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Bunce, Christopher; Khanim, Farhat

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The 'known-knowns', and 'known-unknowns' of extracellular Nm23-H1/NDPK proteins.

Chris M Bunce and Farhat L Khanim

School of Biosciences, University of Birmingham, Birmingham, UK

Abstract

Nucleoside diphosphate kinases (NDPKs/NDK/NME) are a multifunctional class of proteins conserved throughout evolution. Whilst many of the functions of NDPKs have been identified as intracellular, extracellular eukaryotic and prokaryotic NDPK proteins are also detected in multiple systems and have been implicated in both normal physiology and disease. This review provides an overview of where the field stands on our developing understanding of how NDPK proteins get out of cells, the physiological role of extracellular NDPKs, and how extracellular NDPKs may signal to cells. We will also discuss some of the unanswered questions, the 'known-unknowns' that particularly warrant further investigation.

Introduction

The nucleoside diphosphate (NDP) kinase (NDK/NDPK/NME) protein family are amongst the most evolutionarily conserved proteins from prokaryotes through to complex multicellular eukaryotes [1]. NDPKs are small (11-20 kDa) proteins that typically form multimers of dimers as either hexamers or tetramers. In eukaryotes [2, 3], NDPKs mostly form hexamers however some NDPKs from prokaryotes and eukaryotes form tetramers, e.g. rat [4] and Silk worm (*Bombyx mori*) [5]. NDPKs are multifunctional proteins with activities including; catalysing phosphorylation of NDPs to nucleoside triphosphates (NTPs) via a phosphohistidine intermediate, binding DNA and regulating transcription of *c-myc* and other genes, cleaving DNA, and possessing histidine protein kinase activity (reviewed in [6]). Whilst most of the activities described for NDPK proteins are intracellular, NDPK are also released/exported from prokaryotic and eukaryotic cells [7]. Because of the absence of functional motifs common to secreted proteins in either prokaryotes or eukaryotes, the physiological relevance of these extracellular NDPKs (eNDPKs) has been questioned, with some believing that extracellular accumulation is a secondary phenomenon of release following cellular damage. In this brief review we consider the evidence that eNDPKs have important roles during malignant and infectious diseases and identify some areas for future investigation.

Prokaryotic eNDPKs

The function and benefit of secreted NDPKs for free living environmental bacteria was largely unstudied until recently and requires to be addressed. As is often the case, studies have focused on human commensal and pathogenic bacteria. For example, *Mycobacterium tuberculosis* is believed to exploit secreted NDPK in at least two ways. First by modulating macrophage apoptosis as part of an immune avoidance strategy and second, when internalised within macrophages, diminishing free-radical production thereby avoiding intracellular killing [8-10].

The most extensively studied system in the context of bacterial NDPK mediated-host modification is *Porphyromonas gingivalis*, a Gram-negative anaerobic bacterium associated with chronic periodontitis. Like *M. tuberculosis*, *P. gingivalis* is also able to survive within host cells and laboratory based studies at least indicate that *P. gingivalis* NDPK is released from primary infected gingival epithelial cells in which the bacterium is resident [11]. This extracellular translocation of *P. gingivalis* NDPK occurs in the absence of overt damage to the host cell membranes or host cell death [11]. The basis of *P. gingivalis* eNDPK then acting as a virulence factor includes (i) modulation of host cell extracellular ATP (eATP) concentrations resulting in (ii) reduction of host cell eATP/P2X₇ signalling pathways including reduced NLRP3 inflammasome activation [12-14]. Importantly, a recent study

has identified that NDPK released from intracellular *P. gingivalis* localises predominantly to the perinuclear area of infected cells but, upon activation of the host cell by eATP, mobilises to the cell periphery in preparation for translocation to the extracellular space [11]. This observation therefore suggests that the release of *P. gingivalis* NDPK from its host cell is an orchestrated combined host-pathogen response to eATP that results in the depletion of eATP via the catalytic action of the released NDPK protein [15, 16].

Eukaryotic eNDPKs

Extracellular NDPKs are also detected across Eukaryota. One study identified that NDPK from *Leishmania amazonensis* spontaneously accumulates extracellularly in culture [17]. This is a highly selective process unaccompanied by co-release of any other known cytosolic proteins and independent of *L. amazonensis* damage [17]. As shown for both *M. tuberculosis* and *P. gingivalis* NDPKs, the NDPK from *L. amazonensis* diminished macrophage killing in response to eATP [17]. Together these observations provide a collective paradigm where intracellular prokaryote and eukaryote pathogens can propagate their survival and dispersal by limiting their host cell's death in response to local elevation of eATP, arising as a consequence of the inflammatory response to the infection these organisms are mounting [12, 17, 18]. Philosophically this concept has implications for our understanding of the co-evolution of host and parasite. It is reasonable to assume that the re-localisation of foreign NDPK within the host cell and its subsequent translocation to the extracellular space will require pathogen-mediated exploitation of host cell mechanisms.

Drosophila NDPK was first identified in a genetic approach in which developmental mutants were identified as having abnormal wing disks [19-21]. For this reason, the *Drosophila* NDPK is known as AWD. AWD was detected in extracellular vesicles released from *Drosophila* cell lines [22]. Although the function of AWD released in this way remains to be determined, it has also been detected *in vivo* in the haemolymph which serves as the *Drosophila* transport system for other important molecules including nutrients, hormones, defence molecules and mediators of responses to wounding [23].

Similarly the human NDPK, Nm23-H1, can be detected in the blood. Nm23-H1 is one of 10 human NDPKs and until recently has perhaps been the most studied. Interest in Nm23-H1 and its closely related Nm23-H2 was given great impetus by observations by Patricia Steeg and colleagues that these proteins are suppressors of cancer metastasis [24-26]. Elevated Nm23-H1 expression is inversely correlated with metastatic potential in experimental rodent cell cancer models and in human tumours such as breast, ovarian, cervical, bladder, pancreatic and gastric cancer, hepatocellular carcinoma and melanomas [27]. In these settings, overexpression predicts a favourable patient outcome [26-33]. In these tumours, Nm23-H1 accumulates intracellularly as demonstrated using immunohistochemistry [27]. There is also some data that show human breast, colon, pancreas and lung cancer cells have Nm23-H1 and -H2 at their cell surface [34, 35] and can secrete it into the culture media *in vitro* [34, 36, 37]. In addition, laboratory studies in breast cancer suggest that the Nm23 secreted by breast cancer cells may influence breast cancer growth and metastasis [37, 38]. However, studies need to be done to confirm these observations and activities in patients. In contrast, elevated serum levels of Nm23-H1 have been reported in several haematological malignancies [39, 40]. In acute myeloid leukaemia (AML), peripheral T cell lymphoma [41], diffuse large B cell lymphoma (DLBCL) [42], and in Hodgkin lymphoma [43] elevated serum Nm23-H1 positively correlates with disease stage and is a negative prognostic factor for response to treatment [44-46]. This is also the case for neuroblastoma [47].

The role of extracellular Nm23-H1 in haematological malignancies

The elevated levels of Nm23-H1 found in the serum of AML patients or other haematological malignancies may merely reflect the tumour cell origin of the protein. In this scenario, the association of higher levels with poorer prognosis may merely be a surrogate measure of tumour

load, which itself is a negative prognostic indicator in these settings. The alternative model, that elevated extracellular Nm23-H1 levels are a driver of disease activity, required evidence in the same way that eNDPKs have been mechanistically implicated in *M. tuberculosis*, *P. gingivalis* and *L. amazonensis* infections.

In this regard a seminal study by Okabe-Kado *et al* showed that recombinant extracellular Nm23-H1, applied at concentrations commensurate with those that corresponded to poorest prognosis, promoted the survival of cultured human primary AML cells. They further demonstrated that enhanced survival of AML cells in response to Nm23-H1 was associated with secretion of cytokines including IL-1 β , TNF- α and activation of MAPK and STAT signalling pathways [48]. In a complimentary study, Lilly *et al* went on to show that the pro-survival effect of Nm23-H1 on AML blast cells was indirect [49]. Nm23-H1 produced and secreted by AML blast cells bound to more mature monocytoid components of the tumour clone, eliciting production of inflammation associated cytokines, which were able to promote survival and proliferation of the AML blast cells [40]. Lilly *et al* did not measure TNF- α , but did observe both IL-1 β and IL-6 amongst a number of cytokines. Importantly, the combined treatment of AML cells with IL-1 β and IL-6 was able to replicate the indirect actions of Nm23-H1 on AML cells [49]. In a subsequent study, IL-1 β and IL-6 were shown to activate NF κ B signalling and Tyr-705 phosphorylation of STAT3 respectively in KG1a AML cells [40]; indicating that activation of STAT signalling observed by Okabe-Kado *et al* is likely to be an indirect consequence of Nm23-H1. Taken together, the data from these independent laboratories strongly implicate extracellular Nm23-H1 in the biology and progression of AML and as a therapeutic target [39, 40]. As yet, studies investigating the biological significance of elevated Nm23-H1 in the lymphoid haematological malignancies are lacking.

The actions of extracellular Nm23-H1 are not just restricted to malignant haematological cells [50]. Willems *et al* demonstrated that Nm23-H1 (and also Nm23-H2 and -H3), whilst not modulating cell proliferation, were able to alter terminal differentiation of normal haemopoietic stem/progenitor cells promoting erythroid differentiation and suppressing macrophage colonies [51]. In a separate study, immobilised recombinant Nm23-H1 was able to modulate *in vitro* neurite outgrowth from embryonic chick dorsal root ganglia in a concentration dependant manner [52]. In fact, in the presence of high concentrations of Nm23-H1, neurites preferentially grew on Nm23-H1 substrate and turned away from other permissive coatings such as collagen.

Exogenously added Nm23-H1 was also demonstrated to enable serial passage of human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells in the absence of feeder cells or exogenously added growth factors [53, 54]. The Nm23-H1 treated ES and iPS cells maintained pluripotency during the experiments. These studies highlighted the potential of Nm23-H1 as a mechanism for expanding ES and iPS cells.

How do NDPKs exit cells?

Whilst there is now convincing evidence for the role of eNDPK in the infection settings and also in cancers, the mechanisms by which they are secreted has not been fully elucidated [7]. In the case of eNDPKs derived from intracellular pathogens, the proteins have to transverse the barriers of the bacterium envelope and cell wall and the host cell plasma membrane. These mechanisms have yet to be fully elucidated.

Chakrabarty *et al* demonstrated that NDPK, whilst being intracellular in both mucoid and non-mucoid *P. aeruginosa*, was also selectively secreted by mucoid *P. aeruginosa* by a type 1 secretion system [11] and that this secretion was mediated by DXXX motifs in the NDPK carboxy terminus [12]. However, it has been argued that these residues may not fulfil the specified requirements [15].

The secretion of *P. gingivalis* NDPK from gingival epithelial cells was demonstrated to be mediated through interactions with the hemichannel pannexin 1 (PNX1) and the motor molecule myosin 9 [10]. Furthermore, several studies have described interactions between NDPKs and Dynamin, a

family of GTPases involved in membrane-remodelling events e.g. endocytosis and receptor trafficking in drosophila [55-58], *Dictyostelium discoideum* [59], *C.elegans* [60] and human models [61-64]. Some of these studies imply that dynamin may mediate the secretion/export of Nm23-H1 from cells. However, the data is not definitive and further work needs doing in this area.

How do host and pathogen derived eNDPKs communicate with human cells; the case for the NLRP3 inflammasome

Extracellular nucleotides and nucleosides, especially eATP, mediate diverse signalling effects in virtually all organs and tissues impacting upon inflammatory signalling and immune responses especially during infections [41]. Purinergic signalling occurs via a series of nucleotide-selective ligand-gated P2X, metabotropic P2Y and P2Z receptors on macrophages as well as adenosine receptors which are present on many immune cells and also cancers [41, 42]. Purinergic signalling depends upon the levels of extracellular nucleotides/nucleosides which are regulated by (i) the release of endogenous ATP and other nucleotides, and (ii) enzymatic interconversion of nucleosides and nucleotides by extracellular kinases. It is therefore attractive to propose that this is the major route via which eNDPKs can influence host responses.

As discussed above, purinergic signalling can be seen as a unifying paradigm across *M. tuberculosis*, *P. gingivalis* and *L. amazonensis* infections. In the case of *P. Gingivalis*, it seems likely that NDPK released from host cells and its modulation of eATP reduced reactive oxygen species (ROS) levels and diminished NLRP3 inflammasome activation, resulting in the suppression of secretion of the pro-inflammatory cytokine IL1- β from infected cells and immune cells [32, 33]. This contrasts with the studies in AML where exposure of cells to Nm23-H1 clearly drove IL1- β secretion [49, 65] which is a marker widely recognised as a surrogate for NLRP3 inflammasome activation rather than suppression.

In other studies, Dar et al demonstrated that NDPK secreted from intracellular pathogens *Salmonella typhimurium* and *Mycobacterium typhimurium* and the extracellular pathogen *Vibrio cholerae* were able to prevent eATP-induced cytolysis of J774 macrophage cells [8]. In contrast, *Mycobacterium tuberculosis* secreted NDPK enhanced ATP-induced macrophage cell death [66]. Interestingly, the kinase activity of *M. tuberculosis* NDPK was required for this activity which was mediated through the P2Z receptor [66]. This was also the case for *Pseudomonas aeruginosa* NDPK which was demonstrated to be secreted from bacteria, together with other ATP utilising enzymes, and elicited strong cytotoxic effects against murine peritoneal primary macrophages in the presence of eATP [67]. Thus, NDPKs appear to promote survival or enhance cell death of immune cells in response to eATP in different contexts.

The canonical NLRP3 inflammasome pathway in macrophages results in characteristic cytolysis termed pyroptosis, a form of cell death characterised by cell swelling and plasma membrane rupture [68]. However, more recently non-canonical/alternative NLRP3 inflammasome pathways have been shown to induce secretion of IL-1 β from human monocytes in the absence of cell death [69, 70]. Thus, as has been described, the rescue from cell death in response to eATP mediated by NDPKs from *M. tuberculosis*, *P. Gingivalis*, *S. typhimurium*, *M. typhimurium*, *V. cholerae* and *L. amazonensis* is consistent with suppression of the canonical NLRP3 inflammasome pathway in macrophages (or in the case of *P. gingivalis* gingival epithelial cells). In contrast, the Nm23-H1 stimulated IL1- β release from AML patient monocytes in the absence of cell death is more consistent with activation of non-canonical/alternative NLRP3 inflammasome pathways.

At present the promoted cell death of macrophages by *M. tuberculosis* and *P. aeruginosa* NDPKs is difficult to interpret in the context of NLRP3 inflammasome signalling unless they activate canonical inflammasome pathways independently of regulating eATP pools. At present this is difficult to reconcile with the observations given that the kinase activity of *M. tuberculosis* NDPK was required for its cell death promoting activity which was also mediated through the P2Z receptor for eATP [66].

It is important to note that not all the actions of eNDPKs appear to be dependent on kinase activity since the redirected differentiation of haemopoietic stem/progenitor cells and directed outgrowth of neurites from chick embryo dorsal root ganglia were recapitulated by Nm23-H1 H118F mutant recombinant proteins that lack kinase activity [52].

Is there a surface receptor for NDPKs on human cells?

A number of observations have identified Nm23-H1 at the surface of human cells indicating the possibility of surface receptors [34, 35, 40]. Interestingly, both the studies of Okabe-Kado et al and Lilly et al identified that not all primary AML samples respond to exogenously added Nm23-H1 [39, 40]. In the study by Lilly et al, commercial antibodies to Nm23-H1 and flow cytometry identified that ~50 % of primary AML samples contained mononuclear cells that stained positive for Nm23-H1 at their surface at the point of harvest and that these samples corresponded to those AMLs that respond to exogenous Nm23-H1 in vitro [49]. The addition of exogenous recombinant Nm23-H1 then increased staining intensity on these positive samples as measured by flow cytometry [49]. The fact that not all samples contain cells that bound Nm23-H1, either at harvest or after exposure to Nm23-H1 in culture, suggests that the observed binding is not non-specific implying the presence of a selectively expressed receptor. However, this receptor is currently unknown.

Studies by Bamdad and colleagues have identified a cleaved form of the transmembrane receptor MUC1, that they have termed MUC1*, as the putative cell surface receptor for Nm23-H1. These studies were performed predominantly on ES and iPS cells [53, 71]. These observations are potentially important since Nm23-H1 activity may be utilised to propagate large numbers of pluripotent stem cells for therapeutic interventions and urgently need corroborating in independent studies. Importantly, an early study from the Bamdad group identified that cross linking of MUC1* activated MAP kinase signalling cascade and stimulated cell growth [54]. Although this study did not utilise Nm23-H1 to cross link MUC1*, the observation resonates with Nm23-H1 mediated MAPK activation in AML as observed by Okabe-Kado et al [65]. However as we have discussed previously the data of Lilly *et al* indicate that MUC1* is not the Nm23-H1 cell surface receptor in AML [40, 49].

Concluding remarks

As is always the case, the 'known-knowns' of eNDPKs discussed herein highlight a number of 'known-unknowns' (Figure 1). The first of these is how the proteins are translocated from within prokaryotic and eukaryotic cells to their extracellular environments. Although the cumulative evidence is now that this occurs without cell damage, the mechanisms remain very poorly defined (Figure 1). It is possible that a common mechanism occurs across bacteria but equally it is reasonable to assume aspects of the process will vary between gram positive and gram negative organisms. For example, Spooner and Yilmaz have postulated a role for outer membrane vesicle transport of NDPKs from gram negative bacteria [15]. Equally, the process could differ between extracellular and intracellular pathogens. In the case of intracellular pathogen NDPKs, it is reasonable to consider that the export from their host cell utilises the same mechanism that cell may use for its own NDPK export. However, the mechanism(s) by which this occurs remain poorly defined.

Once released, it is as yet uncertain whether intercellular communication via eNDPK occurs exclusively locally, as implied in the case of regulation of host cell activation via eATP, or whether systemic communication at long distances and across different cell and tissue types occurs.

Related to this issue is the 'known-unknown' regarding potential receptors for eNDPKs. Again, in the case of regulating eATP levels, a receptor for NDPK is not required but rather the presence of receptors that perceive ATP or related molecules. Nonetheless, the studies in AML suggest that NDPK must bind to the cell surface to mediate its effect suggesting that a receptor is likely to exist. Such a receptor may then be also important in local eATP if it is required to tether NDPK to the cell surface in order to intimately regulate pericellular eATP concentrations. As described above, a

candidate receptor is MUC1* but this has yet to be corroborated independently and at least one study suggests it is not the receptor in AML. Are there therefore multiple mammalian NDPK surface receptors?

Another concept that has yet to be explored is whether the export systems for NDPKs are reversible or whether surface bound NDPKs can be internalised. If so, NDPKs could have intracellular actions in cells not of their origin, making the opportunities for cell-cell, tissue-tissue and organ-organ communication still greater than currently being discussed.

The studies we have referred to herein have largely focused on human pathogens and human disease processes that have informed our as yet limited understanding of the functions and importance of signalling via eNDPKs. As we have highlighted, the roles of NDPKs in the biology of environmental prokaryotes is a yet to be explored space. For example, can prokaryotes exploit NDPKs for quorum sensing or impacting upon the behaviours of other species, both prokaryotic and eukaryotic in their environment? Finally, in this review we have disregarded functions of NDPKs in plants. This is an emerging field that requires further investigation. As has been the case for non-plant eukaryotic organisms, the early focus has been on intracellular actions of NDPKs [72] but it is reasonable to also predict extracellular roles for NDPKs in Planta.

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Figure Legends

Figure 1: The known-knowns and known-unknowns of extracellular NDPK proteins

The figure summarises the key points discussed in the review and highlights some of what is known about how NDPKs are exported from prokaryotic and eukaryotic cells, and some of the functions of extracellular NDPKs. The left side schematically represents what we know about prokaryotic NDPKs and the right hand panel is a summary of eukaryotic NDPKs. Prokaryotic NDPKs are shown either as tetramers or as hexamers to reflect the variability between prokaryotes whereas the vast majority of eukaryotic NDPK are hexameric. Solid arrows denote actions/mechanisms which have been observed experimentally and published in the literature. Dashed arrows indicate pathways/mechanisms for which there is currently little/no evidence. P2X7R, P2YR and P2ZR are purinergic receptors. Abbreviations: OMV-Outer membrane vesicle, ROS- reactive oxygen species.

Prokaryotic NDPKs

Eukaryotic NDPKs

