

NPP-type ectophosphodiesterases: unity in diversity

Cristiana Stefan, Silvia Jansen and Mathieu Bollen

Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Nucleotide pyrophosphatase/phosphodiesterase (NPP)-type ectophosphodiesterases are found at the cell surface as type-I or type-II transmembrane proteins, but are also found extracellularly as secreted or shedded enzymes. They hydrolyze pyrophosphate or phosphodiester bonds in a variety of extracellular compounds including nucleotides, (lyso)phospholipids and choline phosphate esters. Despite their structurally related catalytic domain, each enzyme has well-defined substrate specificity. Catalysis by NPPs affects processes as diverse as cell proliferation and motility, angiogenesis, bone mineralization and digestion. In addition, there is emerging evidence for non-catalytic functions of NPPs in cell signaling. NPP-type ectophosphodiesterases are also implicated in the pathophysiology of cancer, insulin resistance and calcification diseases, and they hold great promise as easily accessible therapeutic targets.

Introduction

The family of nucleotide pyrophosphatases/phosphodiesterases (NPPs) consists of seven structurally related ecto-enzymes that are numbered according to their order of discovery (Table 1). Until a few years ago, NPPs were only known to hydrolyze pyrophosphate or phosphodiester bonds in (di)nucleotides and their derivatives. However, recent investigations have revealed that other extracellular molecules with a pyrophosphate or phosphodiester bond are also NPP substrates. For example, NPP2 is an extracellular lysophospholipase-D and generates the signaling molecule lysophosphatidic acid from

lysophosphatidylcholine [1]. Likewise, NPP6 [2] and NPP7 [3] are choline phosphate esterases.

Recently, much progress has been made in delineating the complex role of NPPs in various physiological processes, most notably bone mineralization, digestion, and the proliferation and motility of cells. No less exciting is the involvement of NPPs in the pathophysiology of cancer, ectopic mineralization and insulin resistance, which, combined with their extracellular action, makes them suitable targets for therapeutic intervention. Here, we summarize the recent findings on the structural and functional diversity of NPPs. For key references to the early literature of NPPs the reader is referred to other reviews [4,5].

Domain structure and function

Like many other proteins, NPPs have a modular structure. They contain a catalytic domain of nearly 400 residues (Figure 1), with 24–60% identity at the amino acid level between the different human isoforms [6]. This catalytic domain is not related to that of phospholipases [7], Nudix hydrolases [8] or ectonucleotide triphosphate diphosphohydrolases [9], even though they display a partially overlapping activity. In addition to the catalytic domain, NPP4–7 contain a putative N-terminal signal peptide and a C-terminal transmembrane domain. Following the co-translational removal of the signal peptide, NPP4–7 are predicted to be type-I, single-spanning transmembrane proteins with their catalytic domain facing the extracellular space [2,6,10,11]. Accordingly, the C-terminally nicked forms of NPP6 [2] and NPP7 [11] are released extracellularly. NPP1 and NPP3

Table 1. Nomenclature of NPPs

Genes	Proteins	Other protein names	Substrates
<i>ENPP1</i>	NPP1	MAFP, NPPase, NPP γ , NTPPPH, PC-1	Nucleotides and derivatives
<i>ENPP2</i>	NPP2	Autotaxin, Lysophospholipase-D, NPP α , PD-1 α ,	Nucleotides and derivatives, lysophospholipids
<i>ENPP3</i>	NPP3	B10, CD203c, gp130 ^{RB13-6} , NPP β , PD-1 β ,	Nucleotides and derivatives
<i>ENPP4</i>	NPP4	–	Unknown
<i>ENPP5</i>	NPP5	–	Unknown
<i>ENPP6</i>	NPP6	–	Choline phosphate esters
<i>ENPP7</i>	NPP7	Alkaline sphingomyelinase	Choline phosphate esters

^aAbbreviations: B10, the monoclonal antibody that recognizes NPP3 on the apical membrane of rat hepatocyte; CD203c: the cluster of differentiation CD203c that is a basophil-specific activation antigen, and is identical to NPP3; gp130^{RB13-6}, identifies NPP3 as a glycoprotein (gp) with MW of 130 kD recognized by the monoclonal antibody RB13-6 on a subset of neural precursor cells; MAFP, major acidic fibroblast-growth-factor-stimulated phosphoprotein; NPP γ , nucleotide pyrophosphatase phosphodiesterase γ ; NPP α , nucleotide pyrophosphatase phosphodiesterase α ; NPP β , nucleotide pyrophosphatase phosphodiesterase β ; NPPase, nucleotide pyrophosphatase phosphodiesterase; NTPPPH, nucleotide triphosphate pyrophosphohydrolase; PC-1, plasma cell differentiation antigen 1; PD-1 α , phosphodiesterase-1 α ; PD-1 β , phosphodiesterase-1 β .

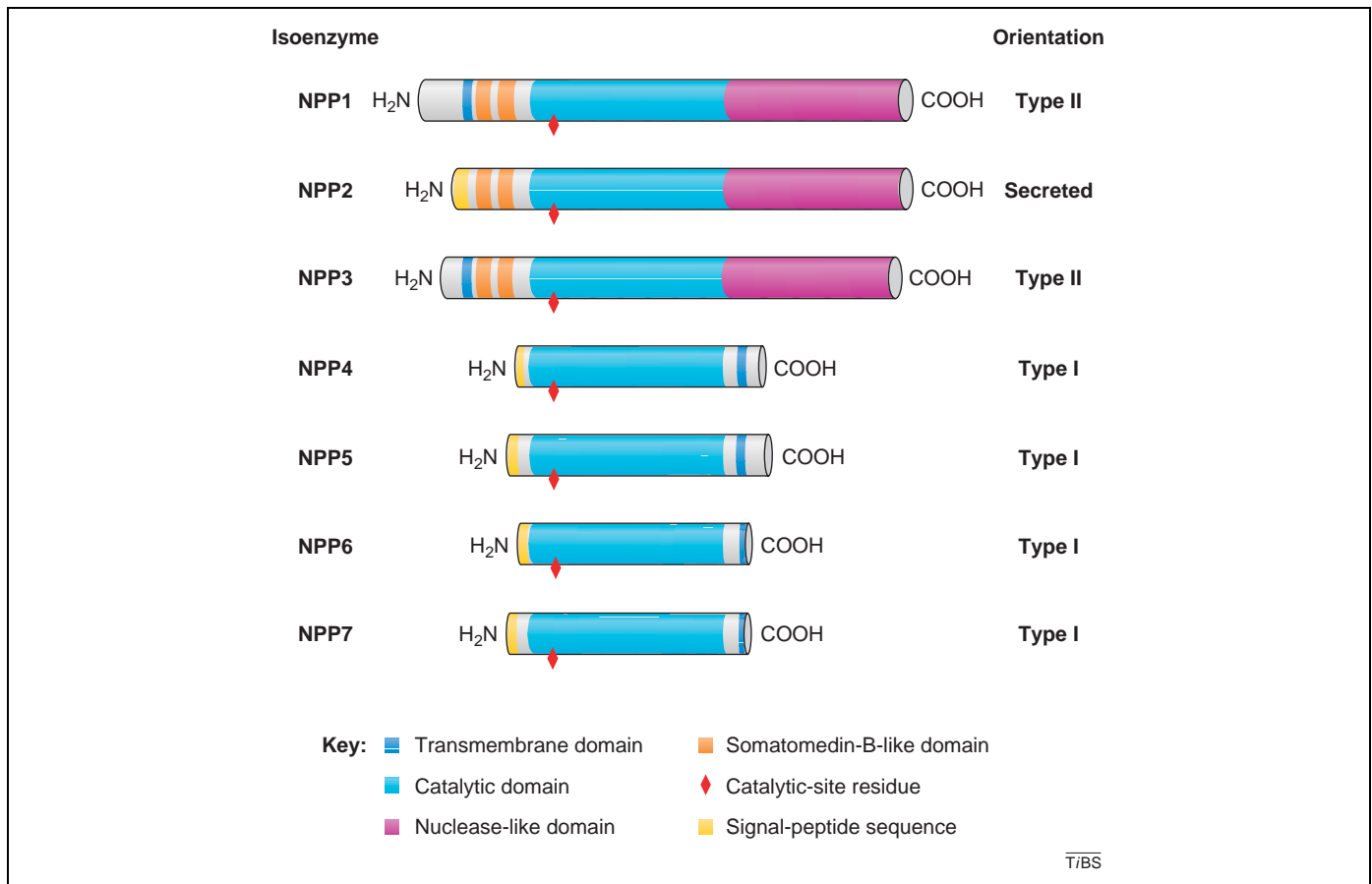


Figure 1. Domain structure of NPPs. The seven NPP-type ectophosphodiesterases have a structurally related catalytic domain. Catalysis occurs via a covalent catalytic intermediate with a conserved threonine residue (serine for NPP6) in the catalytic site. NPP1 and NPP3 contain an N-terminal transmembrane domain and have a type-II orientation. The N-terminal hydrophobic sequence of NPP2 and NPP4–7 is a signal peptide, rather than a transmembrane domain, that mediates uptake in the endoplasmic reticulum during translation. This sequence is removed co-translationally by the signal peptidase, and, therefore, is not part of the mature polypeptides. In contrast to NPP2, which enters the secretory pathway, NPP4–7 contain a C-terminal transmembrane domain that retains them at the plasma membrane with a type-I orientation. Thus, except for NPP2, all NPPs are membrane-bound ecto-enzymes. NPP1–3 contain two N-terminal somatomedin-B like domains and a C-terminal nuclease-like domain, but the exact function of these domains is not yet known. The structures are drawn to scale.

have a transmembrane domain in their N terminus but, because they have a type-II orientation, they are also ecto-enzymes. Furthermore, the cytoplasmic tail of NPP1 contains a di-leucine motif that mediates either basolateral targeting in epithelial cells [12] or targeting to matrix vesicles derived from mineralizing cells [13]. The cytoplasmic domain of NPP3 does not contain a di-leucine motif, which explains why this isoenzyme is targeted apically in polarized cells [14]. Contrary to expectations [4,5,15,16], NPP2 is not a transmembrane protein but is synthesized as a pre-pro-enzyme. Following the removal of the N-terminal signal peptide and further trimming by a furine-type protease, NPP2 is secreted [17]. Other NPP isoenzymes, including NPP1, NPP3 and NPP7, can also be secreted to a variable extent but it is not known whether this results from intracellular proteolysis and/or shedding of their membrane-associated form at the cell surface.

C-terminally to the catalytic domain of NPP1–3 is the nuclease-like domain, which owes its name to the structural similarity with the DNA- or RNA-nonspecific endonucleases [6]. However, this domain is probably not catalytically active because none of the residues that are essential for catalysis by the non-specific endonucleases is conserved in NPP1–3. The ‘tiptoe-walking’ phenotype of mice carrying a naturally occurring nonsense

mutation in the nuclease-like domain of NPP1 is identical to that of NPP1-null mice [18]. The available data do not indicate whether the severe phenotype of this nonsense mutation reflects the importance of the nuclease-like domain for the in-vivo function of NPP1, or simply results from nonsense-mediated decay of the NPP1 transcript. Transient-expression experiments suggested that the translocation of NPP1 from the endoplasmic reticulum to the plasma membrane requires the nuclease-like domain [19]. Also, NPP1 with an incomplete nuclease-like domain is catalytically inactive, possibly due to improper folding or failure to undergo an essential post-translational maturation step. Furthermore, the nuclease-like domain is likely to harbor isoform-specific determinants of catalysis because NPP2 with the nuclease-like domain of NPP1 is inactive [20]. Finally, recent evidence suggests that the nuclease-like domain of NPP2 mediates its anti-adhesion function [21].

Two somatomedin-B-like domains that were initially thought to be involved in homodimerization by disulfide bridges flank the N termini of the catalytic domains of NPP1–3 [19]. However, neither NPP2 nor soluble, extracellular NPP1 forms dimers. Moreover, the crystal structure of the somatomedin-B domain of vitronectin shows that its cysteine residues are all engaged in

intrachain disulfide bonds [22]. Therefore, it is likely that the somatomedin-B-like domains of NPP1–3 function as protein-interaction domains, similarly to the somatomedin-B domain of vitronectin, and that the dimerization of cell-

associated NPP1 and NPP3 is mediated by another N-terminal fragment.

Catalytic mechanism and substrate specificity

The catalytic domain of NPPs has been predicted to fold similarly to that of the superfamily of phospho- or sulfo-coordinating metalloenzymes, which includes alkaline phosphatases [6]. Importantly, the amino acid residues that coordinate two metals in the catalytic site of alkaline phosphatases and their spatial arrangement relative to the residue that forms the catalytic intermediate are conserved in NPPs, which supports the theory of a similar catalytic mechanism. Transposition of the well-studied catalytic mechanism of alkaline phosphatases suggests that catalysis by NPPs occurs in two steps, in accordance with numerous biochemical and mutagenesis data [6,23–25]. During the first step of catalysis, the metal-activated hydroxyl group of the catalytic-site threonine (or serine for NPP6) attacks the phosphate group of the incoming substrate, resulting in the formation of a (di)ester linkage with the phosphate group that also remains bonded, however, with a variable R-group (Figure 2). In the second catalytic step, a water molecule activated by the second metal attacks this intermediate, regenerating the catalytic-site threonine/serine and releasing a phosphorylated product. With the known NPP substrates, the variable R-group can be a hydrogen atom, a nucleotide, a (lyso)lipid or choline (Figure 2). The R-group can also be a non-natural moiety such as a para-nitrophenyl group [23]. It seems likely that this list will expand with the discovery of novel NPP substrates, in particular for the currently substrateless NPP4 and NPP5.

Importantly, some NPP substrates can be hydrolyzed in two different ways, implying catalysis via catalytic intermediates with distinct R-groups. For example, ATP is hydrolyzed by NPP1 into either AMP + PP_i or ADP + P_i via an established nucleotidylated or phosphorylated intermediate, respectively, depending on how the substrate approaches the catalytic site (Figure 2). Thus, what was previously described as ‘autophosphorylated’ NPP1 [4,6] is likely to represent one of its covalent intermediates for ATP hydrolysis. Lysophosphatidylcholine is another NPP substrate that can be hydrolyzed in two distinct ways (Figure 2): either into lysophosphatidic acid and choline by NPP2 (lysophospholipase-D activity) or into a monoacylglycerol and choline phosphate by NPP6 and NPP7 (lysophospholipase-C or choline phosphate esterase activities). The lysophosphatidylated or phosphocholinated catalytic intermediates that are postulated for the latter reactions have not yet been described. However, their existence could be explored by using so-called trapping mutants, that is, NPP mutants that are blocked in the second step of catalysis, as have already been described for NPP1 [6]. A substrate-trapping approach might also be useful for the identification of physiological substrates of NPPs.

Little is known about the determinants for the remarkable substrate specificity of NPPs [23]. Do NPPs have specific binding sites for the R-groups of their substrates or do they somehow restrict the access to their catalytic site? Mutagenesis studies demonstrated

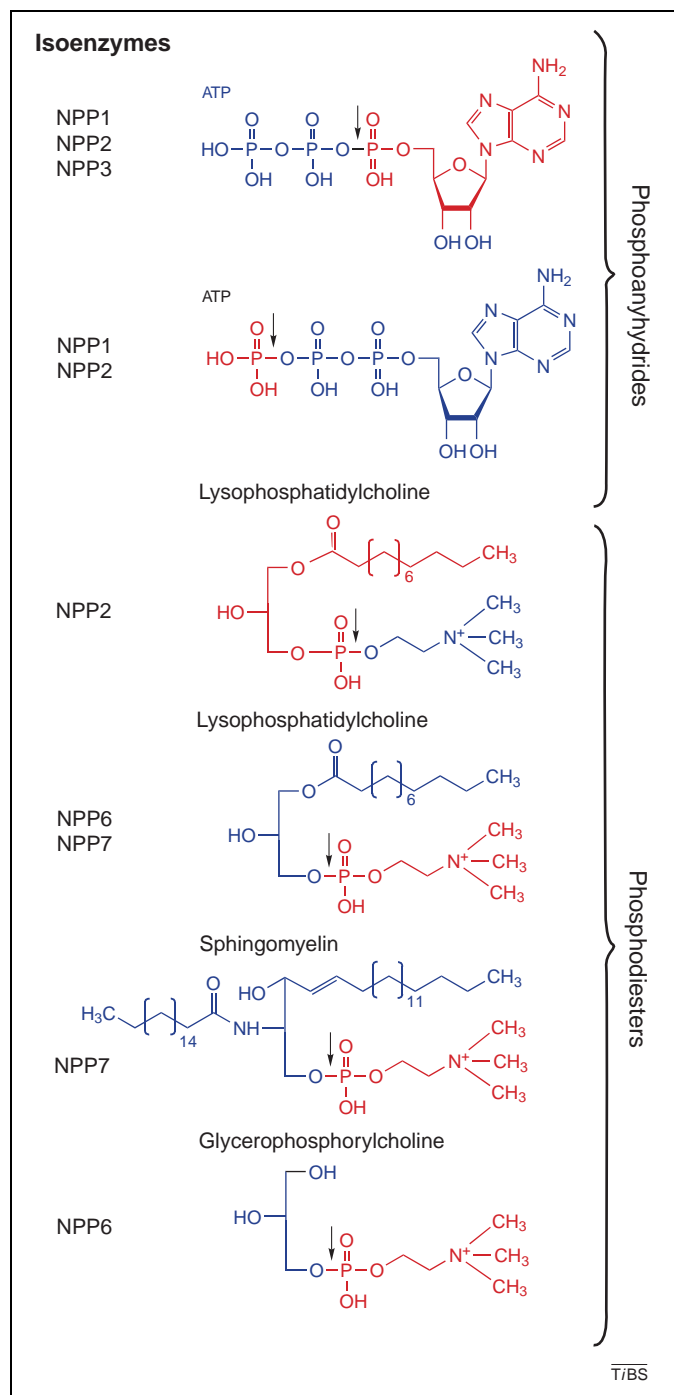


Figure 2. Model substrates for NPPs. The structures of selected phosphoanhydrides or phosphodiester that are substrates of NPPs are shown. The arrows denote the pyrophosphate or phosphodiester bond of the phosphoanhydrides or phosphodiester, respectively, that is hydrolyzed by the indicated NPP isoenzyme(s). Hydrolysis by NPPs occurs through covalent catalysis. The part of the substrates proposed to form a covalent intermediate with the catalytic-site threonine/serine of the NPPs is indicated in red, and consists of a phosphate group and a variable R-group. Hydrolysis of ATP when present at low sub-micromolar concentrations to ADP and P_i, rather than to AMP and PP_i, has been demonstrated for NPP1 and NPP2 but not for other NPPs. Such a reaction also occurs via covalent catalysis, and the ‘autophosphorylated’ NPP1 or NPP2 described in the literature represents the covalent intermediate of a nucleotide phosphohydrolyase reaction. NPP2 functions as a lysophospholipase D, whereas NPP6 and NPP7 both function as a lysophospholipase C. Substrates for NPP4 and NPP5 have not yet been identified.

that isoenzyme-specific residues near the catalytic site determine the relative activities of NPP1 and NPP2 towards nucleotides and lysophosphatidylcholine [20]. However, other determinants of catalytic activity and substrate specificity lie outside the catalytic domain, as demonstrated by domain swapping studies [20]. Intriguingly, NPPs bind to their phosphorylated products (e.g. AMP for NPP1 and lysophosphatidic acid for NPP2) with higher affinity than their substrates do, and inhibit catalysis [4,26]. *In vivo*, such an auto-regulatory mechanism might function to prevent the complete hydrolysis of NPP substrates. This proposal is consistent with observations that NPP2 co-exists with a large excess of its substrate lysophosphatidylcholine. The accumulation of the phosphorylated catalytic intermediate of NPP1 at micromolar ATP concentrations [4] can also be viewed as an auto-regulatory mechanism that prevents the complete hydrolysis of ATP.

NPP functions in health and disease

NPP1 in bone mineralization and soft-tissue calcification

Bone mineralization often begins in matrix vesicles that bud from the plasma membrane of osteoblasts and chondrocytes [27,28]. The crystals of hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] initially form inside the matrix vesicles but eventually burst through the vesicle membrane into the extracellular milieu, where they grow further. The mineralization process is tightly controlled by a balance between the extracellular levels of inorganic phosphate (P_i) and pyrophosphate (PP_i) (Figure 3). P_i is a substrate for mineralization and is generated by the hydrolysis of PP_i by the tissue-nonspecific alkaline phosphatase (TNAP). PP_i also inhibits mineralization by binding to

nascent hydroxyapatite crystals and preventing further incorporation of P_i into these crystals. Importantly, recent work has shown that the functions of extracellular PP_i extend beyond mineral deposition *per se*, to regulation of expression of some genes implicated in mineralization [28–30]. Extracellular PP_i is generated by the NPP1-mediated hydrolysis of extracellular NTPs, and by the intracellular-to-extracellular channeling of PP_i by the transmembrane protein ANK, the product of the progressive ankylosis gene. NPP1 is expressed at the plasma membrane of mineralizing cells but its concentration is at least tenfold higher in the matrix-vesicle membrane fraction. By contrast, ANK is only present in the plasma membrane.

Mineralization is a physiological process in bones and teeth but is prevented in other tissues by the accumulation of extracellular PP_i and/or by the absence of a fibrillar collagen-rich scaffold [31]. Overwhelming experimental and genetic evidence shows that NPP1, by its ability to generate PP_i , is an inhibitor of mineralization *in vivo* (Figure 3). Thus, $ENPP1^{-/-}$ skull-bone osteoblasts show a decreased extracellular PP_i concentration that is associated with hypercalcification [18]. This phenotype can be reverted, for the most part, by the expression of NPP1 but not by the expression of NPP3, which illustrates the isoenzyme specificity of this effect. The stable over-expression of NPP1 is associated with a severely reduced hydroxyapatite deposition [32]. Except for the long bones, NPP1-null mice show a hypermineralization phenotype [33], and the hypomineralization abnormalities of TNAP-null mice are largely absent in the TNAP/NPP1 double-deficient ones [34,35]. The presence of calcified articular cartilage in $ENPP1^{-/-}$ mice [33] is consistent

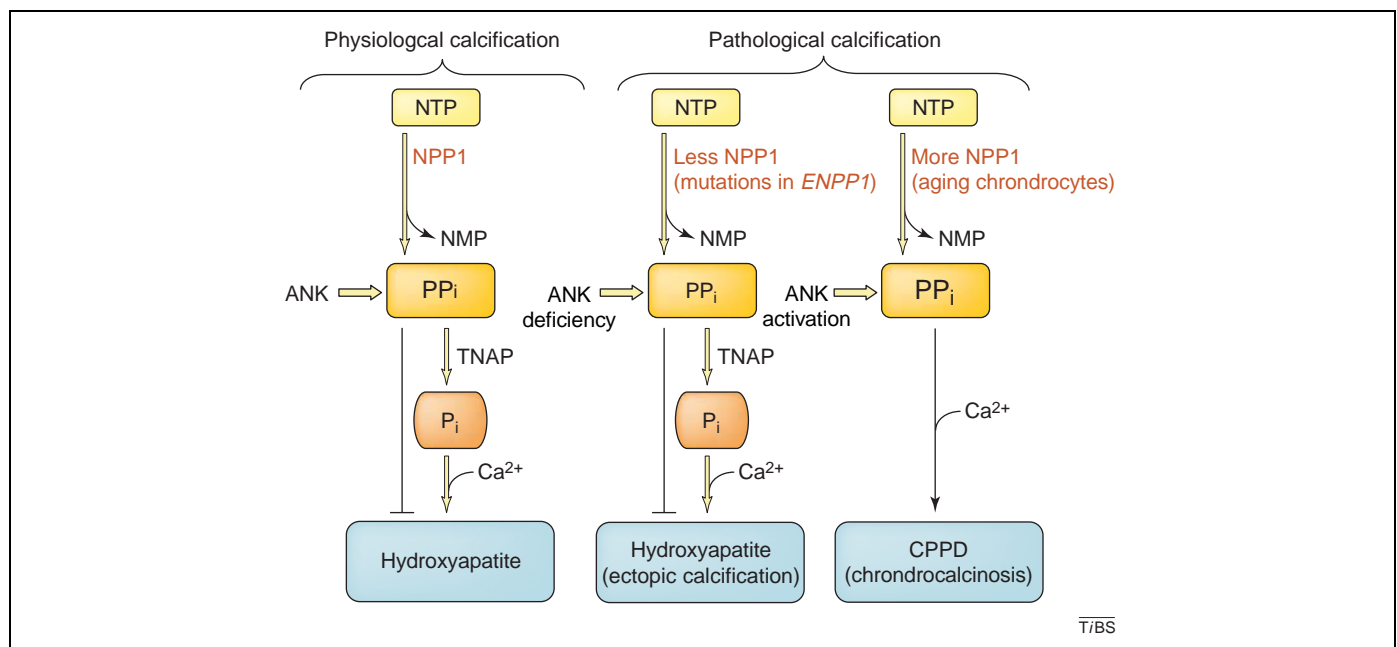


Figure 3. Role of NPP1 in physiological and pathological calcification processes. NPP1 hydrolyzes NTPs extracellularly, and thereby generates PP_i , an inhibitor of hydroxyapatite formation. ANK contributes, too, to the extracellular level of PP_i by functioning as an intracellular-to-extracellular PP_i channel. TNAP hydrolyzes PP_i into P_i , generating the substrate for calcification. Loss-of-function mutations in $ENPP1$, as occur in human infantile arterial calcification, or in ANK, as found in the naturally occurring *ank/ank* mice, result in decreased levels of PP_i and ectopic hydroxyapatite deposition. A hyperactivity of NPP1, as in the age-related chondrocalcinosis, or gain-of-function of ANK, as documented for inherited forms of chondrocalcinosis, leads to increased extracellular concentrations of PP_i and formation of pathological CPPD crystals. Abbreviations: CPPD, calcium pyrophosphate dihydrate; TNAP, tissue-nonspecific alkaline phosphatase; ANK, progressive ankylosis protein; NTP, nucleoside 5' triphosphate; NMP, nucleoside 5' monophosphate.

with studies indicating that articular cartilage, in contrast to growth-plate cartilage, does not normally undergo matrix calcification because its high NPP1 level maintains a high extracellular concentration of PP_i . In further agreement with this notion, many cases of human arterial calcification of infancy, which is characterized by calcification of the internal elastic lamina of large and medium-sized arteries, have been linked to mutations of *ENPP1* [36,37]. NPP1-null mice also suffer from calcification of tendons and ligaments and they are, therefore, models for human ossification of the posterior longitudinal ligament (OPLL), in which compression of the spinal cord results from ectopic ossification of spinal ligaments [33]. Although human OPLL has been associated with *ENPP1* polymorphisms [38], a firm causal relationship has not yet been established. Finally, it should be pointed out that the extent to which NPP1 functions as an inhibitor of mineralization also depends on the expression level of TNAP because it is the activity ratio of both enzymes that determines the extracellular PP_i level [35].

Although PP_i is required to prevent ectopic mineralization, its overproduction leads to deposition of the pathological mineral calcium pyrophosphate dihydrate (CPPD), most often in the articular cartilage (Figure 3). This condition, known as chondrocalcinosis, frequently accompanies age-related osteoarthritis. Considerable evidence implicates NPP1 in the pathophysiology of chondrocalcinosis. Indeed, chondrocytes from CPPD-diseased cartilage express more NPP1 and, consequently, produce more PP_i than the healthy ones [39–41]. Also, the increased NPP1 level in aging chondrocytes promotes apoptosis and the resulting apoptotic bodies, which cannot be cleared up by phagocytes in the non-vascularized articular cartilage, contain NPP1 and have mineralizing properties [39–41].

NPP1 in insulin signaling

NPP1 is not a bone-specific protein and its presence in other cell types, including hepatocytes, adipocytes and fibroblasts [4,5], strongly indicates additional functions for this isoenzyme. These cell types are insulin-sensitive and four major lines of evidence link NPP1 to insulin resistance and type-2 diabetes. First, NPP1 interacts with the insulin receptor and inhibits its autophosphorylation in crude cell fractions and in intact cells [42,43]. However, purified NPP1 has no affinity for the insulin receptor and only decreases the autophosphorylation of the purified receptor by hydrolysis of the kinase substrate ATP [19], indicating that the reported *in-vivo* interaction is indirect and requires at least one additional component. Second, the overexpression of NPP1 in some [44], but not all [19,45], cell types causes impaired insulin signaling by a mechanism that, intriguingly, does not seem to require catalysis by NPP1 [44]. Also, mice that are induced to express human NPP1 in liver cells show insulin resistance and glucose intolerance [46]. Third, NPP1 is overexpressed in cells from insulin-resistant humans [42] and Rhesus monkeys [47], and insulin stimulates the translocation of NPP1 to the plasma membrane [48]. Fourth, a cluster of three single-nucleotide polymorphisms in the 3'-UTR that stabilizes the NPP1 transcript and

results in an increased steady-state level of NPP1 associates with insulin-resistance-related metabolic abnormalities [49]. Moreover, the naturally occurring K173Q variant of NPP1 – wrongly denoted as K121Q in almost all articles on this subject – correlates with an increased prevalence of insulin resistance and type-2 diabetes in large population studies [50]. It has been reported that the K173Q variant of NPP1 displays an increased affinity for the insulin receptor [43], but this has not been a consistent finding either [19]. Clearly, a lot of additional work is needed to elucidate the exact role of NPP1 in insulin signaling.

NPP2 in cell proliferation, migration and survival

As a secreted protein, NPP2 accumulates in body fluids such as plasma [1,51] and cerebrospinal fluid [52], but the cellular sources of these pools of NPP2 are not clearly identified. In addition, local pools of NPP2 with an autocrine or paracrine function exist [21,53]. NPP2 stimulates cell survival, proliferation, contraction and migration (Figure 4), and these effects can all be accounted for by its ability to generate signaling molecules that are collectively referred to as lysophosphatidic acid (LPA), from extracellular lysophosphatidylcholine (LPC) species [1,51,54–56]. LPA binding to specific G-protein-coupled receptors activates multiple signaling pathways [15,16,57], in particular those involving protein kinase B and the small GTPases Ras, RhoA, Rac1 and Cdc42, and accounts for the pleiotropic action of NPP2 and LPA on cell morphology and proliferation. It is possible that NPP2 also has other physiological substrates. One candidate is sphingosylphosphorylcholine, which is an *in-vitro* substrate of NPP2 and yields the cell-motility modulator sphingosine-1-phosphate [58]. At least one effect of NPP2, namely its anti-adhesion effect on oligodendrocytes [21], does not require the catalytic domain but is mediated by the nuclease-like domain, most probably via a pertussis-toxin-sensitive G-protein-coupled receptor, resulting in the dephosphorylation of the focal adhesion kinase and partial disassembly of the focal adhesions (Figure 4).

NPP2 is expressed in many cell lines [4,59], indicating that it has multifunctional capabilities. In an *in-vivo* model for angiogenesis, NPP2 was identified as a novel angiogenic factor that contributes to neovascularization by stimulating migration of smooth muscle cells and to tubule formation by endothelial cells [60]. NPP2 is also released by adipocytes and promotes the proliferation, but inhibits the differentiation, of nearby pre-adipocytes [53,61]. NPP2 is probably also important for the development of the central nervous system because it is first expressed during mouse development in the floor plate of the neural tube [59]. Moreover, NPP2 is the major neurite-retraction factor from the cerebrospinal fluid and might, thus, have a regulatory role in the formation of correct axonal networks [52,62]. Furthermore, independently of its catalytic activity, NPP2 antagonizes the interaction between oligodendrocytes and extracellular-matrix proteins, thereby enabling the oligodendrocytes to move to their target axon and induce myelination [21].

NPP2 has actually been discovered as a tumor-motility stimulating factor that is secreted by melanoma cells [4,5].

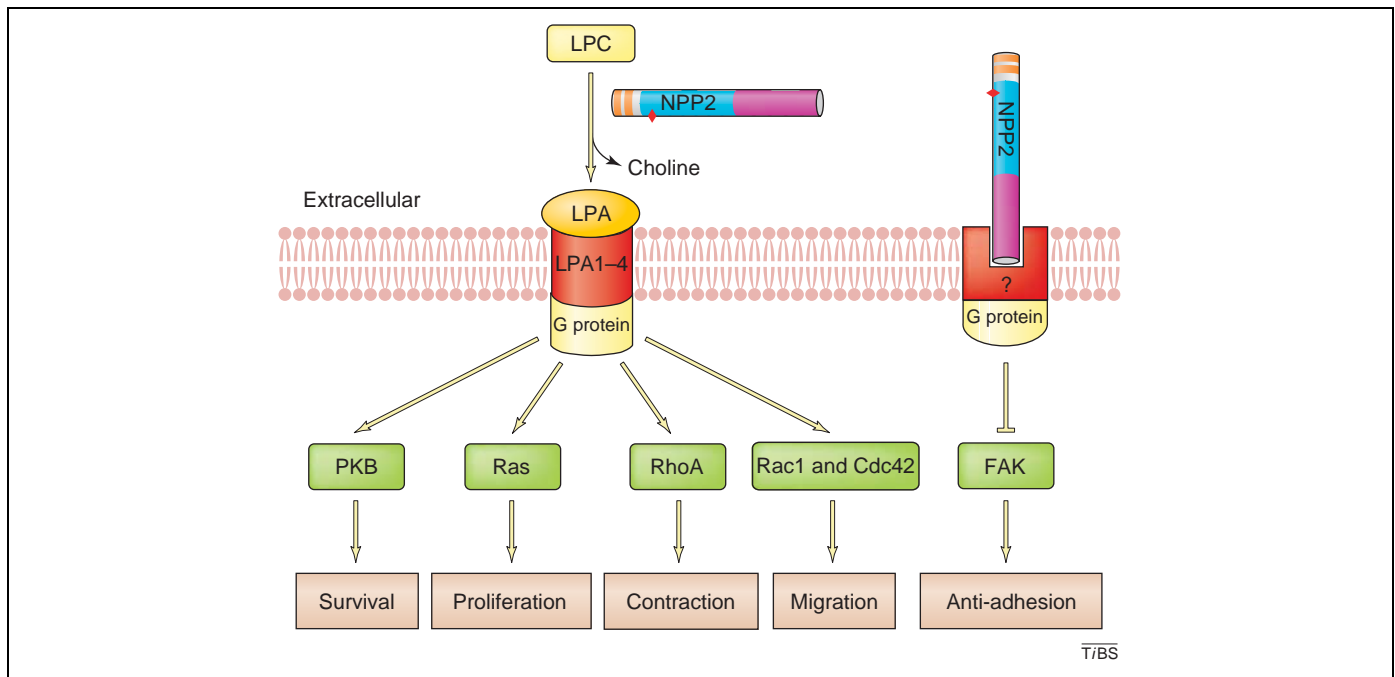


Figure 4. Role of NPP2 in cell morphology and proliferation. NPP2 generates LPA from LPC. The interaction of LPA with specific G-protein-coupled receptors results in the activation of multiple signaling pathways involving PKB and small GTPases (Ras, RhoA, Rac1 and Cdc42) that affect the survival, proliferation, contraction and migration of the cell. PKB promotes survival by the phosphorylation and inactivation of the apoptotic protein Bad, and Ras augments cell proliferation by increasing signaling by MAP kinases. RhoA stimulates the reorganization of the actin cytoskeleton. Finally, Rac1 and Cdc42 promote cell migration via the formation of lamellipodia and filopodia, respectively. It is also suggested that the nuclease-like domain of NPP2 interacts with a still unidentified G-protein-coupled receptor, leading to the dephosphorylation of FAK and loss of components from the focal adhesions. Abbreviations: FAK, focal adhesion kinase; LPA, lysophosphatidic acid; LPA₁₋₄, LPA receptors 1-4; LPC, lysophosphatidylcholine; PKB, protein kinase B.

However, other cancer cell lines also secrete NPP2 together with significant amounts of its substrate lysophosphatidylcholine [1], and it is now established that NPP2 contributes to the metastatic cascade at multiple levels. Indeed, NPP2 stimulates the growth [1] and motility [60,63] of cancer cells, augments the tumorigenicity of *Ras* transformed cells [63] and induces tumor angiogenesis [60,63]. Consistent with a crucial role for NPP2 in metastasis, its expression is increased in various cancers, including breast and lung cancer, and this upregulation correlates with the invasiveness of the cancer cells [64-67]. Moreover, the $\alpha_6\beta_4$ -integrin, which promotes a metastatic phenotype in advanced breast carcinomas, increases NPP2 expression [67]. NPP2 expression is also augmented both during transformation by the oncoprotein *v-Jun* [68] and during infection of Hodgkin lymphoma with the Epstein-Barr virus [69].

Because NPP2-deficient animal models are not yet available, it is difficult to predict how crucial NPP2 is for normal development and/or body homeostasis. LPA, a key product of NPP2-mediated catalysis, interacts with different subsets of the four LPA receptors (denoted LPA₁₋₄) depending on the structure of its hydrophobic tail. Mice lacking an LPA receptor are normal (LPA₂-deficient), show defects in olfaction (LPA₁-deficient), or show delayed embryo implantation (LPA₃-deficient) [70]. However, the phenotype of *ENPP2*^{-/-} mice might not recapitulate the phenotype(s) of specific LPA-receptor-null mice because NPP2 might not generate all LPA species that interact with LPA₁₋₄ equally well [51].

NPP3 and allergies

Exposure to an allergen causes an immediate immunoglobulin E (IgE)-mediated response of basophilic granulocytes (basophils) and mast cells, leading to the release of histamine and other mediators of the allergic reaction. NPP3 is the only molecularly defined marker of both basophils and mast cells, but is not expressed on any other blood cells [71,72]. Preliminary evidence implicates NPP3 in the differentiation of a common progenitor of basophils and mast cells [71]. In addition, NPP3 might have a function in the activation of basophils because it is translocated from an intracellular pool to the plasma membrane within minutes following the challenge of basophils with allergens or anti-IgE antibodies. The measurement of NPP3 expression on the surface of basophils by a flow cytometric method is now advocated as a rapid and sensitive method for the estimation of hypersensitivity to allergens [71,72].

NPPs in digestion

NPP3 is abundantly expressed in the brush border of mature enterocytes in the proximal region of the intestine and has been implicated in the hydrolysis of dietary nucleotides [73]. The intestinal alkaline sphingomyelinase, which was described >35 years ago, was recently identified as NPP7 [3]. It is expressed in the brush border of the proximal intestinal mucosa but can also be released into the intestinal lumen by cleavage of the membrane-associated form with physiological concentrations of trypsin [11]. The activity of NPP7 depends on the presence

of bile acids and increases moderately by trypsinolysis. NPP7 is currently believed to be important for the digestion of dietary and bile-derived choline phosphate esters, including sphingomyelin and lysophospholipids. In addition, hydrolysis of sphingomyelin yields the cell-proliferation inhibitor ceramide. Thus, the decreased level of NPP7, which has been detected in colorectal cancers, familial adenomatous polyposis and ulcerative colitis, might contribute to excessive cell proliferation.

Concluding remarks and future perspectives

Two decades after the cloning of the founding member NPP1, the NPP-type ectophosphodiesterases have finally come of age. In just a few years, the number of known NPP isoenzymes has more than doubled and their (non-)catalytic functions are being unraveled rapidly. Undoubtedly, many NPP functions still remain to be identified, not only for the currently functionless NPP4–6, but also for the better-studied NPP isoenzymes. For example, *in-vitro* evidence implicates NPP1 in the regulation of signaling by extracellular nucleotides and in the recycling of extracellular nucleotides [4,5,74], and *in-vivo* data are beginning to emerge [75]. All well-studied NPP isoenzymes have been linked to the pathophysiology of devastating diseases, which, combined with their attraction as therapeutic targets (Box 1), is likely to draw the attention of drug designers and developers. Although important questions on the structural and functional diversity of NPPs remain (Box 2), the current insights already show that the NPP-type ectophosphodiesterases form a ‘unity in diversity’, yet another testimony of the amazing versatility of nature.

Acknowledgements

The authors' work on NPPs is supported by the Fund for Scientific Research-Flanders (grant G.0082.02). S.J. is a Research Assistant of the Fund for Scientific Research.

Box 1. NPP-type ectophosphodiesterases as therapeutic targets

NPPs are attractive therapeutic targets because they function extracellularly, and effectors (i.e. inhibitors or activators) do not need to be cell permeable. Although our insights into the role of NPPs in the pathophysiology of diseases are not yet complete, one can easily see how NPP effectors could be used therapeutically. Potentially, NPP1 inhibitors can be used to treat or prevent the mineralization defects of hypophosphatasia and chondrocalcinosis, as well as insulin resistance. An activator of NPP1 might be helpful for the treatment of ossification of the posterior longitudinal ligament. The development of NPP2 inhibitors could lead to novel therapies aimed at preventing cancer metastasis. NPP3 is a potential target for the treatment of allergies, although it remains to be explored whether or not the rapid translocation of NPP3 to the plasma membrane of allergen-challenged basophils is a trigger for the allergic response. NPP effectors could take the form of (humanized) antibodies or small-molecule inhibitors or activators that could be obtained by the screening of chemical or biological libraries. In addition, non-hydrolyzable substrate analogs could be developed as inhibitors. Importantly, NPP effectors do not necessarily have to be directed against the catalytic domain because the non-catalytic domains are also important determinants of activity and because NPPs also have non-catalytic functions. For example, an inhibitor of catalysis by NPP1 might be used to treat calcification diseases but is unlikely to interfere with the non-catalytic function(s) of NPP1 in insulin signaling.

Box 2. Outstanding questions

- What are the molecular determinants for the substrate specificity of NPPs?
- What are the ligands for the somatomedin-B-like and nuclease-like domains?
- How and why do NPP1 and NPP3 homo-dimerize, and what is the mechanism for generating extracellular, soluble forms?
- What are the physiological substrates and functions of NPP4–6 and how is NPP1 implicated in nucleotide and insulin signaling?
- Can specific NPP inhibitors or activators be developed and can they be used therapeutically?

References

- 1 Umezū-Goto, M. *et al.* (2002) Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J. Cell Biol.* 158, 227–233
- 2 Sakagami, H. *et al.* (2005) Biochemical and molecular characterization of a novel choline-specific glycerophosphodiester phosphodiesterase belonging to the nucleotide pyrophosphatase/pyrophosphodiesterase (NPP) family. *J. Biol. Chem.* 280, 23084–23093
- 3 Duan, R. *et al.* (2003) Identification of human intestinal alkaline sphingomyelinase as a novel ecto-enzyme related to the nucleotide phosphodiesterase family. *J. Biol. Chem.* 278, 38528–38536
- 4 Bollen, M. *et al.* (2000) Nucleotide pyrophosphatases/phosphodiesterases on the move. *Crit. Rev. Biochem. Mol. Biol.* 35, 393–432
- 5 Goding, J.W. *et al.* (2003) Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. *Biochim. Biophys. Acta* 1638, 1–19
- 6 Gijsbers, R. *et al.* (2001) Structural and catalytic similarities between nucleotide pyrophosphatases/phosphodiesterases and alkaline phosphatases. *J. Biol. Chem.* 276, 1361–1368
- 7 McDermott, M. *et al.* (2004) Phospholipase D. *Biochem. Cell Biol.* 82, 225–253
- 8 Mildvan, A.S. *et al.* (2005) Structures and mechanisms of Nudix hydrolases. *Arch. Biochem. Biophys.* 433, 129–143
- 9 Kishore, B.K. *et al.* (2005) Expression of NTPDase1 and NTPDase2 in murine kidney: relevance to regulation of P2 receptor signaling. *Am. J. Physiol. Renal Physiol.* 288, F1032–F1043
- 10 Ohe, Y. *et al.* (2003) Characterization of nucleotide pyrophosphatase-5 as an oligomannosidic glycoprotein in rat brain. *Biochem. Biophys. Res. Commun.* 308, 719–725
- 11 Wu, J. *et al.* (2004) Pancreatic trypsin cleaves intestinal alkaline sphingomyelinase from mucosa and enhances the sphingomyelinase activity. *Am. J. Physiol. Gastrointest. Liver Physiol.* 287, G967–G973
- 12 Bello, V. *et al.* (2001) Characterization of a di-leucine-based signal in the cytoplasmic tail of the nucleotide-pyrophosphatase NPP1 that mediates basolateral targeting but not endocytosis. *Mol. Biol. Cell* 12, 3004–3015
- 13 Vaingankar, S.M. *et al.* (2004) Subcellular targeting and function of osteoblast nucleotide pyrophosphatase phosphodiesterase 1. *Am. J. Physiol. Cell Physiol.* 286, C1177–C1187
- 14 Meerson, N.R. *et al.* (2000) Intracellular traffic of the ecto-nucleotide pyrophosphatase/phosphodiesterase NPP3 to the apical plasma membrane of MDCK and Caco-2 cells: apical targeting occurs in the absence of N-glycosylation. *J. Cell Sci.* 113, 4193–4202
- 15 Luquain, C. *et al.* (2003) Lysophosphatidic acid signaling: how a small lipid does big things. *Trends Biochem. Sci.* 28, 377–383
- 16 Mills, G.B. and Moolenaar, W.H. (2003) The emerging role of lysophosphatidic acid in cancer. *Nat. Rev. Cancer* 3, 582–591
- 17 Jansen, S. *et al.* (2005) Proteolytic maturation and activation of autotaxin (NPP2), a secreted metastasis-enhancing lysophospholipase D. *J. Cell Sci.* 118, 3081–3089
- 18 Johnson, K. *et al.* (2003) Linked deficiencies in extracellular PP_i and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression. *J. Bone Miner. Res.* 18, 994–1004
- 19 Gijsbers, R. *et al.* (2003) Functional characterization of the non-catalytic ectodomains of the nucleotide pyrophosphatase/phosphodiesterase NPP1. *Biochem. J.* 371, 321–330
- 20 Cimpean, A. *et al.* (2004) Substrate-specifying determinants of the nucleotide pyrophosphatases/pyrophosphodiesterases NPP1 and NPP2. *Biochem. J.* 381, 71–77

- 21 Fox, M.A. *et al.* (2004) Phosphodiesterase-1 α /autotaxin controls cytoskeletal organization and FAK phosphorylation during myelination. *Mol. Cell. Neurosci.* 27, 140–150
- 22 Zhou, A. *et al.* (2003) How vitronectin binds PAI-1 to modulate fibrinolysis and cell migration. *Nat. Struct. Biol.* 10, 541–544
- 23 Gijssbers, R. *et al.* (2003) The hydrolysis of lysophospholipids and nucleotides by autotaxin (NPP2) involves a single catalytic site. *FEBS Lett.* 538, 60–64
- 24 Koh, E. *et al.* (2003) Site-directed mutations in the tumor-associated cytokine, autotaxin, eliminate nucleotide phosphodiesterase, lysophospholipase D, and motogenic activities. *Cancer Res.* 63, 2042–2045
- 25 Wu, J. *et al.* (2005) Functional studies of human intestinal alkaline sphingomyelinase by deglycosylation and mutagenesis. *Biochem. J.* 386, 153–160
- 26 van Meeteren, L.A. *et al.* (2005) Inhibition of autotaxin by lysophosphatidic acid and sphingosine-1-phosphate. *J. Biol. Chem.* 280, 21155–21161
- 27 Terkeltaub, R.A. (2001) Inorganic pyrophosphate generation and disposition in pathophysiology. *Am. J. Physiol. Cell Physiol.* 281, C1–C11
- 28 Harmey, D. *et al.* (2004) Concerted regulation of inorganic pyrophosphate and osteopontin by *Akp2*, *Enpp1*, and *Ank*. *Am. J. Pathol.* 164, 1199–1209
- 29 Johnson, K. *et al.* (2005) Chondrogenesis mediated by PP_i depletion promotes spontaneous aortic calcification in NPP1^{-/-} mice. *Arterioscler. Thromb. Vasc. Biol.* 25, 686–691
- 30 Wang, W. *et al.* (2005) Role of progressive ankylosis gene (*ank*) in cartilage mineralization. *Mol. Cell. Biol.* 25, 312–323
- 31 Murshed, M. *et al.* (2005) Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. *Genes Dev.* 19, 1093–1104
- 32 Johnson, K. *et al.* (1999) Matrix vesicle plasma cell membrane glycoprotein-1 regulates mineralization by murine osteoblastic MC3T3 cells. *J. Bone Miner. Res.* 14, 883–892
- 33 Okawa, A. *et al.* (1998) Mutation in *Npps* in a mouse model of ossification of the posterior longitudinal ligament of the spine. *Nat. Genet.* 19, 271–273
- 34 Hessle, L. *et al.* (2002) Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc. Natl. Acad. Sci. U. S. A.* 99, 9445–9449
- 35 Anderson, H.C. *et al.* (2005) Sustained osteomalacia of long bones despite major improvement in other hypophosphatasia-related mineral deficits in tissue nonspecific alkaline phosphatase/nucleotide pyrophosphatase phosphodiesterase 1 double-deficient mice. *Am. J. Pathol.* 166, 1711–1720
- 36 Rutsch, F. *et al.* (2003) Mutations in *ENPP1* are associated with ‘idiopathic’ infantile arterial calcification. *Nat. Genet.* 34, 379–381
- 37 Ruf, N. *et al.* (2005) The mutational spectrum of *ENPP1* as arising after the analysis of 23 unrelated patients with generalized arterial calcification of infancy (GACI). *Hum. Mutat.* 25, 98–104
- 38 Koshizuka, Y. *et al.* (2002) Nucleotide pyrophosphatase gene polymorphism associated with ossification of the posterior longitudinal ligament of the spine. *J. Bone Miner. Res.* 17, 138–144
- 39 Hashimoto, S. *et al.* (1998) Chondrocyte-derived apoptotic bodies and calcification of articular cartilage. *Proc. Natl. Acad. Sci. U. S. A.* 95, 3094–3099
- 40 Johnson, K. *et al.* (2001) Up-regulated expression of the phosphodiesterase nucleotide pyrophosphatase family member PC-1 is a marker and pathogenic factor for knee meniscal cartilage calcification. *Arthritis Rheum.* 44, 1071–1081
- 41 Johnson, K. *et al.* (2001) The nucleoside triphosphate pyrophosphohydrolase isozyme PC-1 directly promotes cartilage calcification through chondrocyte apoptosis and increased calcium precipitation by mineralizing vesicles. *J. Rheumatol.* 28, 2681–2691
- 42 Maddux, B.A. and Goldfine, I.D. (2000) Membrane glycoprotein PC-1 inhibition of insulin receptor function occurs via direct interaction with the receptor α -subunit. *Diabetes* 49, 13–19
- 43 Costanzo, B.V. *et al.* (2001) The Q allele variant (GLN¹²¹) of membrane glycoprotein PC-1 interacts with the insulin receptor and inhibits insulin signaling more effectively than the common K allele variant (LYS¹²¹). *Diabetes* 50, 831–836
- 44 Goldfine, I.D. *et al.* (1999) Role of PC-1 in the etiology of insulin resistance. *Ann. N. Y. Acad. Sci.* 892, 204–224
- 45 Sakoda, H. *et al.* (1999) No correlation of plasma cell 1 overexpression with insulin resistance in diabetic rats and 3T3-L1 adipocytes. *Diabetes* 48, 1365–1371
- 46 Dong, H. *et al.* (2005) Increased hepatic levels of the insulin receptor inhibitor, PC-1/NPP1, induce insulin resistance and glucose intolerance. *Diabetes* 54, 367–372
- 47 Pender, C. *et al.* (2002) Elevated plasma cell membrane glycoprotein levels and diminished insulin receptor autophosphorylation in obese, insulin-resistant rhesus monkeys. *Metabolism* 51, 465–470
- 48 Menzaghi, C. *et al.* (2003) Insulin modulates PC-1 processing and recruitment in cultured human cells. *Am. J. Physiol. Endocrinol. Metab.* 284, E514–E520
- 49 Frittitta, L. *et al.* (2001) A cluster of three single nucleotide polymorphisms in the 3′-untranslated region of human glycoprotein PC-1 gene stabilizes PC-1 mRNA and is associated with increased PC-1 protein content and insulin resistance-related abnormalities. *Diabetes* 50, 1952–1955
- 50 Abate, N. *et al.* (2005) ENPP1/PC-1 K121Q polymorphism and genetic susceptibility to type 2 diabetes. *Diabetes* 54, 1207–1213
- 51 Tokumura, A. *et al.* (2002) Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. *J. Biol. Chem.* 277, 39436–39442
- 52 Sato, K. *et al.* (2005) Identification of autotaxin as a neurite retraction-inducing factor of PC12 cells in cerebrospinal fluid and its possible sources. *J. Neurochem.* 92, 904–914
- 53 Ferry, G. *et al.* (2003) Autoaxin is released from adipocytes, catalyzes lysophosphatidic acid synthesis, and activates preadipocyte proliferation. *J. Biol. Chem.* 278, 18162–18169
- 54 Hama, K. *et al.* (2004) Lysophosphatidic acid and autotaxin stimulate cell motility of neoplastic and non-neoplastic cells through LPA₁. *J. Biol. Chem.* 279, 17634–17639
- 55 Jung, I.D. *et al.* (2002) Cdc42 and Rac1 are necessary for autotaxin-induced tumor cell motility in A2058 melanoma cells. *FEBS Lett.* 532, 351–356
- 56 Lee, H.Y. *et al.* (2002) Autotaxin promotes motility via G protein-coupled phosphoinositide 3-kinase γ in human melanoma cells. *FEBS Lett.* 515, 137–140
- 57 Moolenaar, W.H. *et al.* (2004) The ins and outs of lysophosphatidic acid signaling. *Bioessays* 26, 870–881
- 58 Clair, T. *et al.* (2003) Autotaxin hydrolyzes sphingosylphosphorylcholine to produce the regulator of migration, sphingosine-1-phosphate. *Cancer Res.* 63, 5446–5453
- 59 Bächner, D. *et al.* (1999) Developmental expression analysis of murine autotaxin (ATX). *Mech. Dev.* 84, 121–125
- 60 Nam, S.W. *et al.* (2001) Autotaxin (NPP-2), a metastasis-enhancing motogen, is an angiogenic factor. *Cancer Res.* 61, 6938–6944
- 61 Simon, M. *et al.* (2005) Lysophosphatidic acid inhibits adipocyte differentiation via lysophosphatidic acid 1 receptor-dependent down-regulation of peroxisome proliferator-activated receptor γ 2. *J. Biol. Chem.* 280, 14656–14662
- 62 Fukushima, N. (2004) LPA in neural cell development. *J. Cell. Biochem.* 92, 993–1003
- 63 Nam, S.W. *et al.* (2000) Autotaxin (ATX), a potent tumor motogen, augments invasive and metastatic potential of *ras*-transformed cells. *Oncogene* 19, 241–247
- 64 Yang, S.Y. *et al.* (2002) Expression of autotaxin (NPP-2) is closely linked to invasiveness of breast cancer cells. *Clin. Exp. Metastasis* 19, 603–608
- 65 Kehlen, A. *et al.* (2004) Expression, regulation and function of autotaxin in thyroid carcinomas. *Int. J. Cancer* 109, 833–838
- 66 Umezono-Goto, M. *et al.* (2004) Lysophosphatidic acid production and action: validated targets in cancer? *J. Cell. Biochem.* 92, 1115–1140
- 67 Chen, M. and O’Connor, K.L. (2005) Integrin α 6 β 4 promotes expression of autotaxin/ENPP2 autocrine motility factor in breast carcinoma cells. *Oncogene* 24, 5125–5130
- 68 Black, E.J. *et al.* (2004) Microarray analysis identifies autotaxin, a tumour cell motility and angiogenic factor with lysophospholipase D activity, as a specific target of cell transformation by v-Jun. *Oncogene* 23, 2357–2366
- 69 Baumforth, K.R. *et al.* Induction of autotaxin by the Epstein–Barr virus promotes the growth and survival of Hodgkin’s lymphoma cells. *Blood* (in press)

- 70 Ye, X. *et al.* (2005) LPA₃-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature* 435, 104–108
- 71 Bühring, H. *et al.* (2004) The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. *Int. Arch. Allergy Immunol.* 133, 317–329
- 72 Ebo, D.G. *et al.* (2004) *In vitro* allergy diagnosis: should we follow the flow? *Clin. Exp. Allergy* 34, 332–339
- 73 Scott, L.J. (1997) Biochemical and molecular identification of distinct forms of alkaline phosphodiesterase I expressed on the apical and basolateral plasma membrane surfaces of rat hepatocytes. *Hepatology* 25, 995–1002
- 74 Vollmayer, P. *et al.* (2003) Hydrolysis of diadenosine polyphosphates by nucleotide pyrophosphatases/phosphodiesterases. *Eur. J. Biochem.* 270, 2971–2978
- 75 Johnson, K. *et al.* (2004) Mediation of spontaneous knee osteoarthritis by progressive Chondrocyte ATP depletion in Hartley guinea pigs. *Arthritis Rheum.* 50, 1216–1225

Have you contributed to an Elsevier publication?

Did you know that you are entitled to a 30% discount on books?

A 30% discount is available to ALL Elsevier book and journal contributors when ordering books or stand-alone CD-ROMs directly from us.

To take advantage of your discount:

1. Choose your book(s) from www.elsevier.com or www.books.elsevier.com

2. Place your order

Americas:

TEL: +1 800 782 4927 for US customers

TEL: +1 800 460 3110 for Canada, South & Central America customers

FAX: +1 314 453 4898

E-MAIL: author.contributor@elsevier.com

All other countries:

TEL: +44 1865 474 010

FAX: +44 1865 474 011

E-MAIL: directorders@elsevier.com

You'll need to provide the name of the Elsevier book or journal to which you have contributed. Shipping is FREE on pre-paid orders within the US, Canada, and the UK.

If you are faxing your order, please enclose a copy of this page.

3. Make your payment

This discount is only available on prepaid orders. Please note that this offer does not apply to multi-volume reference works or Elsevier Health Sciences products.

For more information, visit www.books.elsevier.com