ACTA ABP BIOCHIMICA POLONICA

Vol. 58, No. 2/2011
137–147
on-line at: www.actabp.pl

Review

Phosphorylation of basic amino acid residues in proteins: important but easily missed

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Reversible phosphorylation is the most widespread posttranslational protein modification, playing regulatory role in almost every aspect of cell life. The majority of protein phosphorylation research has been focused on serine, threonine and tyrosine that form acid-stable phosphomonoesters. However, protein histidine, arginine and lysine residues also may undergo phosphorylation to yield acid-labile phosphoramidates, most often remaining undetected in conventional studies of protein phosphorylation. It has become increasingly evident that acid-labile protein phosphorylations play important roles in signal transduction and other regulatory processes. Beside acting as high-energy intermediates in the transfer of the phosphoryl group from donor to acceptor molecules, phosphohistidines have been found so far in histone H4, heterotrimeric G proteins, ion channel KCa3.1, annexin 1, P-selectin and myelin basic protein, as well as in recombinant thymidylate synthase expressed in bacterial cells. Phosphoarginines occur in histone H3, myelin basic protein and capsidic protein VP12 of granulosis virus, whereas phospholysine in histone H1. This overview of the current knowledge on phosphorylation of protein basic amino-acid residues takes into consideration its proved or possible roles in cell functioning. Specific requirements of studies on acid-labile protein phosphorylation are also indicated.

Keywords: basic amino acids, posttranslational modification, phosphorylation, acid-labile, base-stable, phosphoramidate

Received: 05 March, 2011; revised: 05 April, 2011; accepted: 30 April, 2011; available on-line: 27 May, 2011

INTRODUCTION

The great diversity of the proteome, in comparison to the relatively small number of genes, is achieved mainly by posttranslational protein modifications of which over 100 are known, with phosphorylation being the most widespread. It is estimated that up to 30% of proteins in a mammalian cell are phosphorylated at any time (Cohen, 2000). Reversible protein phosphorylation affects every basic cellular process, including metabolism, growth, division, differentiation, motility, organelle trafficking, membrane transport, muscle contraction, immunity, learning and memory (Manning *et al.*, 2002; 2002a). Abnormal phosphorylation events are implicated in many disease states. Phosphorylation and dephosphorylation are catalyzed by protein kinases and phosphatases, respectively, responding to different stimuli and thus the two reactions being separately controlled events. Considering the chemistry of the bond between phosphate and an amino acid side chain, phosphoramino acids include phosphomonoesters (serine, threonine and tyrosine), phosphoramidates (histidine, arginine and lysine), acylphosphates (aspartate and glutamate), and thiophosphate (cysteine). Reversible, multisite phosphorylation of protein Ser, Thr and Tyr residues mediates numerous signal transduction pathways in eukaryotic (Cohen, 2000) and prokaryotic (Deutscher & Saier, 2005) cells. Histidine and cysteine phosphorylation are well known critical processes involved in a bacterial phosphoenolpyruvate-dependent carbohydrate transport system (Meadow *et al.*, 1990; Stadtman, 1994). Aspartate residue acts as a phosphate acceptor in P-type ATPases (Post & Kume, 1973) and certain class of phosphotransferases (Collet *et al.*, 1998). Histidine and aspartate phosphorylations are engaged in two-component and multi-component phospho-relaying signalling systems in bacteria, fungi and plants (Stock *et al.*, 2000; Kruppa & Calderone, 2006; Grefen & Harter, 2004), involved in linking an extracellular stimulus, such as changing osmolarity, oxygen, nitrogen, phosphorus or ethylene levels, to gene-regulating events. Such systems have not been discovered in higher eukaryotes, nevertheless it is becoming increasingly evident that histidine phosphorylation plays important regulatory roles also in mammalian cellular signal transduction (Matthews, 1995; Klumpp & Krieglstein, 2002; Besant & Attwood, 2005; Steeg *et al.*, 2003; Tan *et al.*, 2002; Kimura *et al.*, 2000; Kowluru, 2003; 2008). Reports regarding the presence and significance of arginine and lysine phosphorylation in proteins are sparse and mainly concern histones. We have not found any reports about glutamate phosphorylation in proteins.

The majority of protein phosphorylation research has been focused on Ser, Thr and Tyr, i.e. phosphomonoesters that are acid-stable and may be studied by methods involving acidic treatments commonly used in phosphoprotein and phosphopeptide analysis. In contrast, phosphoramidates, i.e. phosphohistidine, phosphoarginine and phospholysine, are susceptible to hydrolysis under acidic conditions, and therefore these posttranslational modifications generally are overlooked in conventional studies of protein phosphorylation.

The objectives of the present paper are: (i) to overview our current knowledge on the occurrence of phos-

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Abbreviations: GPRC, G protein-coupled receptor; KCa3.1, potassium channel; NDPK, nucleoside diphosphate kinase; PHP or PHPT-1, phosphohistidine phosphatase

Table 1. Proteins phosphorylated on histidine residue(s)

Proteins	References
Intermediate forms of enzymes	
Bacterial phosphoenolpyruvate-sugar phosphotransferase system (PTS)	Meadow *et al.*, 1990; Stadtman, 1994
Two-component and multi-component phospho-relay signalling systems in bacteria, fungi and plants	Stock *et al.*, 2000; Grefen & Harter, 2004; Kruppa & Calderone, 2006
Nucleoside diphosphate kinases	Kowluru & Metz, 1994; Wålinder, 1968; Kimura *et al.*, 2000
Heat shock protein Hsp70	Lu *et al.*, 2006
Proteasome 20 S	Yano *et al.*, 1999
Succinyl-CoA synthetase	Boyer *et al.*, 1962
ATP-citrate lyase	Williams *et al.*, 1985; Robertson *et al.*, 1988; Krivanek & Novakova, 1991
Human prostatic acid phosphatase	Ostrowski 1978; McTigue & Van Etten, 1978
Glucose-6-phosphatase	Feldman & Butler, 1969; Ghosh *et al.*, 2004
6-Phosphofructo-2-kinase/fructose-2,6-bis-phosphatase	Pilkis *et al.*, 1983; Mizoguchi *et al.*, 1999
Phosphoglycerate mutase	Rose, 1970
Phospholipase D superfamily	Gottlin *et al.*, 1998
Most likely more proteins	Lott *et al.*, 2006
Proteins phosphorylated by protein histidine kinases	
Histone H4	Chen *et al.*, 1974
Heterotrimeric G-proteins	Wieland *et al.*, 1991; 1993
Potassium channel KCa3.1	Srivastava *et al.*, 2006
Annexin 1	Muimo *et al.*, 2000
P-selectin	Crovello *et al.*, 1995
Thymidylate synthase	Frączyk *et al.*, 2009

phorylated basic amino acid residues (His, Arg, Lys) in proteins and the role of such modifications in cell functioning, and (ii) to indicate methodological limitations of studies on phosphorylation of basic amino acids.

PROTEINS PHOSPHORYLATED ON HISTIDINE

There are two biologically relevant phosphohistidine isomers — the phosphoryl group may be linked to N-1 (N^{π}) or N-3 (N^{τ}) of the His residue (Attwood *et al.*, 2007 and references there, Fig. 1). The phosphohistidine phosphoramidate bond has a large negative standard free energy (ΔG^{o} = ~ –12 to –14 kcal/mol) of hydrolysis and therefore is less stable than phosphoester bonds in phosphohydroxyamino acids in proteins (ΔG^{o}= –6.5 to 9.5 kcal/mol (Hultquist, 1968; Stock *et al.*, 1990). However, in proteins the stability of phosphoramidate bond depends on the neighboring amino-acid residues (Waygood *et al.*, 1985; Kim *et al.*, 1993; Lott *et al.*, 2006). It has also been postulated that at acidic pH in the cellular microenvironment, the labile nature of this bond might be used in a system that requires an on/off switch without the need for participation of protein phosphatases (Klumpp & Krieglstein, 2002). It should be noted, however, that a mammalian protein histidine phosphatase (PHP) has been identified (Ek *et al.*, 2002; Klumpp *et al.*, 2002). It is estimated that about 6% of protein phosphorylation in eukaryotes concerns histidine residues, thus this modification is much more frequent than tyrosine phosphorylation (Matthews, 1995). In most cases protein phosphohistidine residues are found in intermediate forms of enzymes whose catalytic activities involve transfer of high-energy phosphoryl groups to other molecules *via* a phosphohistidine intermediate, however, there is also another category of phosphohistidine-containing proteins: those that do not autophosphorylate, but are phosphorylated by protein histidine kinases (Table 1).

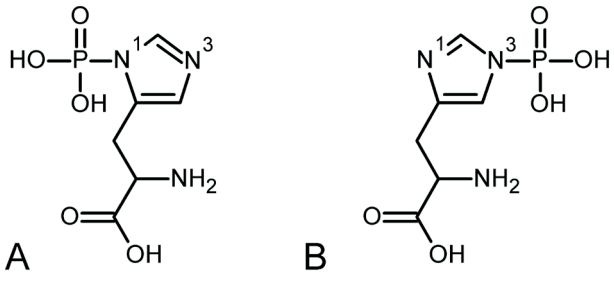

Figure 1. Structures of *N*-1-phosphohistidine (A) and *N*-3-phosphohistidine (B)

In this chapter we focus on the latter group of proteins, emphasizing the possible regulatory role of this posttranslational modification.

Histone H4

Histones are small basic proteins associated with DNA. The four histones termed H2A, H2B, H3 and H4 form an octameric protein core around which DNA is wound in the basic nucleosome structure of chromatin. Another member of the histone family, histone H1, is

bound to the outside of the core and "locks" the DNA into position. Various posttranslational modifications of histones, such as acetylation, methylation, phosphorylation, ribosylation and ubiquitination may affect transcription (for review, see Khorasanizadeh, 2004). Histone H4 undergoes acetylation on Lys5,8,12 and 16, phosphorylation on Ser1 and phosphorylation on His18 and His75. It was the first vertebrate protein identified to contain phosphorylated histidine residues. The formation of histone H4 histidine phosphate has been observed *in vivo* 18 h after partial hepatectomy in rats and correlated with DNA synthesis (Chen *et al.*, 1974). The first and well characterized histone H4 histidine kinase was purified from yeast (Huang *et al.*, 1991). *In vitro* it phosphorylates specifically His75, but not His18, to form *N*-1-phosphohistidine. Other histone H4 histidine kinases have been detected in regenerating rat liver (Smith *et al.*, 1973; Chen *et al.*, 1977; Tan *et al.*, 2004), in fetal rat and human liver (Tan *et al.*, 2004), human hepatocarcinoma tissue (Tan *et al.*, 2004), Walker-256 carcinoma cells (Smith *et al.*, 1974), pancreatic β-cells (Kowluru, 2002) and thymus (Besant & Attwood, 2000). The histone kinases from regenerating rat liver and Walker-256 carcinoma cells appear to phosphorylate His75 and His18 in histone H4, respectively, resulting in formation of *N*-1-phosphohistidine in the former and *N*-3-phosphohistidine in the latter case. The histone H4 histidine phosphorylation appears to be also an *in vivo* phenomenon, as proteolytic digestion of phosphorylated histone fractions from regenerating rat liver after *in vivo* administration of ^{32}P-labelled sodium phosphate showed H4 phosphohistidine (Chen *et al.*, 1974). Interestingly, in regenerating rat livers, phosphohistidine was not formed on *de novo* synthesized H4 molecules, but exclusively on those preexisting before a peak of DNA synthesis, as was shown in livers of rats injected with [^{3}H]histidine and $^{32}P_i$ 18 h after partial hepatectomy (Chen *et al.*, 1977). The turnover of the P-N linkages in H4 was apparently rapid, with an approximate life-time of 2 h (Chen *et al.*, 1977). These data, coupled with the coincidence of the increase in H4 kinase activity with DNA synthesis and cell proliferation (Chen *et al.*, 1974), suggest a physiological role of histone H4 histidine phosphorylation during DNA replication. It is possible that this modification, occurring at the time when histones are displaced from DNA during replication, prevents premature formation of nucleosome complexes during DNA synthesis (Besant *et al.*, 2003). Nickel and copper were reported to bind to His18 of histone H4 (Zoroddu *et al.*, 2000), pointing to the possibility of a more general carcinogenesis pathway. Nickel was found to decrease histone H4 Lys12 acetylation in mammalian cells, which may represent a positional effect of His18 phosphorylation (Broday *et al.*, 2000). If the His18 phosphorylation alters H4 acetylation, it may be expected to affect gene expression pattern.

Unfortunately, even when histidine phosphorylation is detected, it is not a trivial task to identify its biological consequences, therefore no conclusive evidence has been found so far in support of these postulated roles of H4 histidine phosphorylation.

As regards the reverse reaction, i.e. histidine dephosphorylation, histone H4 phosphorylated by yeast histidine kinase on His75 has been found to be a substrate for Ser/Thr protein phosphatases 1, 2A and 2C from rabbit skeletal muscles (Kim *et al.*, 1993), rat liver and spinach leaves (Matthews & Mackintosh, 1995), but nothing is known yet on enzymatic histone H4 histidine dephosphorylation *in vivo*.

Heterotrimeric G-proteins

Numerous G-proteins, comprising the α, β and γ subunits, coupled to a large variety of transmembrane receptors (G protein-coupled receptor, GPRC), are components of the most widely used signalling system in mammalian cells (Wettschureck & Offermanns, 2005). Binding of an extracellular agonist (biogenic amines, amino acids, ions, lipids, peptides, proteins and other agents) to GPRC causes conformational changes of the receptor protein which in turn transduces this information to the respective heterotrimeric G protein (Gilman, 1987). Receptor-mediated activation of the G protein α-subunit involves an exchange of bound GDP for GTP and dissociation of the GTP-bound α-subunit, with both the activated α-subunit and the βγ dimer capable of regulating downstream targets (adenylate cyclase, phosphodiesterase and several forms of phospholipases) to bring about biological responses, such as, among others, proliferation, cell survival, differentiation, migration, angiogenesis, metastasis.

Recent data suggest that there are also alternative, GPRC-independent ways of G protein activation. One of them uses a special class of proteins (activators of G-protein signalling, AGS) directly interacting with G-protein α-subunits and βγ dimers and activating this signalling pathway (Blumer *et al.*, 2007), and another involves phosphorylation of histidine residue in the protein. The G protein β subunit of heterotrimeric G proteins has been found to be phosphorylated on histidine in various tissues (Wieland *et al.*, 1993; Kowluru *et al.*, 1996; Cuello *et al.*, 2003; Hippe *et al.*, 2003). Apparently, nucleoside diphosphate kinase (NDPK) B forms a complex with the G protein and phosphorylates its β subunit at His266. The high-energy phosphate can then be transferred onto GDP and the formed GTP subsequently activates stimulatory $G\alpha_s$ and inhibitory $G\alpha_i$ proteins (Wieland *et al.*, 1991; 1992; 1993; Cuello *et al.*, 2003; Hippe *et al.*, 2003). It is interesting that nucleoside diphosphate kinases (NDPKs) have long been thought to be housekeeping enzymes that solely catalyze, through a high-energy P-His118 intermediate, the transfer of terminal phosphate groups from 5'-triphosphate to 5'-diphosphate nucleosides, and thus play a key role in nucleotide metabolism. In mammalian tissues NDPKs are encoded by the *nm23* gene family. Various combinations of subunits in heterohexamers yield nine isoforms. Two of them, NDPK A and NDPK B (also known as Nm23H1 and Nm23H2, respectively), have been identified to serve as protein kinases to phosphorylate various substrates on His, Asp, Ser and Thr residues (Engel *et al.*, 1995; reviewed in Steeg *et al.*, 2003; Besant & Attwood, 2005; Klumpp & Krieglstein, 2009) and increasingly appear to act as signalling molecules (reviewed in Kimura *et al.*, 2000; 2003; Roymans *et al.*, 2002; Kowluru, 2008; Wieland *et al.*, 2010; Mehta & Orchard, 2010).

In cardiomyocytes, classical activation of $G\alpha_s$ and $G\alpha_i$ proteins through GPRCs regulates intracellular cAMP levels that control myocardial contractility by activation of the protein kinase A pathway and alteration in Ca^{2+} transients (Bers, 2002; Rockman *et al.*, 2002). The GPRC-independent activation of a G protein signalling pathway involving G protein β subunit His266 phosphorylation appears to have a physiological role in regulation of basal cAMP production. Recombinant Gβγ dimer, carrying His266Leu mutation on β subunit (deficient in intermediate Gβ phosphorylation, but not interfering with the heterotrimers activation *via* classical receptor-agonist mechanism), integrated into heterotrimeric G

proteins and overexpressed in rat cardiomyocytes, suppressed basal cAMP formation by up to 55%, as compared with wild-type Gβγ. A similar effect was obtained by siRNA-mediated NDPK knockdown (Hippe *et al.*, 2007). It is also worth adding that in the membrane fraction of failing myocardium a relative enrichment for NDPK was observed, although the total NDPK level appeared unchanged. Increased membrane-associated NDPK levels run parallel with the progression of cardiac hypertrophy induced by chronic β-adrenergic receptor stimulation (Lutz *et al.*, 2003). It has been speculated that this may lead to enhanced formation of basal cAMP and thereby contribute to progression of the disease (Engelhardt & Rochais, 2007).

A mechanism for alternate, GPRC-independent activation of trimeric G proteins in the pancreatic β-cell, has also been proposed by Kowluru and coworkers (Kowluru 2008; 2003; Kowluru *et al.*, 1996). In the context of physiological, glucose-induced insulin secretion, one of the histone H4-phosphorylating histidine kinases or NDPK phosphorylates the Gβ subunit on a histidine residue. This phosphate, in turn, is relayed to the GDP bound to the Gα subunit to yield an active GTP-bound G protein. It is proposed that such a mechanism is similar to the classical *ping-pong* mechanism of activation of NDPK. Following this, the Gα subunit dissociates and both GTP-bound Gα and the Gβγ dimer regulate various effector proteins. However, some experimental data argue against a direct transfer of the phosphate from P-His266 in the Gβ subunit onto Gα-bound GDP (Hohenegger *et al.*, 1996) and recent evidence suggests that the high-energy phosphate is specifically transferred onto free GDP, locally forms GTP which binds to and thereby activates the respective G protein α subunit (discussed in Wieland, 2007). On one hand, G protein β subunit may serve only as a simple high energy phosphate relay, but on the other, His266 phosphorylation may alter G protein β subunit function in a yet to be determined fashion.

The first identified eukaryotic phosphohistidine phosphatase (PHP) (Hermesmeier & Klumpp, 1999; Ek *et al.*, 2002; Klumpp *et al.*, 2002) was found to specifically dephosphorylate P-His in the Gβ subunit (but not NDPK B) of the retinal G protein transducin, as well as phosphorylated Gβ in membranes of H10 cells. Additionally, stable overexpression of PHP in H10 cells led to a strong reduction of phosphate incorporated into Gβ, but not into NDPK, in those cells (Mäurer *et al.*, 2005). Thus PHP might be an endogenous regulator of NDPK-dependent G protein activation.

Potassium channel KCa3.1

KCa3.1 is a component of an intermediate conductance Ca^{2+}-activated K^+ channel, expressed in T and B cells, epithelial cells and smooth muscle cells. By mediating the efflux of K^+, the channel functions to keep a negative membrane potential, which is required to maintain a favorable electrochemical gradient for Ca^{2+} influx. An increase in cytosolic Ca^{2+} triggers events leading to the transcription of a number of genes. KCa3.1 channels are rapidly upregulated after T cell activation, and are required for maximal Ca^{2+} influx and proliferation during the reactivation of naive T cells (Cahalan *et al.*, 2001).

Aside from the requirement of binding of Ca^{2+} to the calmodulin bound to carboxy terminus of KCa3.1, the channel also needs phosphatidylinositol 3-phosphate PI(3)P for activation. The effect of PI(3)P is mediated *via* 14 amino acids in the same carboxy terminus of KCa3.1 (Srivastava *et al.*, 2006a). Recent elegant studies of Srivastava and coworkers have shown that phosphorylation of a histidine residue of the channel protein regulates its activation in CD4 T cells (Srivastava *et al.*, 2006). Screening of a yeast two-hybrid library demonstrated the binding of nucleoside diphosphate kinase B to KCa3.1 carboxy terminus containing the 14 amino acids (the same as those necessary for PI(3)P action). Immunoprecipitation experiments confirmed the specific interaction of endogenous NDPK B and endogenous KCa3.1 in human CD4 T cells. NADPK B expression in CHO cells overexpressing KCa3.1 increased the channel amplitude, as assessed by whole-cell patch-clamp experiments. In addition, the kinase activity of NDPK B was crucial for this activation because expression of a mutated NADPK B (His118, required for the kinase activity, was replaced by Asn) did not activate KCa3.1. The target for NDPK B phosphorylation turned out to be His358 within the 14 amino acid carboxy terminus of KCa3.1 metioned above, as shown in inside/out membrane patches. Experiments with the use of siRNA to silence NDPK B led to a marked inhibition of KCa3.1 channel activity, Ca^{2+} influx and proliferation of human CD4 T lymphocytes and provided genetic evidence that NADPK B is required for full activation of the channel and subsequent reactivation of CD4 T cells. According to the authors (Srivastava *et al.*, 2006), the 14 amino acids of the KCa3.1 carboxy terminus account for the distinct regulation by PI(3)P by binding and recruiting NDPK B to the KCa3.1 channel. Once bound, NDPK B directly phosphorylates His358, which relieves the inhibitory effect of these 14 amino acid on the channel activity, leading to channel activation. However, the relationship between PI(3)P, NDPK B and KCa3.1 activation requires further studies.

Srivastava and coworkers have also provided evidence for negative regulation of CD4 T cells through dephosphorylation of His358 in KCa3.1 by protein histidine phosphatase PHPT-1 (Srivastava *et al.*, 2008). PHPT-1 is the phosphatase demonstrated to dephosphorylate P-His266 in G protein β subunit and ATP-citrate lyase, named there PHP (Klumpp & Krieglstein, 2005, 2009). Using the same methods as for studying His358 phosphorylation of KCa3.1 by NDPK B, the authors demonstrated PHPT-1 to inhibit KCa3.1 channel activity, with the enzyme's phosphatase activity being necessary for this inhibition. They showed direct binding of PHPT-1 and KCa3.1 in HEK 293 cells and direct inhibition of KCa3.1 channel activity in isolated membrane patches. *In vitro* PHPT-1 dephosphorylated KCa3.1 P-His358, phosphorylated by NDPK B, but not NADPK B autophosphorylated on His118. Silencing PHPT-1 by siRNA in primary CD4 T cells led to increased KCa3.1 channel activity and T cell receptor-dependent stimulated calcium influx and proliferation.

Even though an attempt to detect histidine-phosphorylated KCa3.1 *in vivo*, in cells labelled with orthophosphate, has failed (Srivastava *et al.*, 2008), it seems that so far the KCa3.1 channel is the best example of regulation of a biological function in mammalian cells by reversible histidine phosphorylation.

Annexin 1

Annexin 1 is a member of a superfamily consisting of 13 calcium- or calcium- and phospholipids-binding proteins sharing high biological and structural

homology (Raynal & Pollard, 1994). Each member of the superfamily has a core domain where calcium- or phospholipids-binding consensus lies. The N-terminus, unique to each annexin, with varying amino-acid sequence and length, is thought to be responsible for the biological activity and specific function of these proteins. Their main biochemical feature is, in theory, the binding to phospholipid membranes in a calcium-dependent manner. Annexin 1, the first described member of this superfamily, originally known as macrocortin (Blackwell *et al.*, 1980), is ascribed many important roles, among them membrane aggregation, antiinflammatory function, fagocytosis, regulation of cell proliferation, differentiation and apoptosis, and possible contribution to tumor development and progression (reviewed in Lim & Pervaiz, 2007; and Rescher & Gerke, 2004). The 49-amino acid N-terminus of annexin 1 is the regulatory region, undergoing phosphorylation on serine, threonine and tyrosine residues (Raynal & Pollard, 1994; Rothhut, 1997; Solito *et al.*, 2006; Dorovkov & Ryazanov, 2004; Mulla *et al.*, 2004) and proteolysis (Seemann *et al.*, 1997).

There is also a report on annexin 1 histidine residue(s) phosphorylation, with GTP or ATP as phosphate donor (Muimo *et al.*, 2000). The annexin 1 His phosphorylation, in contrast to other posttranslational modifications of the protein, occurs on the C-terminus, which localizes it to the core domain. As the proposed functions of the core include membrane organization and aggregation (Donnelly & Moss, 1997; Gerke & Moss, 1997), an altered charge on histidine may affect these functions. It is also possible that phosphohistidine may initiate annexin 1 cleavage and thus generate active peptide fragments, or lead to further degradation of the protein (Muimo *et al.*, 2000).

Annexin 1 histidine phosphorylation is inhibited by cAMP, shown to bind to the protein C-terminus at a site adjacent to conserved His103, alter annexin 1 membrane aggregating and abolish its ability to act as a calcium channel in artificial lipid bilayers (Cohen *et al.*, 1995; Muimo *et al.*, 2000). On the other hand, cAMP may also bind directly to the histidine kinase and allosterically regulate its activity or act through a yet unknown pathway (Muimo *et al.*, 2000).

Phosphorylation of histidine residue has recently been proposed as a latent common mechanism for signaling of intracellular chloride concentration to proteins within the apical membrane of respiratory epithelia (Treharne *et al.*, 2006). In the apical fraction from sheep tracheal epithelium Muimo and coworkers have identified the first protein underlying the phosphorylation cascade, i.e. NDPK, whose Cl^--dependent phosphorylation precedes phosphorylation of most other histidine-phosphorylated proteins in this system, such as annexin 1 (Muimo *et al.*, 1998; 2000). Annexin 1 and NDPK do not undergo phosphorylation in an ion-dependent manner when purified to homogeneity but require interactions within apical membrane environment to become chloride "sensors". Moreover, phosphatases could also play a role, because phosphatase inhibiton with phosphorothioate nucleotide analogues (but not with classical phosphatase inhibitors, such as okadaic acid) alter the net of phosphoprotein profile of the apical membrane (Treharne *et al.*, 2006).

Thus the presence of a novel regulatory pathway, involving histidine residue phosphorylation and annexin 1, is suggested.

P-selectin

P-selectin is an adhesion receptor of platelets and endothelial cells, which mediates the interaction with neutrophils and monocytes (Larsen *et al.*, 1989). The protein resides in the membrane of α granule, but upon platelet activation and degranulation is rapidly translocated to the plasma membrane, where it becomes activated and in turn activates certain metabolic pathways and morphological changes. P-selectin is composed of a lectin domain, an epidermal growth factor domain, a series of consensus repeat domains, a transmembrane region and a C-terminal cytoplasmic tail (Johnston *et al.*, 1989). Platelet activation is accompanied by phosphorylation of the cytoplasmic tail of P-selectin on Ser, Thr and Tyr residues (Crovello *et al.*, 1993; Fujimoto & McEver, 1993). Rapid phosphorylation and selective dephosphorylation of specific amino acids may be important for P-selectin function and signal transduction within the platelets (Crovello *et al.*, 1993).

Crovello and coworkers have demonstrated that P-selectin phosphorylation during activation of human platelets by thrombin or collagen includes also a transient generation of both P-His771 and P-His773 on the C-terminal tail (Crovello *et al.*, 1995) that was unnoticed during previous P-selectin phosphorylation research. Thrombin is known to initiate a distinct signalling pathway through the thrombin receptor (Coughlin, 1994), with the thrombin-induced platelet activation being rapid and complete in 60 s, whereas collagen activates platelets *via* an independent mechanism involving binding to, among other factors, glycoprotein Ia-IIa and α2β1 integrin receptor. Collagen-induced platelet activation is less potent, with the duration time of the order of several minutes. The kinetics of phosphorylation/dephosphorylation of histidine residues in P-selectin from thrombin- or collagen-induced platelets paralleled the rate of respective platelet activation and was simultaneous with Ser/Thr/Tyr phosphorylation. Since P-selectin is unlikely to undergo autophosphorylation on histidines because of its short cytoplasmic tail, the histidine kinase(s) and phosphatases(s) involved remain to be identified.

The function of transient histidine phosphorylation in P-selectin, and possibly in other proteins, during platelet activation remains to be elucidated.

Thymidylate synthase

Thymidylate synthase catalyzes *de novo* synthesis of thymidylate, necessary for DNA replication and repair, and thus cell proliferation (reviewed in Carreras and Santi, 1995; Costi *et al.*, 2005). Thymidylate synthase isolated from L1210 mouse leukemia parental and 5'-fluorodeoxyuridine-resistant cells showed significant difference in sensitivity to the slow-binding inactivation by 5-fluoro-dUMP which was not due to the corresponding gene mutations (Cieśla *et al.*, 2006). In view of a previous report on possible phosphorylation of the enzyme in cultured rat cells (Samsonoff *et al.*, 1997), such a modification was sought by conventional mass spectrometry of isoelectric focusing fractions of native proteins of the two enzyme forms. Surprisingly, phosphorylation of Ser10 and Ser16 was found, but only in the resistant cell enzyme (Frączyk *et al.*, 2009), although staining of proteins separated by SDS/PAGE with the Pro-Q® Diamond Phosphoprotein Gel Stain showed phosphorylation of the enzyme forms from both cell lines (Cieśla *et al.*, 2006). One possibile explanation of this apparent

discrepancy is that at least the enzyme from the parental cells is phosphorylated on basic amino acids, which escaped detection by the former procedure. A similar problem appeared during studies of four recombinant enzyme preparations (human, rat, mouse and *Trichinella spiralis* thymidylate synthases), whose phosphorylation could be demonstrated by Pro-Q® Diamond staining, but not by conventional MS analysis. However, ^{31}P NMR spectra showed the presence of phosphohistidine residues in phosphorylated fraction enriched from each of the recombinant enzyme preparations (Frączyk *et al.,* 2009). Interestingly, the phosphorylation of the enzyme's His residue(s) affected not only its catalytic (by lowering the V_{max}^{app} values), but also non-catalytic (by affecting repression of the enzyme's own mRNA *in vitro* translation) properties (Frączyk *et al.*, 2009).

The possible function(s) of the phosphorylation of thymidylate synthase is of obvious interest but, as with many other phosphorylated proteins (Lienhard, 2008), further studies are needed to learn more about it.

PROTEINS PHOSPHORYLATED ON ARGININE AND LYSINE

While there has been ample research concerning protein arginine and lysine modifications, such as methylation, acetylation, citrullination, SUMOylation and ubiquitination, especially with reference to histones, where combinatorial action of these posttranslational modifications regulates critical DNA processes, including replication, repair and transcription (reviewed in Khorasanizadeh, 2004), protein Arg and Lys phosphorylation has been studied considerably less frequently. Additionally, beside experimental difficulties in phosphoramidate research, phosphoarginine and phospholysine appear to be present much less frequently than phosphohistidine. Nevertheless, several sporadic and one well-documented report have evidenced the occurence of phosphoarginine and phospholysine in proteins. A few kinases and phosphatases that act on arginine and lysine residues have been found in various eukaryotic tissues (Ohmori *et al.*, 1993; Wong *et al.*, 1993; Kumon *et al.*, 1996; for review, see Matthews, 1995, Besant *et al.*, 2009).

Histones H1 and H3

Considering the presence of phosphoramidates in histones, not only histidine (in histone H4; see above) but also arginine and lysine (Fig. 2) were found phosphorylated. Acid-labile histone H1 phosphates were formed *in vivo* 18 h after partial hepatectomy in rats, which correlated with the onset of DNA synthesis (Chen *et al.*, 1974). Regenerating rat liver nuclei contain two histone kinases producing acid-labile histone phosphates, one active at pH 6.5, and the other at pH 9.5. The former kinase activity remained approximately constant during liver regeneration, whereas the latter increased at 12 h, reached a peak at 18 h, and began to decline 24 h after partial hepatectomy. Analysis of proteolytic digests of histone H1, isolated from regenerating liver revealed the presence of N^{ε}-phospholysine. Administration of [^{3}H]lysine and $^{32}P_i$ during liver regeneration showed that phospholysine was formed in some new and, presumably, some preexisting H1 molecules (Chen *et al.*, 1977). Analysis of the formation of acid-labile (P-N) and acid-stable (P-O) linkages in nuclear proteins demonstrated the two processes to be equally common (Chen *et al.*, 1974, 1977). The biological role of histone H1 Lys phosphorylation is not known. It may have functions similar to those postulated for other types of histone phosphates, e.g. contribution to gene activation, DNA replication or mitosis. Acidic non-histone proteins have also been found highly phosphorylated during liver regeneration, yielding both acid-labile (Lys and His) and acid-stable forms (Chen *et al.*, 1974).

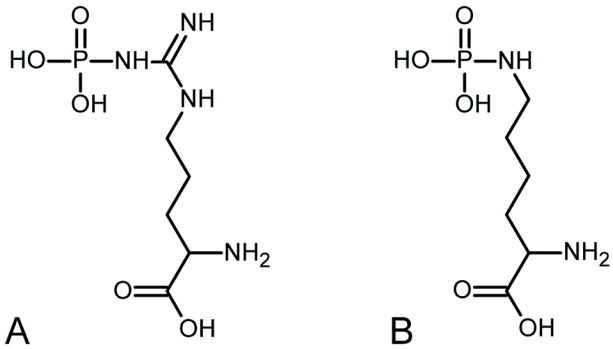

Figure 2. Structures of N^{ω}-phosphoarginine (A) and N^{ε}-phospholysine (B).

Ser10 and Ser28 residues in the N-terminal region of histone H3 have been proposed as mitosis-specific sites of phosphorylation involved in chromatin condensation (Shibata *et al.*, 1990). However, those authors destroyed any potential phosphoramidate linkages by precipitating the phosphorylated protein with trichloroacetic acid. Wakim and Aswad (1994) showed histone H3 to undergo phosphorylation by a Ca^{2+}-calmodulin-dependent kinase on four arginine residues (Arg2, 128, 129 and 131), three of them located within the C-terminus. It has been demonstrated by *in vivo* incorporation of ^{32}P into H3 in rat heart endothelial cells that phosphorylation of basic amino acid occurred in quiescent but not in dividing cells (Wakim *et al.*, 1995). A Ca^{2+}-calmodulin-dependent kinase, while present in nearly equal amounts in both quiescent and dividing cells, was activated 20–100-fold in quiescent cells. Those authors proposed that phosphorylation of histone H3 was involved in cell cycle exit in eukaryotes.

Other proteins

Phosphorylation on serine and threonine residues in myelin basic protein has been studied in a number of laboratories. Smith and coworkers examined the phosphorylation of myelin basic protein under pH-neutral conditions and reported the occurrence of not only base-stable but also acid-labile phosphoryl bonds in myelin, the latter connected with the presence of phosphoarginine and phosphohistidine (Smith *et al.*, 1976).

Phosphoarginine and phosphoserine were found in basic internal core protein, VP12, of granulosis virus infecting the Indian meal moth *Plodia interpunctella* (Wilson & Consigli, 1985). The cyclic-nucleotide-independent protein kinase catalyzing the transfer of phosphate to both arginine and serine residues of VP12 has been localized to purified viral capsids. The authors believed this kinase to play a significant role in the viral replication cycle (Wilson & Consigli, 1985).

An arginine-specific protein kinase tightly bound to DNA has been found in rat liver. The enzyme autophosphorylated and also phosphorylated a single chromosom-

al 11-kDa protein (also tightly bound to DNA) and viral capsidic protein VP12 (Levy-Favatier *et al.*, 1987).

IDENTIFICATION OF PHOSPHOHISTIDINE, PHOSPHOARGININE AND PHOSPHOLYSINE IN PROTEINS

Several standard methods are employed to detect phosphorylated proteins, including sample fractionation, affinity purification, gel separation, immuno-based techniques, kinase/phosphatase activity assay, and various analytical procedures including mass spectrometry and ^{31}P NMR (for review, see Ross, 2007; Matthews, 1995; Fujitaki & Smith, 1984; Besant & Attwood, 2010). Phosphomonoesters (P-Ser, P-Thr, P-Tyr) are stable under acidic conditions and labile under alkaline conditions, except for phosphotyrosine, whereas phosphoramidates (P-His, P-Arg, P-Lys) are extremely acid-labile but relatively base-stable, except for arginine in hot alkali (Fujitaki & Smith, 1984; Klumpp & Krieglstein, 2002). Additionally, in contrast to phosphomonoesters, all phosphoramidates are unstable when exposed to neutral hydroxylamine or pyridine (DiSabato & Jencks, 1961; Duclos *et al.*, 1991). Histidine phosphorylation is inhibited by diethylpyrocarbonate and other histidine-specific reagents. Phosphocysteine is labile in slightly acidic conditions but is quite stable at very high or very low pH (Pigiet & Conley, 1978). Acylphosphates (P-Asp, P-Glu) are, on the other hand, labile at either pH extreme (Koshland, 1952) and are cleaved by hydroxylamine (DiSabato & Jencks, 1961). These features must be taken into account while using various techniques for phosphoprotein studying and may be employed for discrimination among phosphoproteins containing different types of phosphoamino acids. Since the majority of conventional techniques involve acidic treatment, they have to be adapted for use for phosphoramidate identification or several alternative experimental approaches may be utilized: (i) partial alkali hydrolysis to reduce the relative abundance of base-labile P-Ser and P-Thr (Chen *et al.*, 1974; Besant & Attwood, 1998), (ii) dialysis methods of assaying for acid lability (Fujitaki *et al.*, 1981), (iii) extraction methods for determination of phosphomonoesters to phosphoramidates ratio (see Fujitaki & Smith, 1984 and references there), (iv) neutral or basic polyacrylamide gel electrophoresis (Hardison & Chalkley, 1978; Fujitaki & Smith, 1984), (v) staining with commercially available phosphoprotein-specific fluorescent stain Pro-Q® Diamond, (vi) HPLC (Steiner *et al.*, 1980; Matthews & Wei, 1991; Zu *et al.*, 2007), (vii) reversed-phase column chromatography (Tan *et al.*, 2003), (viii) reversed-phase TLC (Besant *et al.*, 2000), (ix) thin-layer silica chromatography (Hess *et al.*, 1988), (x) thin-layer electrophoresis (Besant & Attwood, 2000), (xi) mass spectrometry (Medzihradszky *et al.*, 1997; Besant *et al.*, 2000; Zu *et al.*, 2007; Kleinnijenhuis *et al.*, 2007; Lapek *et al.*, 2011), (xii) ^{31}P-NMR (Fujitaki *et al.*, 1981). Both acidic conditions and high temperature should be avoided throughout the whole process of phosphorylation analysis. This means that neutral pH is favorable also during purification of the specific protein. On the other hand, applying gentler methods for detection of phosphorylation, such as ECD-MS, should improve the results (Kleinnijenhuis *et al.*, 2007; Kowalewska *et al.*, 2010).

Another option is to use, at least in *in vitro* studies, more stable analogues of phosphorylated basic amino acids, e.g. thiophosphoramidate analogue such as thiophosphohistidine, in which one of the oxygen atoms is replaced by the less electronegative sulfur atom. While substitution of sulfur for oxygen seems to cause a negligible steric/electronic perturbation to phosphohistidine, thiophosphohistidine is much more stable than phosphohistidine (Lasker *et al.*, 1999; Pirrung *et al.*, 2000; Ruman *et al.*, 2009; 2010). Thiophosphorylation can be carried out either by the use of an engineered protein kinase, capable of consuming ATPγS (Allen *et al.*, 2005; Carlson *et al.*, 2010), or by chemical reaction with thiophosphoramidate (Lasker *et al.*, 1999; Pirrung *et al.*, 2000; Ruman *et al.*, 2009; 2010). The latter method leads to a specific modification of the N-3 atom in the histidine side chain.

The usage of stable analogues of phosphorylated basic amino acids should help to solve another problem hampering studies on phosphoramidate-modified proteins. Since 1981, when the first antiphosphoamino acid antibody, capable of recognizing phosphotyrosine-containing proteins, was obtained from serum of rabbits immunized with benzonyl phosphonate conjugated to keyhole limpet hemocyanin (Ross *et al.*, 1981), this new tool has been widely used in immunodetection of protein phosphorylation. Moreover, in 1991 phosphorylation state-specific (phospho-specific) antibodies were produced in rabbits following immunization with phosphorylated peptides of an amino-acid sequence identical with that encompassing the target protein phosphorylation site (Czernik *et al.*, 1991). The latter created the possibility of specific recognition of the phospho-protein of interest. It should be noted, though, that commercial antiphosphoamino acid antibodies enabling qualitative and quantitative immunoassay of phosphoproteins as well as their concentration by immunoprecipitation or affinity chromatography, are only available thus far for specific proteins phosphorylated on hydroxy amino acids. Until very recently attempts to develop antibodies selective to a hapten containing phosphohistidine have been failing, presumably due to hydrolysis of such an immunogen being too fast to raise a strong immune response. Consequently, application of non-hydrolysable analogues of phosphorylated basic amino-acids appeared hopeful in this respect. One example of such a phosphohistidine analogue was phosphofurylalanine (Schenkels *et al.*, 1999) but to our knowledge no reports appeared on its use as a hapten, and another was a pyrrole derivative analogous to phosphofurylalanine, that was successfully used to raise antibodies that were, unfortunately, selective for the analogue but not for phosphohistidine (Attwood *et al.*, 2007). Only last year were Kee *et al.* (2010) successful in designing and synthesizing two phosphoryltriazolylalanine isomers as stable analogues of the two phosphohistidine isomers (1-P-His and 3-P-His). Of note is that both analogues were applied with success in solid-phase peptide synthesis and a synthetic peptide obtained this way, containing 3-P-His analogue, was used as an immunogen to raise rabbit polyclonal antibody selectively recognizing 3-P-His in full length histone H4 protein.

Of note, many commercially available anti-phosphotyrosine IgGs recognize also phosphohistidine. Taking into account that phosphohistidine may be 10 to 100 times more abundant than phosphotyrosine, this might provide a means to identify phosphohistidine in proteins. On the other hand, this phenomenon may also be misleading in the case of studies of phosphotyrosine.

Further problems with detecting phosphohistidine, phosphoarginine and phospholysine may result from a lack of commercially available standards (except for N^{ω}-phosphoarginine), although appropriate synthetic methods have been reported (Zetterquist & Engström, 1967; Marcus & Morrison, 1964; DeLuca *et al.*, 1963).

It is also important to bear in mind that denaturing buffers and phosphatase inhibitors normally used to suppress enzyme activity during protein extraction will most likely not guard against hydrolysis of phosphohistidine. The potential methodological obstacles encountered by researchers working on acid-labile protein phosphorylation are well presented in several previous reviews (Matthews, 1995; Wei & Matthews, 1990; Klumpp & Krieglstein, 2002; 2009; Besant & Attwood, 1998; 2005; 2009; Besant *et al.*, 2003; 2009; Steeg *et al.*, 2003; Attwood *et al.*, 2007; Ross, 2007; Kowluru, 2008; Zu *et al.*, 2009).

In view of the difficulties in the analysis of phosphorylation of basic amino acids, the usage of *in silico* simulations should be considered useful, for example to predict the influence of phosphorylation on protein properties. Such a study with the use of molecular dynamics simulations allowed revealing the mechanism of the influence of Ser124 phosphorylation on the catalytic activity of human thymidylate synthase (Jarmuła *et al.*, 2010), and the same approach could be applied to study the consequences of phosphorylation of basic amino-acid residues, taking advantage of the force-field parameters calculated recently for phosphohistidine (Kosinsky *et al.*, 2004; Homeyer *et al.*, 2006).

CONCLUSIONS

Although the presence of phosphorylated basic amino-acid residues in proteins was recognized long time ago, it is often missed because of the short life-time and acid lability of the phosphoramidate bond and a lack of dedicated tools, such as specific antibodies, and therefore poorly studied. Surprisingly, for a long time phosphorylation of histidine (as well as lysine and arginine) residues in proteins and its role in signal transduction have been investigated and appreciated in prokaryotes but believed to be absent from eukaryotes, while phosphorylation of protein hydroxyamino acid (Ser, Thr, and Tyr) residues has been extensively studied in eukaryotes but believed not to occur in prokaryotes. As it now appears obvious that both types of protein phosphorylation play important roles in the functioning of both prokaryotic and eukaryotic cells, new tools and methods are necessary to intensify the studies on phosphorylation of protein basic amino-acid residues.

Acknowledgements

Supported by the Ministry of Science and Higher Education (grant number N N401 024 036).

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