubation of the synaptic membrane with [γ-^{32}P]GTP was immunoprecipitated by an antibody specific for the G protein β-subunits. The phosphorylation was stable against treatment with NaOH but sensitive to treatment with heat, HCl, and hydroxylamine. Moreover, treatment of the membranes with the histidine-modifying agent diethyl pyrocarbonate resulted in a loss of phosphate incorporation. Later, Wieland and co-workers performed phosphoamino acid analysis on the phosphorylated β-subunits and showed phosphohistidine to be the only phosphoamino acid present (*52*). Thus a β-subunit of a G protein is phosphorylated on a histidine residue. Further evidence in support of this conclusion is the finding by Wieland and co-workers that incubation of the $\beta\gamma$-subunits of retinal rod G protein, transducin, with [^{35}S]GTPγS and rod outer segment membranes resulted in the formation of thiophosphorylated β-subunits (*53*). This process is not apparently autophosphorylation by the β-subunits, or phosphorylation of β-subunits by γ-subunits, since phosphorylation did not occur in the absence of rod outer segment membranes (*53*). This was also found to be the case for the phosphorylation of G protein β-subunits associated with cell membranes from a variety of tissues derived from a number of mammalian sources (*52*). This suggests that there is a protein histidine kinase present in many mammalian cell membranes that is capable of phosphorylating the β-subunits of G proteins. In the case of HL-60 cells, it was found that the thiophosphorylation of the β-subunit of the G protein was upregulated by formyl peptide receptors activated by fMet-Leu-Phe (*54*). Wieland and co-workers have also studied what the purpose of this phosphorylation might be (*51*−*55*). These workers showed that incubation of thiophosphorylated transducin β-subunits platelet membranes resulted in the activation of G protein-mediated processes (*55*). In addition, incubation of thiophosphorylated transducin β-subunits with GDP in the presence of HL-60 membranes resulted in the production of GTPγS, which led to the suggestion that GDP bound to G protein α-subunits was phosphorylated (*53*). This suggestion was supported by the finding that thiophosphorylated β-subunits can thiophosphorylate GDP when incubated with GDP-bound transducin α-subunits to form GTPγS (*55*). In addition, incubation of the γ-^{32}P-phosphorylated membrane protein in HL-60 membranes with GDP resulted in dephosphorylation of the putative G protein and the formation of [γ-^{32}P]GTP (*53*). Thus, it would appear that a membrane-bound histidine protein kinase is able to phosphorylate the β-subunits of G proteins. Since this results in a "high-energy" phosphoramidate bond, the phosphoryl group can thus be easily transferred from the phosphohistidine of the β-subunit to GDP from a G protein α-subunit (possibly while still bound to the α-subunit) and thus resulting in an activated GTP-bound G protein α-subunit. Hence, this process provides an alternate means of activating trimeric, receptor-linked G proteins, or maintaining them in an activated state. As far as the authors of this review are aware, the protein histidine kinase(s) responsible for the phosphorylation of G protein β-subunits has not yet been purified and characterized.

With a growing interest in two-component histidine kinases in prokaryotes and plants, together with robust research effort exploring the newly discovered histone H4-phosphorylating histidine kinases from yeast and *P. polycephalum* identified by Matthews' group at the University of California (Davis, CA) (*4*, *13*, *30*, *41*), some level of interest in histidine kinases was maintained throughout the 1980s. However, it was not until the 1990s that researchers once again began to search for evidence of histidine kinases in mammalian cells. Two proteins designated p36 and p38 were identified independently and found to be phosphorylated on a histidine residue.

The Japanese group of Motojima and Goto identified a membrane-associated protein they designated p36 in rat liver and rat hepatoma Fao cells, whose phosphorylation on histidine was induced by the action of peroxisome proliferators such as clofibrate (*56*−*58*). Phosphoamino acid analysis, involving alkaline hydrolysis and thin-layer chromatography, was used to demonstrate histidine phosphorylation of p36. The associated kinase was also found to be in the membrane fraction of the tissue extract, and although not purified to homogeneity, it was reported to have a molecular mass between 70 and 75 kDa (*58*). This kinase was found to be resistant to genestein [a tyrosine kinase inhibitor that also inhibited yeast histidine kinase activity (*59*)] (*58*) and was shown in vitro to be activated by both Ras and GTP (*56*). Speculation about a role for p36 histidine phosphorylation in cell signaling was briefly alluded to in this work (*56*), with the suggestion that p36 may be involved somewhere downstream of a signaling pathway that involves Ras.

At about the same time, Hedge and Das reported a p38 protein from rat liver plasma membrane, which was also phosphorylated on histidine and was in some respects similar to p36 described above (*60*). Like that of p36, p38 phosphorylation is also enhanced by p21Ras. Glucagon was also reported to enhance p38 phosphorylation, both in vivo and in vitro (*61*). Another similarity with p36 was the ability of the kinase responsible for its phosphorylation to utilize both ATP and GTP as the phosphate donor. Phosphoamino acid identification was initially based solely on the acid lability of phosphorylated p38 fixed within a polyacrylamide gel. While this acid lability does suggest phosphohistidine, it does not rule out the presence of phospholysine, phosphoarginine, or, as the authors themselves recognize, acyl phosphates (phosphoaspartate and phosphoglutamate) (*61*). The study of histidine phosphorylation of p38 also involved the use of the histidine-modifying reagent diethyl pyrocarbonate (DEPC) to determine histidine phosphorylation (*60*). While this method has commonly been used in determining the involve-

ment of histidine as a site for phosphorylation, caution must be used in interpreting the results of such experiments since DEPC is capable of modifying other amino acids such as tyrosine (*62*). To date, there is no solid evidence to show that p36 and p38 are identical. p38 has been identified as ornithine transcarbamylase (an enzyme involved in the arginine synthesis pathway) via a cDNA library screening using a p38 antibody (*5*), whereas p36 has never been definitively identified. What the biological roles of the phosphorylation of p36 and p38 are remain unclear, although p38 expression has been shown to be negatively correlated with liver cell division (*63*). For both p36 and p38, neither the sites nor stoichiometries of phosphorylation were reported. Again, as far as we are aware, there have been no further studies to determine what the protein histidine kinase(s) is that is responsible for the these phosphorylations.

Further support for the existence of mammalian histidine kinases stems from the research of Noiman and Shaul, who produced one publication in which they described the use of DEPC treatment, acid lability, and phosphoamino acid analysis to detect phosphohistidine-containing phosphoproteins when cellular extracts from various rat tissues were incubated with [γ-^{32}P]ATP (*64*). Two proteins in particular, p37 and p38, were reported to contain phosphohistidine. The phosphorylation of p43 on a serine or threonine residue(s) was suggested to be dependent on a histidine kinase on the basis of the inhibition of its phosphorylation by DEPC. However as noted above, the results of experiments in which DEPC is used as a histidine-modifying reagent are not always easily interpretable. While pH−stability, DEPC studies and phosphoamino acid analysis of these proteins suggest the existence of histidine kinases, none of the proteins mentioned in this article or their putative kinases have ever been identified or studied further.

Stimulation of human platelets by thrombin or collagen resulted in the phosphorylation of the cytoplasmic part of the membrane protein P-selectin (*65*). Phosphoamino acid analysis of the phosphorylated P-selectin showed the presence of phosphoserine, phosphothreonine, phosphotyrosine, and phosphohistidine (*65*). A phosphorylated cytoplasmic tail peptide obtained by tryptic cleavage of phosphorylated P-selectin and Edman degredation of the peptide revealed H771 and H773 to be phosphorylation sites. A functional biological role for this histidine phosphorylation of P-selectin in platelet signal transduction pathways and identification of the putative histidine kinase has yet to be determined.

The beginning of a new century has brought with it a continuation of research into histidine phosphorylation and histidine kinases in mammalian cells. The isolation of a 37 kDa phosphoprotein from the apical membrane of ovine tracheal epithelia was recently found to be phosphorylated on histidine (*66*). This phosphoprotein identified as annexin I was found to be phosphorylated on histidine using ATP or GTP, with the site of phosphorylation being localized to the carboxy-terminal fragment of the protein (*66*). The results of this study implicate annexin I as a component of an intracellular signaling system in which the intracellular Cl^- concentration regulates its phosphorylation on histidine (*66*−*68*). However, annexin I does not autophosphorylate (*66*), unlike those histidine kinases involved in bacterial two-component environmental sensors (*69*), but appears to be phosphorylated by the action of a separate protein histidine kinase. AMP and cAMP appear to regulate the phosphorylation of annexin I, although it is not clear whether this regulation is via binding of these molecules directly to annexin I which influences the availability of a histidine residue(s) for phosphorylation (*66*) or via effects on the histidine kinase. Muimo and co-workers convincingly demonstrated that the effects of cAMP on annexin I phosphorylation were not mediated via protein kinase A. No stoichiometry of annexin I phosphorylation was reported. Identification and characterization of the histidine kinase responsible for annexin I phosphorylation is eagerly awaited.

Another group investigating mammalian histidine kinases is currently re-examining a bacterial-like histidine kinase thought to possess only serine kinase activity. Two mammalian enzymes, branched-chain α-ketoacid dehydrogenase kinase and pyruvate dehydrogenase kinase (BCKDHK and PDHK, respectively), contain prototypical two-component histidine kinase motifs (*70*, *71*). BCKDHK and PDHK are each part of a mitochondrial enzyme complex involved in the regulation of the oxidative decarboxylation of the branched-chain α-ketoacids derived from leucine, isoleucine, valine, and pyruvate. In vitro, both of these enzymes are known to autophosphorylate on a serine residue, but there has also been conjecture about whether BCKDHK also has intrinsic histidine kinase activity (*70*, *72*). Recent work from a laboratory at the University of California at San Francisco studying mammalian, bacterial-like histidine kinases has solid evidence to validate BCKDHK as a histidine kinase (C. W. Turck at the Department of Medicine, personal communication). This group has used a combined approach of histidine and serine point mutations, two-dimensional phosphopeptide mapping, and phosphoamino acid analysis to show that BCKDHK autophosphorylates in vitro on serine and histidine residues. They have also examined structural similarities between the ATP-binding motif of BCKDHK and that of the "Bergerat fold" family of proteins to which two-component histidine kinases belong (*73*). On the strength of this structural similarity to other members of the Bergerat fold protein family, they have identified an inhibitor that was able to inhibit the kinase activity of both BCKDHK and other known two-component histidine kinases. Overall, this comprehensive study of BCKDHK illustrates the idea that bacterial-like histidine kinase activity may exist in mammalian cells. From an evolutionary standpoint, the fact that BCKDHK is a mitochondrial enzyme and has bacterial-like histidine kinase motifs makes sense with respect to the hypothesis put forward by evolutionary biologists of a primordial symbiosis of a bacterial-like cell becoming what we now recognize as a mitochondrion.

5. Supporting Evidence: Protein Histidine Phosphatases

If histidine kinases participate in mammalian cellular signaling pathways, one might expect there also to be complementary phosphatases capable of catalyzing the hydrolysis of phosphohistidine in phosphoproteins. The establishment of the existence of mammalian histidine phosphatases has added some weight to the possibility that histidine kinases could be involved in cellular signaling pathways. Wong et al. (*74*) have identified histidine/lysine phosphatase activity in the soluble fractions of various rat tissues after chromatography on DE-52 cellulose columns. Using synthetic polyphosphohistidine as a substrate, two

polyphosphohistidine phosphatases were identified, the first of which was stimulated 2.5−3.5-fold by Mg^{2+}. Similar studies by Ohmori et al. (*75*, *76*) have identified both a 30 kDa 6-phospholysine phosphatase and a dual 150 kDa 6-phospholysine/3-phosphohistidine phosphatase from rat brain. The dual-specificity enzyme was capable of catalyzing the hydrolysis of both 3-phosphohistidine and 6-phospholysine with comparable V_{max} and K_m values, but neither enzyme was capable of catalyzing the hydrolysis of phosphocreatine or N^{ω}-phosphoarginine (*75*). In addition, however, the dual-specificity phosphatase was capable of efficiently catalyzing the hydrolysis of AMP, GMP, and *p*-nitrophenylphosphoryl, whereas the 6-phospholysine phosphatase was not (*75*). This suggests that the dual-specificity phosphatase is a very broad specificity phosphatase. This phosphatase did not appear to be dependent on Mg^{2+} for activity (*75*) and was later shown to behave like an acid phosphatase (*76*). None of the phosphatases described by Wong et al. and Ohmori et al. have been definitively identified, and although these authors speculated that the phosphatases might act as phosphoprotein phosphatases, their ability to catalyze the dephosphorylation of such substrates was not tested.

Other phosphohistidine phosphatases have also been reported in rat liver (*57*, *77*). The first of these is a soluble 45 kDa protein phosphatase that dephosphorylates the p36 protein substrate (mentioned above) and was isolated, together with the histidine kinase, from the membrane-bound fraction of rat liver (*57*). This phosphatase has a requirement for Mg^{2+} and is resistant to 100 μM okadaic acid, suggesting that it belongs to the protein phosphatase 2C (PP2C) family (*78*).

An excellent study of what were traditionally thought of as serine/threonine phosphatases has led to the discovery that protein phosphatases 1, 2A, and 2C from yeast are also protein histidine phosphatases (*79*). The activities of protein phosphatases 1 and 2A against histone H4 phosphorylated on histidine by the yeast histidine kinase (*30*) and phosphorylated phosphorylase *a* (phosphoserine) were compared. It was found that the phosphorylated histone H4 was at least as good a substrate as phosphorylase *a*, indicating that the phosphohistidine hydrolase function of these phosphatases is a major part of their activity (*79*). Also using histone H4 phosphorylated on histidine by the yeast histidine kinase (*30*), these protein phosphatases were tested along with the phosphatase inhibitors okadaic acid and inhibitor-1 for the ability to dephosphorylate H4 (*5*, *79*). Conclusions drawn from these experiments suggest that these phosphatases may play an important role in the regulation of signaling pathways that use phosphohistidine. This idea is re-enforced by experiments involved in the detection of protein histidine phosphatases in rat liver and spinach cell extracts (*77*), in which members of the family of protein phosphatases 1 and 2A from these divergent eukaryotic sources were shown to dephosphorylate histone H4 phosphorylated specifically on histidine.

Even though protein histidine phosphatases clearly exist, the need for histidine phosphatases to dephosphorylate phosphoproteins containing phosphohistidine in vivo remains to be explored. It is possible that like in the "two-component" histidine kinase systems the phosphoryl group is removed by an intramolecular transfer to an aspartate residue on the same or another protein, or like the phosphorylated G protein β-subunit described above, the phosphoryl group is used to phosphorylate GDP, or other small molecules. The acid lability of phosphohistidine may obviate any need for a specific phosphatase given the right intracellular conditions where the pH of a particular microenvironment or organelle within the cell is acidic. All this aside, it is encouraging to know that both histidine kinases and phosphatases exist and may indeed act in concert to control signal transduction pathways.

6. Methodological Advances

One of the reasons for the lack of studies that focus on mammalian histidine kinases has been the technical difficulties in detecting such proteins, along with the acid lability of its product. Many of the classical techniques employed determining kinase activity utilize acidic treatments such as TCA precipitation (*80*). While some assays had been designed to facilitate the detection of acid-labile phosphorylation, such as phenol extraction (*29*) and gel-based assays (*41*), many of these techniques were laborious and/or difficult to apply quantitatively. In 1990, Wei and Matthews had developed a method that was selective for the detection of alkali-stable protein phosphorylation (*81*). Essentially, after incubation of the substrate protein and kinase preparation with [γ-^{32}P]ATP, the reaction mixture is then subjected to mild alkaline hydrolysis. This results in the hydrolysis of phosphoserine and phosphothreonine present in phosphoproteins in the reaction mixture, thus reducing the contribution of these common phosphoamino acids to the total amount of phosphate present in phosphoproteins. The proteins in the reaction mixture are then adsorbed onto a nylon, Nytran membrane. The membrane is then washed in an alkaline solution, usually containing ATP and pyrophosphate, to remove any bound [γ-^{32}P]ATP or $^{32}P_i$. After the membrane has dried, the amount of radioactivity present on the membrane is determined by scintillation counting (*81*). This assay provides a relatively quick means of screening samples for the ability to phosphorylate proteins in an alkali-stable manner. In our laboratory, we have extended this method to assay acid-labile phosphorylation of proteins by splitting reaction mixtures and acid-treating one-half followed by neutralization with alkali and the addition of an equal concentration of salt to the other half. The samples are then applied to Nytran membranes and treated in the usual way. While both types of assays are indicative of histidine phosphorylation, it is still important that phosphoamino acid analysis be performed to confirm this. Fujitaki and Smith have also reported on methods allowing for the detection of phosphoramidate-containing proteins, including ^{31}P NMR and various methods of phosphoamino acid analysis involving such techniques as HPLC and paper electrophoresis (*82*).

In addition, techniques for preparation and purification of the various isoforms of phosphohistidine have been refined (*83*, *84*). Furthermore, the separation of phosphohistidine from phosphotyrosine and other phosphoamino acids has been refined using thin layer electrophoresis (*32*), reverse phase TLC (*85*), and HPLC (*84*). These methodological developments have led to the identification and characterization of the yeast histidine kinase (*30*). Some of the technical difficulties confronted as a consequence of the acid-labile

nature of phosphohistidine have been overcome by methods which aim at increasing its stability. This has been achieved by replacing the double-bonded oxygen atom with a sulfur atom in the group of phosphohistidine (*86*). Thiophosphorylated histidine can easily be synthesized using either $PSCl_3$ (*86*) or thiophosphoramidate (*87*). Thiophosphohistidine is at the same time a close molecular analogue of phosphohistidine while being much more stable to hydrolysis. The stabilization of phosphohistidine by thiophosphorylation would allow for the analysis of proteins containing putative histidine phosphorylation sites while withstanding the acidic environments employed by conventional biochemical techniques. It may be possible to use radiolabeled ATPγS as a substrate in many histidine kinase reactions, GTPγS having already been shown to be a substrate for the kinase that phosphorylates the G protein β-subunit (*51*−*55*).

Other techniques which will aid in the detection of histidine kinase activity include in-gel kinase assays and mass spectrometry. In-gel kinase assays provide a means of detecting kinase activity and determining the molecular masses of the kinases. Samples containing the kinase activity are separated by SDS−PAGE on a gel in which the protein substrate is incorporated into the gel matrix. The separated proteins in the gel are then renatured, and the gel is incubated with the components of the kinase reaction, including [γ-^{32}P]-ATP. The gel is then washed and subjected to autoradiography or phosphorimaging in which the bands of the substrate protein which have been phosphorylated and which correspond to bands of kinases are visualized. The yeast histone H4 histidine kinase has been detected by an in-gel kinase assay in which histone H4 was the substrate protein incorporated into the gel (*32*). Preparations of cellular extracts from porcine thymus have been shown to contain histidine kinase activity and multiple histone H4 histidine kinases (ref *32* and unpublished data from P. V. Attwood and C. W. Turck; see section 3 above). Under the conditions used for the in-gel kinase assays, the histidine kinases from both yeast and thymus have been shown not to undergo autophosphorylation (*32*). Recently, mass spectrometry has been used to detect synthetic peptides containing phosphohistidine (*88*) and to detect phosphohistidine in synthetic preparations of that phosphoamino acid (*85*).

7. Discussion

Over the past 30 years or so, there has been a series of transient contributions to research in the area of histidine phosphorylation and histidine kinases in mammalian cells, which have usually been in the form of one to three or four publications by individual research groups. In the past decade or so, a more sustained interest in eukaryotic histidine kinases and phosphatases in general has been maintained primarily by the efforts of Matthews and co-workers. In more recent years, our laboratory and that of Turck have sought to study eukaryotic and mammalian histidine kinases and further refine the associated research techniques.

From the evidence presented in this review, we hope that readers will agree that there are such enzymes as protein histidine kinases in mammalian cells. Most of them appear to be of the classical protein kinase type, although some such as PDHK and BCKDHK may be related to the two-component histidine kinases common in bacteria and lower eukaryotes.

Once we have concluded that protein histidine kinases exist in mammalian cells, the question of why there is a need for such enzymes arises, given that there are many examples of hydroxyamino acid protein kinases and their complementary phosphatases. The answer to this question probably lies in the chemistry of the N−P bond in phosphohistidine; the free energy of hydrolysis of this bond is much higher than that of the phosphoester bond in the phosphohydroxyamino acids. This enables the phosphoryl group from phosphohistidine to be readily transferred to other molecules to form, for example, acyl phosphates, phosphoesters, and phosphoanhydrides. This is in fact the foundation of the two-component histidine kinase signaling systems evident in bacteria and lower eukaryotes. The mammalian system in which a membrane-bound histidine kinase phosphorylates the β-subunit of a G protein is described in section 4. When this phosphorylated subunit binds to the inactive G protein α-subunit−GDP complex, the phosphoryl group from the phosphohistidine of the phosphorylated β-subunit is transferred to the GDP to form GTP which activates the α-subunit. This can provide a mechanism of keeping the G protein in its active state (and hence maintaining cellular responses) without the need for the G protein to interact again with an activated cellular receptor protein. One can easily imagine other systems in which a protein histidine kinase phosphorylates a protein which is then able to bind to another protein and transfer its phosphoryl group to an amino acid residue on that protein. This could be, for example, a serine residue or an aspartate residue, and as a result of this phosphorylation, some function of the protein is regulated. In the case where an aspartate residue is phosphorylated, phosphoryl transfer to another protein is possible. Thus, for example, a membrane-bound protein histidine kinase, which may be a receptor protein, or be regulated by a receptor, phosphorylates a cytosolic protein. This phosphorylated protein is then able to migrate to the nucleus, bind to a nuclear protein, perhaps a transcription factor, and regulate its activity, i.e., very much paralleling the two-component histidine kinase systems. Thus, phosphorylation of the effector protein is dependent on the highly specific binding with the phosphohistidine-carrying protein, which may in turn be a process regulated by other factors.

Mammalian histidine kinases such as BCKDHK may be a remnant of mammalian evolution from bacterial origins. There is the possibility that primitive two-component environmental systems evolved in mammalian cells so that kinases phosphorylate proteins on histidine and then pass them on to another protein. This idea of histidine kinase evolution has been considered in a recent article by Koretke et al. (*89*), where the evolution of two-component histidine kinases is examined via a bioinformatics approach. The possibility of a distant evolutionary relationship between two-component histidine kinases and other eukaryotic protein kinases is inferred through a phylogenetic tree, which has at its origin an ancestral "protokinase". The phylogenetic tree described in this study is bifurcated early in the evolutionary process with one branch leading to two-component histidine kinases encompassed within the Bergerat fold family of proteins.

Where protein histidine kinases function in cellular signaling systems similar to those described above, the need for histidine phosphatases to dephosphorylate phosphoproteins

containing phosphohistidine in vivo is questionable, although they could form an integral part of the signaling systems to act to regulate the size of the pool of phosphistidine-containing proteins. In some cases also, the acid lability of phosphohistidine may obviate any need for a specific phosphatase given the right intracellular conditions where the pH of a particular microenvironment or organelle within the cell is acidic. All this aside, it is encouraging to know that both histidine kinases and phosphatases exist and may indeed act in concert to control cellular signaling pathways.

Continued development of methodologies for the detection and assay of protein histidine kinases and their phosphorylated protein products will be required to investigate their biological roles. Anti-phosphotyrosine antibodies have been important tools in the study of the involvement of tyrosine kinases in mammalian cellular signaling pathways, and similarly, the production of anti-phosphohistidine antibodies would be significant for histidine kinases. This raises the question of the necessity of raising antibodies to both the 1- and 3-phospho forms of phosphohistidine. Both 1- and 3-phosphohistidines seem to exist in mammalian phosphoproteins, and it is likely that two sets of antibodies would be required. We have made some unsuccessful attempts in our laboratory to produce such antibodies and are not aware of any successful attempts.

It may be possible to refine mass spectrometric techniques that have been developed to study protein phosphorylation by serine/threonine and tyrosine kinases for the study of histidine kinases. In this way, we can identify phosphorylation sites on target proteins and look for sequence determinants around phosphorylated histidines with a view of understanding how histidine kinases recognize their substrates and searching for other potential cellular protein substrates. Much of the work that has been carried out to detect mammalian histidine kinase action has been qualitative in nature, and there is much yet to be done in many of the enzymic systems reported in this article, in terms of the determination of stoichiometries and identification of sites of phosphorylation of the substrates.

Most important in advancing this field of research is the need to purify, clone, and sequence a mammalian histidine kinase which is different from the type of enzyme represented by PDHK and BCKDHK, which are related to the two-component histidine kinases. With this sequence information, it will then be possible to mine the sequence and genomic databases for other potential members of the histidine kinase family. This has been done to some extent using the recently determined crystal structure of BCKDHK (*90*). However, only the two-component-like histidine kinases BCKDHK and PDHK have so far been identified.

The research into histidine kinases and phosphatases reviewed in this article provides enough evidence to indicate that histidine phosphorylation not only exists in mammalian cells but in some cases also plays a role in cell signaling. By comparison to its hydroxyamino acid kinase counterparts, the field of mammalian histidine kinase research is still in its infancy, despite the period of time since its inception. This is in part due to the inherent technical problems associated with the chemistry of this type of phosphorylation. However, with each publication more information is slowly giving rise to ways that overcome some of the problems faced with the acid-labile nature of phosphohistidine.

With the current developments in proteomics, together with our accumulated knowledge of histidine phosphorylation and histidine kinases, identifying mammalian histidine kinases should become easier. We are at an exciting moment in history, as the new wave of protein research opens up the floodgates of possibilities. The detection of phosphohistidine in mammalian proteins has now become well-established; the next logical progression is to identify the associated kinases. Hopefully, the resurgence of interest in mammalian histidine kinases over the past decade will continue into the future. It is a case of watch this space.

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