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The actions of NME1/NDPK-A and NME2/NDPK-B as protein kinases

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Abstract

Nucleoside diphosphate kinases (NDPKs) are multi-functional proteins encoded by the NME (Non Metastatic cells) genes, also called NM23. NDPKs catalyze the transfer of γ-phosphate from nucleoside triphosphates to nucleoside diphosphates by a ping-pong mechanism involving the formation of a high energy phospho-histidine intermediate. Growing evidence shows that nucleoside diphosphate kinases (NDPKs), particularly NDPK-B, can additionally act as a protein histidine kinase. Protein kinases and phosphatases that regulate reversible O-phosphorylation of serine, threonine and tyrosine residues have been studied extensively in many organisms. Interestingly, other phosphoamino acids histidine, lysine, arginine, aspartate, glutamate and cysteine exist in abundance but remain understudied due to the paucity of suitable methods and antibodies. The N-phosphorylation of histidine by histidine kinases via the two- or multi-component signaling systems is an important mediator in cellular responses in prokaryotes and lower eukaryotes, like yeast, fungi, and plants. However, in vertebrates knowledge of phosphohistidine signaling has lagged far behind and the identity of the protein kinases and protein phosphatases involved is not well established. This article will therefore provide an overview of our current knowledge on protein histidine phosphorylation particularly the role of nm23 as a protein histidine kinase.

Protein Serine, Threonine and Tyrosine Phosphorylation

In animals the most well recognized forms of protein phosphorylation are those in which the phosphoserine, phosphothreonine or phosphotyrosine are formed on the target protein in reactions catalyzed by Ser/Thr or Tyr protein kinases. The phosphorus of the phosphoryl group is bonded to the hydroxyl oxygen in these phosphoamino acids, which are thus phosphoesters (Fig. 1). These reactions involve the transfer of the gamma-phosphoryl group from ATP to the hydroxyl oxygen of the side chain of Ser, Thr or Tyr. The phosphorylation of target proteins results in a change in protein activity through conformational changes in the protein structure induced by the insertion of the relatively large, negatively charged phosphoryl group. Alternatively, the phosphoamino acid acts as a docking site to allow other proteins to bind to the phosphoprotein (e.g. proteins containing SH2 domains can bind via these domains to phosphoproteins containing phosphotyrosine). Many aspects of cellular function in animals are regulated by signaling pathways involving Ser/Thr and Tyr protein kinases. In addition these pathways also involve phosphoprotein phosphatases which catalyze the hydrolysis of the phosphoamino acids in phosphoproteins, thus reversing the effects of phosphorylation on protein activity and protein-protein interaction. It is only relatively recently that similar protein kinases and phosphoprotein phosphatases were found to occur in bacteria (for reviews see Pereira *et al.* 2011 and Chao *et al.* 2014)[1, 2].

Protein Histidine Phosphorylation

(i) Bacteria, fungi and plants

Before the discovery of the occurrence of Ser/Thr and Tyr protein kinases in bacteria it was thought that the predominant form of protein phosphorylation to occur in these organisms was histidine phosphorylation, catalyzed by protein histidine kinases.

In phosphohistidine, the phosphorus of the phosphoryl group is bonded to one of the nitrogens in the imidazole ring of the side chain of histidine to form a phosphoramidate (Fig. 1). Since the

imidazole ring of histidine contains two nitrogens, there are two possible isomers of phosphohistidine, 1-phosphohistidine and 3-phosphohistidine, both of which occur biologically (Fig. 1).

In bacteria and lower eukaryotes, protein histidine kinases are parts of the two-component histidine kinase systems where the histidine kinase is usually part of a membrane-bound receptor, which is activated on receipt of an extracellular stimulus by the sensor domain. This results in the autophosphorylation of a conserved histidine residue in the dimerisation domain of the receptor (see Fig. 2 in Attwood, 2013[3]). The phosphoryl group is then transferred from the phosphohistidine to a conserved aspartate residue in the regulatory domain of a response regulator protein in a reaction catalyzed by this protein (Fig. 1). This activates the response regulator protein, which is most commonly a transcription factor, which then induces the ultimate cellular response to the original extracellular stimulus. There are more complex systems involving such histidine kinases in which there are multiple phosphoryl transfers between the histidine kinase and the response regulator protein in a phosphorelay system involving proteins/domains in which phosphoryl transfer occurs alternately to aspartate and histidine (see Fig. 3 in Attwood, 2013[3]). These systems occur in plants and fungi, as well as bacteria. It is the thermodynamic instability of phosphohistidine (as well as that of phosphoaspartate – a phosphoanhydride) compared to the phosphoester phosphoamino acids that facilitates these phosphoryl transfer reactions (Table 1). In addition, in bacteria, Lu et al. showed that NDP kinase was capable of phosphorylating histidine residues in the two-component histidine kinases EnvZ and CheA in Escherichia coli.[4] However, since this phosphorylation only occurred when ADP was present in the reaction mixture, Levit et al demonstrated that, independent of any protein-protein phosphotransfer mechanism by NDPK, contaminating ADP in the reactions (and NDP kinase) may have been responsible for generation of ^{32}P-ATP required for autophosphorylation of the bacterial His kinases.[5]

Another system in bacteria that involves histidine phosphorylation is the phosphoenolpyruvate/sugar phosphotransferase system in which a phosphoryl group initially derived from phosphoenolpyruvate, and following transfer between different protein components of the system, is finally used for phosphorylation of the transported sugar molecule[6].

(ii) Animals

This type of phosphoryl transfer between proteins does not appear to occur in animal signalling systems that use Ser/Thr and Tyr protein kinases. Nor is there any direct evidence of the occurrence of two-component histidine kinase systems in animals, although as noted by Attwood (2013),[3] there are a number of animal proteins in sequence databases that contain the conserved amino acid sequences associated with both histidine kinase and response regulator proteins in two-component histidine kinase systems. What little is known about protein phosphorylation by histidine kinases in animals suggests that the main purpose of this form of phosphorylation is similar to that in the Ser/Thr and Tyr kinase systems i.e. changing protein activity and possibly protein-protein interaction (see below).

If protein histidine phosphorylation is playing similar roles to serine, threonine and tyrosine phosphorylation in animals, one would expect there to be one or more phosphatases to catalyse the dephosphorylation of phosphohistidine-containing phosphoproteins. In fact, there is some question as to the requirement for a specific protein phosphohistidine phosphatase (PHP) since it has been shown that some protein phosphoserine/phosphothreonine phosphatases are also very efficient PHPs, e.g. PP1, PP2A and PP2C.[7, 8] However, a mammalian PHP (or PHPT1) has been discovered[9, 10] that shows high specificity towards proteins and peptides containing phosphohistidine and no activity towards peptides containing phosphoserine, phosphothreonine or phosphotyrosine[6]. Later though, PHPT1 was found to have a somewhat wider specificity, as it was also found to catalyse the hydrolysis of phosphoramidate[11] and also phospholysine, but not

phosphoarginine[12]. A number of metabolic enzymes do make use of the thermodynamic instability of phosphohistidine to catalyse phosphoryl transfer reactions via an enzymic, active site phosphohistidine intermediate e.g. phosphoglycerate mutase[13] ATP-citrate lyase[14] and nucleoside diphosphate kinase (NDPK)[15]. It has been suggested that the apparent phosphorylation of both succinate thiokinase and ATP-citrate lyase by NDPK[16, 17] may be explained as autophosphorylation events in these enzymes since both enzymes have a reactive histidine residue in their active sites which undergoes autophosphorylation in the course of their normal catalytic cycles.[5, 18] However, this does not explain the histidine phosphorylation of aldolase C by Nm23-H1 or rat liver NDPK[19] and other mammalian proteins as detailed below.

Animal Protein Histidine Kinases

(i) Histone H4 Histidine Kinase(s)

Amongst the earliest described animal (mammalian) histidine kinases is histone H4 histidine kinase. This was discovered in regenerating rat liver nuclei[20, 21] and in Walker 256 carcinosarcoma cells[22]. Later, it was shown that a similar histone H4 histidine kinase was present in porcine thymus nuclei[23] and in human hepatocellular carcinoma tissue and human foetal liver [17] Histone H4 histidine kinase activity was also found in *Physarum polycephalum*[24] and *Saccharomyces cerevisiae*[25]. Thus, this histidine kinase activity is associated with cellular proliferation in normal and cancerous cells, however it is not clear how histone H4 histidine phosphorylation is linked to these process. In addition, a histone H4 histidine kinase has never been fully characterized and identified from any source.

(ii) Nucleoside Diphosphate Kinase

NDPK is normally regarded to fulfill a metabolic role in cells by maintaining the balance of nucleoside triphosphates by catalyzing the transfer of a phosphoryl group from a nucleoside

triphosphate (most commonly ATP) to a nucleoside diphosphate via an active site phosphohistidine intermediate (Fig. 2). Recently however, the -A and -B isoforms of NDPK have been reported to act protein histidine kinases, in which the phosphoryl group from the active site phosphohistidine of NDPK is transferred to a histidine residue in another protein. NDPK-A has been reported to phosphorylate ATP-citrate lyase[26] and annexin A1 [27, 28] whilst NDPK-B phosphorylates the beta subunit of trimeric G-proteins, [29] the potassium channel – $K_{Ca}3.1$[30] and the calcium channel – TRPV5.[31]

Histidine phosphorylation and TRPV5 calcium channels

Cai et al provided evidence that TRPV5 is regulated by reversible histidine phosphorylation [31]. The authors demonstrated that NDPK-B, but not NDPK-A, regulates the epithelial Ca^{2+} channel the transient receptor potential-vanilloid-5 (TRPV5); activation of TRPV5 requires phosphorylation of His711 in the C-terminus by NDPK-B whereas dephosphorylation by PHPT1 inactivated the channel. Furthermore, knockdown of NDPK-B by siRNA resulted in decreased TRPV5 channel activity, and in addition, NDPK-$B^{-/-}$ mice fed a high-Ca^{2+} diet showed increased urinary Ca^{2+} excretion. The findings that NDPK-B and PHPT1 regulate TRPV5 and Ca^{2+} reabsorption support the idea that reversible histidine phosphorylation plays many important but as yet-unknown roles in mammalian cellular function.

NDPK and tumour metastasis suppression

In the early 1990s, it became evident that enhanced cancer metastasis was linked to reduced expression of a gene named nm23, which turned out to be identical to human Nme1, also called Nm23-H1 or NDPK-A. Differential expression of Nme1 was found in experimental melanoma metastasis whereby Nme1 was more highly expressed in poorly metastatic sublines of the K-1735 murine melanoma than in related, highly metastatic sublines.[32] Transfection of Nme1 into a highly

metastatic melanoma line significantly reduced metastasis but had no effect on primary tumor size.[33] The metastasis suppressor activity of Nme1 has been demonstrated in many cancers except leukemias, lymphomas, and neuroblastoma.[34, 35] Multiple aspects of metastasis have been implicated in Nme1 suppression including cell adhesion[36-38], motility and signalling[39-45], and proteolysis[46]. As discussed by Kaetzel *et al.* 2014, Nme1 promotes genomic stability in melanoma through direct interactions with at least two DNA repair pathways, nucleotide excision repair and double-strand break repair.[38] However, to date, the mechanism and function of Nme1 underlying its tumor metastasis suppressor activity remains to be established.

The question of which of the biological activities of Nme1 is associated with suppression of metastasis has been the subject of investigation over a number of years. Freije *et al.*[47] and Wagner *et al.*[48] used the approach of examining the effects of overexpression of wild-type and site-directed mutant forms of Nme1on human breast carcinoma cell motility. Freije *et al.*[47] showed that the loss of protein kinase activity in the two mutants (P96S and S120G) correlated with the inability of these mutants to suppress cell motility. Both of these mutant forms of Nme1 retained normal autophosphorylating activity[47]. Wagner *et al*.[48]showed that the failure of these two mutant forms of Nme1 to suppress motility was most strongly correlated with a reduced ability to phosphorylate glutamate or aspartate residues on a 43 kDa protein from bovine brain rather than the ability to phosphorylate histidine residues on succinate thiokinase or ATP-citrate lyase. Later work by Hartsough *et al.*[40], associated the metastasis suppressor activity of Nme1 with its ability to phosphorylate the kinase suppressor of Ras on serine residues. More recently, Wu *et al.*[49] showed that two serine mutants of Nme1 (S44A and S120G) were unable to suppress tyrosine phosphorylation of STAT3 which is involved in the upregulation of expression of metalloproteinase 9, an important metastatic factor. However, the H118F mutant of Nme1 still maintained the ability to suppress STAT3 phosphorylation. In this instance at least, the autophosphorylation ability and protein kinase function of Nme1 does not appear to be directly connected with the effect on STAT3

phosphorylation. Thus much remains to be investigated concerning the relationship between the protein kinase activity of Nme1 in general, and its protein histidine kinase activity in particular, and its anti-metastatic effect.

Histidine phosphorylation and Ca^{2+}-activated potassium channels

$K_{Ca}3.1$ is a Ca^{2+}-activated K^+ channel of an intermediate conductance which is expressed in T and B cells, smooth muscle and epithelial cells. The channel functions to keep a negative membrane potential by mediating the efflux of K^+, which is required to maintain a favourable electrochemical gradient for Ca^{2+} influx. Activation of $K_{Ca}3.1$ requires not only binding of phosphatidylinositol 3-phosphate PI(3)P and Ca^{2+}/calmodulin to 14 amino acids in the carboxyl terminus but also phosphorylation by nucleoside diphosphate kinase (NDPK-B) at His358 in the same cytoplasmic domain [30, 50]. Among the four calcium-activated K_{Ca} potassium channels (KCa2.1, KCa2.2, KCa2.3 and $K_{Ca}3.1$; also called SK1–4), $K_{Ca}3.1$ is unique as it possesses a His residue at 358, which requires phosphorylation for activation. Using a yeast two-hybrid library screen, Srivastava *et al*, demonstrated binding of NDPK-B to the 14 amino acids carboxyl terminus of $K_{Ca}3.1$ and showed that phosphorylation of H358 of $K_{Ca}3.1$ regulates its activation in $CD4^+$ T cells.[30] The specificity and relevance of the NDPK-B/ $K_{Ca}3.1$ interaction was confirmed by biochemical, molecular and functional approaches: a) immunoprecipitation involving endogenous NDPK-B and $K_{Ca}3.1$ proteins in human $CD4^+$ T cells, b) whole-cell patch-clamp experiments showing increased amplitude in CHO cells overexpressing NDPK-B and $K_{Ca}3.1$ proteins, c) NDPK-B knockdown showed inhibition of $K_{Ca}3.1$ channel activity, Ca^{2+} influx and proliferation of human $CD4^+$ T cells. Srivastava *et al* have also demonstrated that the protein histidine phosphatase PHPT1 dephosphorylates pHis 358 of $K_{Ca}3.1$ leading to negative regulation of $CD4^+$ T cells.[51] Interestingly, that PHPT1 failed to dephosphorylate autophosphorylated NDPK-B (pHis118) [52] provided evidence for the potential existence of other PHPs apart from Ser/Thr phosphatases that

can also randomly dephosphorylate pHis proteins as shown by a previous report.[7] In this regard, Panda *et al*. recently provided confirmation that other phosphatases exist that may specifically interact with and target phosphohistidine residues on particular proteins via specific binding motifs; they showed that phosphoglycerate mutase 5 (PGAM5) dephosphorylates NDPK-B autophosphorylated on H118, thereby inhibiting subsequent phosphorylation and activation of $K_{Ca}3.1$ and resulting in decreased TCR-mediated Ca^{2+} influx and proinflammatory cytokine production in $CD4^+$ T cells. [53] These authors also observe that PGAM5 function as a histidine phosphatase is restricted to NDPK-B pHis 118 and that a 12 aa motif N-terminal to the PGAM domain mediates binding to NDPK-B, but PGAM is unable to dephosphorylate NDPK-A pHis118 or the $K_{Ca}3.1$ pHis358 and inhibit its activity; both NDPK-A and $K_{Ca}3.1$ lack the cognate motif for PGAM5 (conversely NDPK-B lacks the binding motif for PHPT1)[51]. Although counterintuitive at first glance, since the 3D - structure of human NDPK-A/ADP shows only a single NTP/NDP binding,[54] this suggests that phosphorylated His118 in the NDPK active site is exposed and accessible to PGAM5. It is worth noting that PGAM5 belongs to the histidine phosphatase superfamily, a large functionally diverse group of proteins that share a common catalytic domain, designated as the PGAM domain and functions as phosphotransferases and/or phosphohydrolases; and share a conserved catalytic core centred on a histidine which becomes phosphorylated during the course of the reaction.[55] Most members of the superfamily possess phosphoglycerate mutase activity and are involved in regulating glucose metabolism. On the other hand, some family members such as PGAM5, which do not possess PGAM activity, have been shown to function as protein phosphatases specific for phospho-Ser/Thr (pSer/pThr) residues. Thus, Panda *et al* provide evidence for transfer of phosphate from the autophosphorylated NDPK-B H118 active site and subsequent phosphorylation of His105 within the PGAM5 active site.[53] However, the reaction stoichiometry is unclear from this study and whether the transferred phosphate is retained for further relay to yet-unknown target(s) or finally released as Pi; nonetheless, the study provides more

evidence for His/His phospho-relay signalling networks, largely underappreciated in eukaryotic systems. Additionally, it is also as yet unclear whether PGAM5-activity against pSer/Thr results in similar phosphorylation of PGAM at H105 or Pi release, since phosphoester bonds have low Delta G degrees of hydrolysis, compared to the phosphoramidate (P-N) bond of phosphohistidine (Table 1).

NDPK and CFTR chloride channels

In epithelia, chloride (Cl^-) channels play a prominent role in fluid and electrolyte transport. Of particular importance is the cystic fibrosis (CF) transmembrane conductance regulator Cl^- channel (CFTR, a cAMP/ATP and/or GTP regulated chloride channel) with mutations of the CFTR encoding gene causing cystic fibrosis. Using a two-hybrid screen and immunoprecipitation analysis, Klein et al recently provide evidence that CFTR and $K_{Ca}3.1$ can exist in a dynamic complex in epithelia and demonstrate that physical interaction occurs between cytosolic domains of both proteins; both the N-terminal fragment M1-M40 and part of the calmodulin binding domain (residues L345-A400) of $K_{Ca}3.1$ were found to interact with the nucleotide binding domain NBD2 segment (G1237-Y1420) and C- terminal region of CFTR (residues T1387- L1480) [56].

Previous work demonstrated that in apical membranes from airway epithelia, NDPK histidine phosphorylation is regulated by both chloride and cation concentration *in vitro* and provided evidence not only for NDPK involvement but for potential His/His phospho-relay signalling in chloride secretion and epithelial function[27, 28, 57].

Although NDPK-A and B have previously been shown to exist as a heterohexamer, increasing evidence suggests that despite their highly homologous nature (and their genes lie adjacent to one another), the cellular actions of NDPK-A and -B isoforms differ substantially in airway epithelia. For example, NDPK-B is predominantly cytosolic in airway epithelia while NDPK-A is present in both the cytosol and membrane fractions [58]. The precise distinction between cytosolic and

particulate NDPK-A is as yet unknown but recent evidence also shows that membrane bound NDPK-A interacts with AMP-activated protein kinase (AMPK) [59, 60]. Although both NDPK-A and AMPK exist in the cytosol and membrane compartments, the two proteins do not always exist in a complex in epithelia suggesting the existence of a specific mechanism or post-translational modification and/or binding partner (unpublished observations).

Interestingly, both NDPK-A and B interact with CFTR either directly or indirectly through associated proteins [58, 60]. This interaction with CFTR occurs independently of each isoform, by different mechanisms and with different cellular end points. King et al provided evidence that NDPK-A, AMPK, and CFTR exist in a plasma membrane-associated complex in airway epithelia, whereby AMPK phosphorylates CFTR and inhibits PKA-stimulated CFTR channel gating. Although the precise mechanisms remain unclear, it was observed that a) AMPK catalytic function enhances NDPK-A auto-phosphorylation at His-118 *in vitro,* b) *Xenopus* oocytes co-expressing AMPK and CFTR with wild type, but not the NDPK-A catalytically inactive H118F mutant or various Ser-120 NDPK-A mutants, inhibit CFTR conductance in two-electrode voltage clamp studies indicating that NDPK-A catalytic function plays an integral role in the inhibition of CFTR by AMPK.[60] The observation that AMPK enhances NDPK-A auto-phosphorylation is in agreement with a previous work in human bronchial epithelial cell line 16HBE14o- (enhancement does not occur in CF bronchial epithelial cells (CFBE41o^{-}) cell lines [59, 61]. In contrast, others have provided evidence that during nutrient stress, AMPK phosphorylates NDPK at S120, thereby mediating inhibition of NDPK activity. AMPK is a key energy sensor that regulates metabolism to maintain cellular energy balance [62]. The functional interaction between CFTR, AMPKα1 and NDPK-A, is independent of NDPK-B.

Following cAMP/PKA activation, NDPK-B translocates from the cytosol to the apical membrane in airway epithelia to form a functionally relevant complex with CFTR. The precise mechanism leading to NDPK-B relocation to the apical membrane is still unclear but interaction with CFTR

occurs through a distinct domain of NDPK-B involving amino acids 36 - 56 while the nucleotide binding domain 1 (NBD1, aa 351– 727) of CFTR constitutes an NDPK-B interaction site. Interestingly, although both CFTR and NDPK-B exist in other cellular compartments, the cAMP/PKA induced interaction is restricted to the apical membrane and highlights a particular role for NDPK-B in epithelial cell chloride secretion. A peptide derived from NDPK-B 36–54 aa sequence, but not its NDPK-A equivalent, not only disrupts the interaction between NDPK-B and CFTR but also reduces chloride currents in epithelia.

Histidine phosphorylation and heterotrimeric G-protein signalling

Stimulated G-protein–coupled receptors (GPCRs) catalyze the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) and the dissociation of the G-protein heterotrimer, allowing both the GTP-bound Gα subunit and free βγ complexes to signal to downstream effectors including adenylate cyclase, phosphodiesterase, phospholipase C or ion channels to bring about a wide variety of physiological processes. Recent evidence shows that His/His phospho-relay signalling provides an alternative receptor-independent way of heterotrimeric G protein activation [29, 63]. NDPK-B, but not NDPK-A, forms complexes with Gβγ resulting in phosphorylation of histidine residue His266 on the G protein β subunit of heterotrimeric G proteins and leading to formation of GTP and to an increase in cAMP synthesis. Cells expressing an inactive NDPK-B mutant (H118N) exhibit decreased cAMP formation. In cardiomyocytes, heterotrimeric G proteins are key regulators of myocardial contractility. SiRNA-mediated NDPK-B knockdown and overexpression of a βγ-dimer mutant carrying a single amino acid substitution at His-266 in the β1 subunit (Gβ1H266Lγ_2) led to reduced receptor-independent Gαs activation, suppressed basal cAMP formation and basal contractility in intact cardiomyocytes [64]. However, the β-adrenoceptor (βAR) agonist isoproterenol-induced increase in cAMP formation was not affected by Gβ1H266Lγ_2.

Mäurer *et al* have also demonstrated that eukaryotic phosphohistidine phosphatase (PHP, also called PHPT1) specifically dephosphorylates Gβ (pHis266), but not NDPK-B (pHis118), in reconstituted systems and in H10 cells membranes [65] suggesting that PHPT1 might be a candidate for an endogenous regulator of basal NDPK-dependent G protein activation. It has also been observed that in plasma membranes of hearts from patients with severe congestive heart failure, but not in patients treated with a βAR antagonist, a 3-fold elevated content and activity of NDPK is detected and that chronic treatment of rats with isoproterenol, induces an increase in plasma membrane bound NDPK-B [66, 67]. This suggests that NDPK-B membrane content may be under the control of β-adrenergic signalling and that NDPK may play an important role in heart failure and hypertrophy. *Abu*-Taha *et al* show that NDPK-C-mediates targeting of NDPK-A and -B isoforms to the plasma membrane and formation of the NDPK-B/G proteins complex in cardiomyocytes and is thus a critical regulator of β-adrenoceptor/cAMP signaling and cardiac contractility [68].

In conclusion, currently the well-studied cellular functions of NDPKs as protein kinases is the regulation of G protein activation and of ion channels by NDPK-B. Among the important questions that remain to be addressed is how NDPK can function both as a nucleoside phosphotransferase and a histidine protein kinase. The crystal structure of human NDPK-A/ADP shows a single NTP/NDP binding pocket that in this conformation does not appear to be accessible to protein substrates.[54] It has been suggested that residues of the substrate proteins of NDPK may play a role in catalyzing phosphoryl transfer from pHis118 of NDPK-B and in this regard, Attwood and Wieland have recently suggested various models of structures of NDPK-B and target proteins.[6] However, it is also clear that other as yet uncharacterised protein factors are required for interaction and phosphoryl transfer to occur.[29, 30, 68]

In view of the high sequence similarity between NDPK-A to C (66-88%)[26], protein-protein interactions most likely form the basis for target selection and distinction between the various

NDPKs. As demonstrated for CFTR, $K_{Ca}3.1$ and PGAM5 binding with NDPK-B, the notion that protein-protein interactions modulate the specific molecular actions of NDPK[59] remains current and is likely to grow in importance particularly as more targets for NDPKs are identified, e.g. by new advances in phosphohistidine proteomics[69, 70].

Disclosure/Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Figure Legends

Figure 1. Structures of the side chains of: **a.** phosphoserine; **b.** phosphothreonine; **c.** phosphotyrosine; **d.** 1-phosphohistidine; **e.** 3-phosphohistidine; **f.** phosphoaspartate.

Figure 2 The reaction mechanism of the normal metabolic reaction catalyzed by NDPK. This shows the initial transfer of gamma phosphoryl group from a nucleoside triphosphate (XTP) to a histidine residue in the active site of NDPK, (NDPK-His) to form NDPK-phosphohistidine (NDPK-His-P) and XDP. The nucleoside diphosphate (YDP) then binds in the active site of NDPK and the phosphoryl group is transferred to it from the phosphohistidine to YDP to form YTP.

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