The hydrolysis of lysophospholipids and nucleotides by autotaxin (NPP2) involves a single catalytic site

Rik Gijsbers[a], Junken Aoki[b], Hiroyuki Arai[b], Mathieu Bollen[a,*]

[a] *Afdeling Biochemie, Faculteit Geneeskunde, University of Leuven, Herestraat 49, B-3000 Leuven, Belgium*
[b] *Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

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Abstract Autotaxin (NPP2) is a tumor cell motility-stimulating factor that displays both a nucleotide pyrophosphatase/phosphodiesterase activity and a recently described lysophospholipase D activity. The hydrolysis of nucleotides is a metal-assisted reaction that occurs via a nucleotidylated threonine in the catalytic site. We show here that the catalytic site threonine and the metal-coordinating residues are also essential for the hydrolysis of lysophospholipids. In comparing the substrate specificity of NPP2 and the closely related NPP1 and NPP3, we found that only NPP2 displayed a lysophospholipase D activity, whereas NPP1 and NPP3 had a much higher nucleotide pyrophosphatase activity.

Key words: NPP; Nucleotide pyrophosphatase/phosphodiesterase; Lysophospholipase D; Autotaxin; Cell motility; Catalytic mechanism; PC-1

1. Introduction

Nucleotide pyrophosphatases/phosphodiesterases (NPPs) are ubiquitous membrane-associated or secreted ectoenzymes that release nucleoside 5′-monophosphate from a variety of nucleotides and nucleotide derivatives [1]. The mammalian NPP family comprises five members but only three of these (NPP1–3) have been studied in some detail. NPP1 has a regulatory function in bone mineralization and this stems from its ability to convert ATP into AMP and PP_i, the latter being an inhibitor of calcification [2]. A loss of functional NPP1 is associated with hypermineralization abnormalities such as osteoarthritis and ossification of the posterior longitudinal ligament of the spine [3,4]. NPP2 or autotaxin was originally identified as an autocrine motility-stimulating factor that is secreted by melanoma cells [5,6]. Later, NPP2 was also shown to augment the carcinogenic and metastatic potential of *ras*-transformed cells [7,8]. These effects of NPP2 cannot be explained by the hydrolysis of nucleotides but have recently been attributed to an intrinsic lysophospholipase D activity, resulting in the hydrolysis of extracellular lysophosphatidylcholine (lysoPC) into lysophosphatidic acid (lysoPA) and choline [9,10]. LysoPA, via G protein-coupled receptors, elicits hormone and growth factor-like responses in almost every cell type and orchestrates chemotaxis, invasiveness and proliferation of cancer cells [10–13]. The third NPP isoform, NPP3, has also been shown to affect the morphology and invasive properties of fibroblasts and glioma cells but the underlying mechanism is unknown [14].

NPPs belong to a superfamily of phospho/sulfo-coordinating metalloenzymes that also includes alkaline phosphatases [15,16]. Based on structural similarities with better characterized members of this superfamily we have identified residues that are essential for the nucleotide pyrophosphatase/phosphodiesterase reaction [16]. The recent description of NPP2 as a lysophospholipase D prompted us to examine whether lysophospholipids and nucleotides, which both contain a phosphodiester bond, were hydrolyzed by the same or by different catalytic sites. The present study shows that both enzyme activities of NPP2 are mediated by a single catalytic site. Surprisingly, in spite of the structural similarities of NPP1–3, only NPP2 acted as a lysophospholipase D, whereas NPP1 and NPP3 were much better nucleotide pyrophosphatases/phosphodiesterases.

2. Materials and methods

2.1. Reagents

Snake venom phosphodiesterase I from *Crotalus atrox* (crude dried venom), monoclonal myc tag antibodies (clone 9E10), monoclonal hemagglutinin (HA) tag antibodies (clone HA-7), *p*-nitrophenylphosphate (*p*NPP), *p*-nitrophenyl thymidine 5′-monophosphate (*p*NP-TMP) and C14:0 lysoPC were purchased from Sigma. Bis(*p*-nitrophenyl)phosphate (bis-*p*NPP) was obtained from Aldrich.

2.2. NPP constructs

The mouse NPP1 coding sequence (residues 35–905), obtained from the pSVL/PC-1 plasmid [17], was subcloned in a modified pEGFP-N_1 vector (Clontech), thereby fusing mouse NPP1 to an HA tag (YPYDVPDYA) at its N-terminal end and to enhanced green fluorescent protein (EGFP) at its C-terminal end. The cDNAs for rat NPP2β and rat NPP3, the latter obtained from Dr. H. Deissler (University of Essen, Germany), were similarly subcloned in this modified EGFP-N_1 vector. To construct C-terminally myc-tagged NPP isoforms, the cDNA encoding EGFP was cut using *Bam*HI-*Not*I and replaced by an adapter encoding the amino acid residues 410–419 (EQKLISEEDL) of the human c-*myc*. Point mutations in the catalytic domain of rat NPP2β were introduced with the QuickChange site-directed mutagenesis protocol (Stratagene). All polymerase chain reaction amplifications were conducted using Pwo-proofreading polymerase (Roche Diagnostics). All mutations and subcloning steps were verified by DNA sequencing.

*Corresponding author. Fax: (32)-16-34 59 95.
E-mail address: mathieu.bollen@med.kuleuven.ac.be (M. Bollen).

Abbreviations: NPP, nucleotide pyrophosphatase/phosphodiesterase; *p*NPP, *p*-nitrophenylphosphate; bis-*p*NPP, bis(*p*-nitrophenyl)phosphate; *p*NP-TMP, *p*-nitrophenyl thymidine 5′-monophosphate; lysoPC, lysophosphatidylcholine; lysoPA, lysophosphatidic acid

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2.3. Expression of wild-type and mutant NPPs

COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and 100 U/ml each of penicillin and streptomycin. Cells were transfected at 30–40% confluence, using the FuGene™6 reagent (Roche Diagnostics). Transiently overexpressed NPP proteins were harvested after 48–72 h. The cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in 20 mM Tris–HCl at pH 7.5, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 0.3 M NaCl and 0.2% Triton X-100. After centrifugation (5 min at 5000×*g*) the supernatant was used for immunoprecipitation of the myc-tagged fusions with monoclonal myc tag antibodies and protein A-TSK (Affiland). The immunoprecipitates were washed once with 0.25 M LiCl and twice with ice-cold Tris-buffered saline (TBS), resuspended in TBS and used for Western analysis or assayed for specified enzymatic activity. Secreted NPP1–3 were immunocaptured in the medium using myc tag antibodies (clone 9E10) and subsequently precipitated using protein A-TSK.

2.4. Western analysis

Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 7.5% tricine gels, proteins were electroblotted onto polyvinylidene difluoride membranes (Amersham Bioscience) at 39 V in 50 mM Tris base supplemented with 50 mM boric acid (pH 8.3). Aspecific binding sites were blocked using 0.2% (v/v) Triton X-100 and 5% (w/v) powdered milk in PBS. Myc-tagged fusion proteins were detected by clone 9E10 monoclonal antibodies, HA-tagged proteins were visualized by clone HA-7 monoclonal antibodies or clone 12CA5 monoclonal antibodies. Peroxidase-labelled secondary antibodies were used for chemiluminescent detection.

2.5. Enzymic activities of NPPs

The nucleotide pyrophosphatase/phosphodiesterase activity was measured with *p*NP-TMP or ATP as substrates [15]. The NPP isoforms were incubated at 37°C in the presence of 100 mM Tris–HCl at pH 8.9 and either 5 mM *p*NP-TMP or 50 μM [γ-^{32}P]ATP. The hydrolysis of *p*NP-TMP was stopped by a 10-fold dilution in 3% (w/v) trichloroacetic acid. Subsequently, the reaction mixture was neutralized with 60 μl 5 N NaOH and the formed *p*-nitrophenol (*p*NP) was quantified colorimetrically at 405 nm. The hydrolysis of ATP was arrested by the addition of 100 mM EDTA. One μl of the reaction mixture was analyzed by thin-layer chromatography on polyethyleneimine cellulose plates (Merck). Nucleotides and degradation products were separated by ascending chromatography in 750 mM KH_2PO_4 at pH 3.0. Radioactive spots were visualized by autoradiography. The nucleotidylated intermediate, formed during the hydrolysis of 50 μM [α-^{32}P]ATP, was trapped according to Blytt et al. [18], with slight modifications [16]. Following SDS–PAGE, the trapped intermediate was visualized by autoradiography.

Bis-*p*NPP and *p*NPP were also tested as substrates for NPP1–3. The NPP isoforms were incubated in 100 mM Tris–HCl at pH 8.9 and either 5 mM bis-*p*NPP or *p*NPP for 2.5 h at 37°C. Subsequently, the formed *p*NP was quantified colorimetrically at 405 nm.

The lysophospholipase D activity was determined as described by Umezu-Goto et al. [10]. Equal amounts of the immunoprecipitated NPPs were incubated for 12 h at room temperature with 2 mM C14:0 lysoPC from egg (Sigma) in 100 mM Tris–HCl at pH 8.9, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 500 mM NaCl and 0.05% Triton X-100. The liberated choline was detected by an enzymic colorimetric method using choline oxidase, horseradish peroxidase and TOOS reagent (*N*-ethyl-*N*-(2-hydroxy-3-sulfoproryl)-3-methylaniline) as a hydrogen donor. The purple color was quantified at 540 nm using a 96-well plate reader.

3. Results

3.1. NPP2 hydrolyzes nucleotides and lysophospholipids in the same catalytic site

In accordance with recently published data [9,10], NPP2 expressed in COS-1 cells with a C-terminal myc tag and precipitated from the culture medium with myc tag antibodies displayed a lysophospholipase D activity (Fig. 1). The catalytic site threonine, which is nucleotidylated during the nucleotide pyrophosphatase/phosphodiesterase reaction, also appeared to be essential for the lysophospholipase D activity of NPP2. Indeed, the T207A mutant had no detectable lysophospholipase D activity whereas the T207S mutant only showed 15% of the wild-type activity. Since the catalytic site threonine has also been shown to be essential for the motility-stimulating properties of NPP2 [7,19], these data are further evidence for a contribution of the lysophospholipase D activity to motility stimulation.

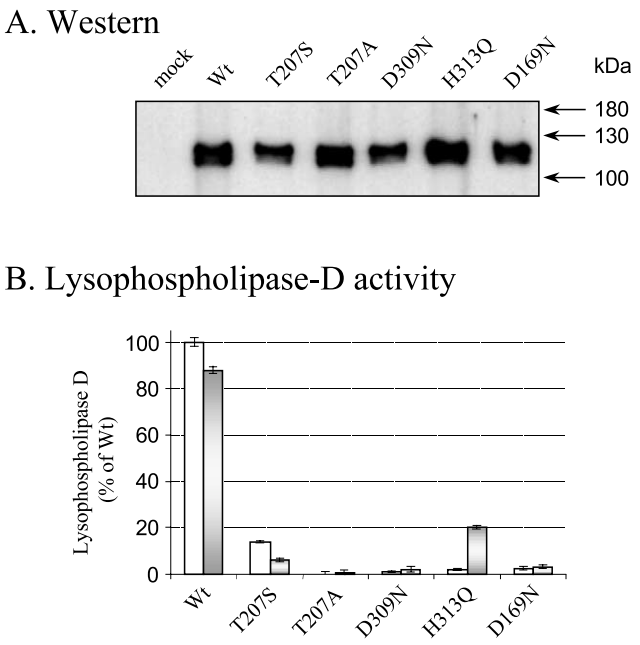

Fig. 1. The lysophospholipase D activity of catalytic site mutants of NPP2. Rat NPP2 or the indicated mutants were expressed in COS-1 cells as fusions with a C-terminal myc tag, and immunoprecipitated from the culture medium with myc tag antibodies. D309N and H313Q are predicted to coordinate Me1, whereas D169N is believed to coordinate Me2. Equal amounts of immunoprecipitated fusion proteins, as judged by Western analysis with myc tag antibodies (A), were used for the assay of lysophospholipase D using lysoPC as substrate in the absence (open bars) or presence (shaded bars) of 1 mM of $ZnCl_2$ (B). The results represent the means ± S.E.M. of six independent assays. The myc tag antibodies did not precipitate any detectable lysophospholipase D activity from mock-transfected cells (data not shown). Wt, wild-type.

We have previously identified residues that coordinate two metals in the catalytic domain of NPP1 [16]. These residues are also conserved in NPP2 and we found that mutation of residues involved in the coordination of either Me1 (D309N, H313Q) or Me2 (D169N) completely abolished the nucleotide pyrophosphatase/phosphodiesterase (not shown) and lysophospholipase D activities of NPP2 (Fig. 1). However, the H313Q mutant still showed 20% of the wild-type activity when assayed in the presence of 1 mM $ZnCl_2$, which is strong evidence that the effect of this mutation stems from the loss of a metal rather than from gross conformational changes.

The above data indicate that NPP2 hydrolyzes nucleotides and lysophospholipids by the same mechanism in a single catalytic site. In further agreement with this proposal, the hydrolysis of both substrates by NPP2 was severely reduced by metal chelators such as EDTA (data not shown) or EGTA [11,20]. Both activities were also severely compromised by the histidine acylator diethylpyrocarbonate (not shown), in keeping with the role of histidines (e.g. H313, see Fig. 1) in catal-

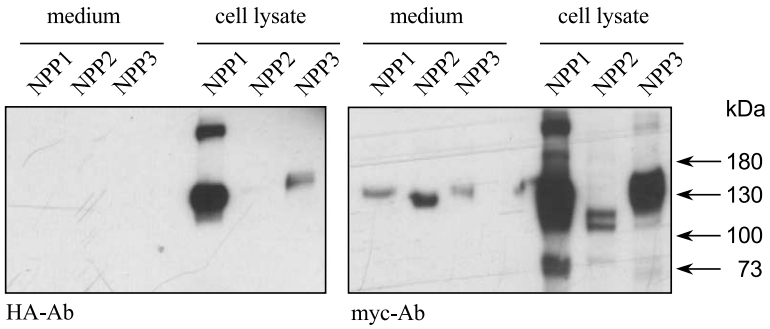

Fig. 2. The expression of NPP1–3 as membrane-associated and secreted proteins in COS-1 cells. NPP1–3 were expressed in COS-1 cells as fusion proteins with both an N-terminal HA tag and a C-terminal myc tag. The expression levels of the NPPs in the cell lysate and the culture medium were quantified by Western analysis with HA tag (left panel) and myc tag (right panel) antibodies. Equal volumes (10 μl) of cell lysate and culture medium were applied.

ysis [16]. Furthermore, both activities were similarly blocked by vanadate (not shown).

3.2. Substrate specificity of NPP1–3

Since NPP1–3 are structurally closely related and have all independently been reported to hydrolyze nucleotides, this raised the possibility that NPP1 and NPP3, like NPP2, also displayed a lysophospholipase D activity. The report that NPP3 [14], like NPP2, affects cell motility and invasive properties was also suggestive for an overlapping substrate specificity. To compare the substrate specificity of NPP1, NPP2 and NPP3, the rodent isoforms were expressed in COS-1 cells, with both an N-terminal HA tag and a C-terminal myc tag. All three isoforms were detected both in the medium and in cell lysates (Fig. 2). However, whereas NPP1 and NPP3 were mainly present in the cell lysates, NPP2 was predominantly detected as a soluble protein in the culture medium. The soluble NPP isoforms could not be detected with HA tag antibodies, in keeping with reports that soluble NPPs are generated from the membrane-associated forms by the proteolytic removal of the N-terminus [6,21,22].

Following immunoprecipitation of the soluble and membrane-associated NPPs with myc tag antibodies, the respective isoforms were used for a comparative analysis of substrate specificities. At similar concentrations of the membrane-associated (not shown) or soluble NPPs, as determined by Western analysis (Fig. 3A), only NPP2 displayed a lysophospholipase D activity (Fig. 3B). On the other hand, the classical nucleotide phosphodiesterase substrate, *p*NP-TMP, was readily hydrolyzed by NPP1 but was a three- and six-fold poorer substrate for NPP3 and NPP2, respectively (Fig. 3C). Similarly, whereas NPP1 efficiently hydrolyzed ATP into AMP and PP_i (Fig. 3D), NPP3 was less active towards this substrate and the ATP-hydrolyzing activity of NPP2 was barely detectable. The latter data were corroborated by findings that the adenylated catalytic intermediate in the presence of imidazole could be readily visualized for NPP1 (Fig. 3D), whereas the covalent intermediate of ATP hydrolysis by NPP3 and NPP2 was only detected at prolonged exposure times (not shown). Another substrate that has classically been used to assay nucleotide pyrophosphatases/phosphodiesterases is bis-*p*NPP [23]. This is an unusual substrate in that it is neither a nucleotide nor a lysophospholipid derivative. Surprisingly, NPP2 was able to hydrolyze bis-*p*NPP whereas both NPP1 and NPP3 were totally ineffective (Fig. 3E). One of the products of the hydrolysis of bis-*p*NPP is *p*NPP, a classical in vitro substrate for alkaline phosphatases. In spite of the structural and catalytic similarities between NPPs and alkaline phosphatases, none of the NPPs was able to hydrolyze *p*NPP (not shown).

We also tested all of the above-mentioned substrates with snake venom phosphodiesterase I. Snake venom phosphodiesterases from different sources have previously been demonstrated to display catalytic properties similar to those of NPP1 and NPP3 [24,25]. Surprisingly, all of the above substrates, except *p*NPP, were readily hydrolyzed by the snake venom phosphodiesterase I. Also, when [α-^{32}P]ATP was used as a substrate, the adenylated catalytic intermediate could be visualized in the presence of imidazole (not shown). Thus, snake venom phosphodiesterase I appears to have a much broader substrate specificity than the mammalian NPP1–3.

4. Discussion

We have shown here that the lysophospholipase D and nucleotide pyrophosphatase/phosphodiesterase activities of NPP2 are similarly affected by mutation of catalytic site residues (Fig. 1) and by metal-chelating and histidine-modifying agents. This is strong evidence that both activities stem from the same catalytic site. The only structural feature that all known NPP substrates have in common is a diester- or pyrophosphate-bonded phosphate group (Fig. 3). Thus, the catalytic site of NPPs appears to be capable of hydrolyzing phosphodiester and pyrophosphate bonds in a broad range of substrates. Interestingly, whereas the snake venom phosphodiesterase I hydrolyzed all tested substrates, the mammalian

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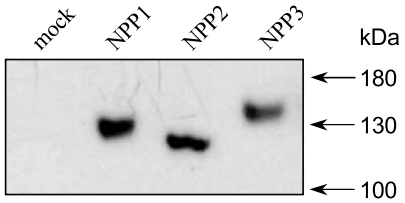
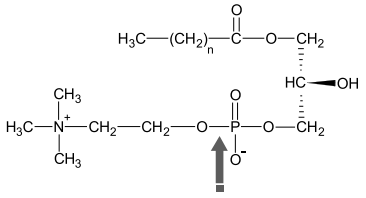
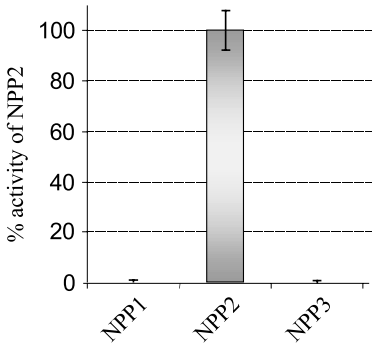
Fig. 3. The substrate specificity of NPP1, NPP2 and NPP3. The secreted NPP fusions (Fig. 2) were immunoprecipitated with myc tag antibodies. Equal amounts of the immunoprecipitated proteins, as judged by Western analysis with myc tag antibodies (A), were used for the assay of enzymic activities with lysoPC (B), *p*NP-TMP (C), [γ-^{32}P]ATP (D) or bis-*p*NPP (E) as substrates. The results represent the means ± S.E.M. of three to six independent assays. Panel D also shows the adenylated catalytic intermediate of the hydrolysis of [α-^{32}P]ATP that was trapped in the presence of imidazole. Similar data were obtained with the NPPs immunoprecipitated from cell lysates (not shown). The figure also shows the structure of the used NPP substrates; the phosphodiester or pyrophosphate bonds that are hydrolyzed by NPPs are indicated by an arrow.

A. Western

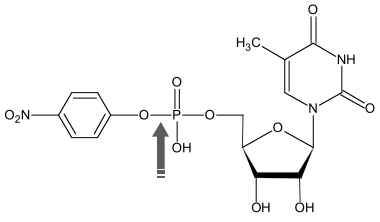


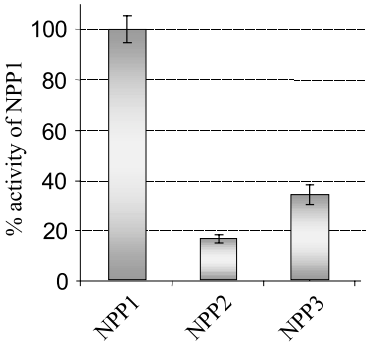
B. lysoPC hydrolysis



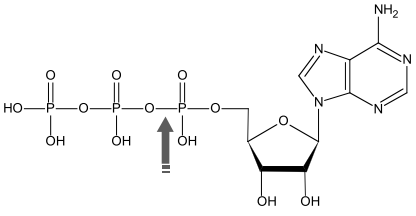


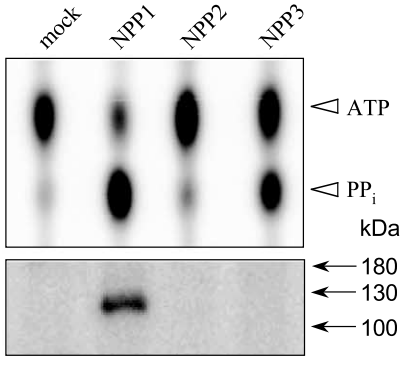
C. *p*NP-TMP hydrolysis



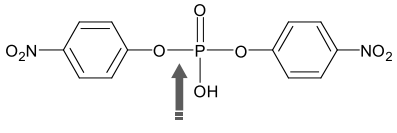


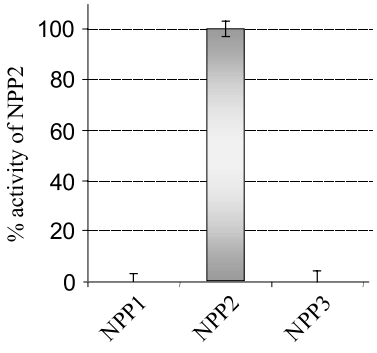
D. ATP hydrolysis





E. bis-*p*NPP hydrolysis





NPPs showed a remarkable substrate specificity (Fig. 3). It is reassuring that the observed in vitro substrate specificities correspond nicely to known biological functions of NPPs. Thus, NPP1 is by far the best ATP-hydrolyzing isoform in vitro (Fig. 3) and convincing evidence shows that NPP1 also hydrolyzes this substrate in vivo (see Section 1). Similarly, the cell motility effects of NPP2 have been attributed to its lysophospholipase D activity and we found that NPP2 is the only NPP isoform with this activity.

It is currently unknown what determines the substrate specificity of the NPPs. Since we have compared NPP1–3 from the same source, it can be excluded that the observed differences in substrate specificity stem from the use of different expression systems or from the use of soluble versus membrane-associated enzymes. It therefore seems likely that the substrate specificity of NPP1–3 is determined by substrate recognition motifs. For example, NPP1 and NPP3 contain a so-called GxGxxG motif that is also found in protein kinases and other nucleotide-binding proteins, and that may well be involved in the recognition of nucleotides as substrates [1]. Interestingly, NPP2 lacks this motif which could explain why this isoform is a poor nucleotide pyrophosphatase/phosphodiesterase. We speculate that NPP2 contains a lysophospholipid-binding motif.

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