Functional characterization of the non-catalytic ectodomains of the nucleotide pyrophosphatase/phosphodiesterase NPP1

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The ubiquitous nucleotide pyrophosphatases/phosphodiesterases NPP1–3 consist of a short intracellular N-terminal domain, a single transmembrane domain and a large extracellular part, comprising two somatomedin-B-like domains, a catalytic domain and a poorly defined C-terminal domain. We show here that the C-terminal domain of NPP1–3 is structurally related to a family of DNA/RNA non-specific endonucleases. However, none of the residues that are essential for catalysis by the endonucleases are conserved in NPP1–NPP3, suggesting that the nuclease-like domain of NPP1–3 does not represent a second catalytic domain. Truncation analysis revealed that the nuclease-like domain of NPP1 is required for protein stability, for the targeting of NPP1 to the plasma membrane and for the

expression of catalytic activity. We also demonstrate that 16 conserved cysteines in the somatomedin-B-like domains of NPP1, in concert with two flanking cysteines, mediate the dimerization of NPP1. The K173Q polymorphism of NPP1, which maps to the second somatomedin-B-like domain and has been associated with the aetiology of insulin resistance, did not affect the dimerization or catalytic activity of NPP1, and did not endow NPP1 with an affinity for the insulin receptor. Our data suggest that the non-catalytic ectodomains contribute to the subunit structure, stability and function of NPP1–3.

Key words: endonuclease, nucleotide pyrophosphatase/phosphodiesterase (NPP), PC-1, somatomedin B.

INTRODUCTION

Nucleotide pyrophosphatases/phosphodiesterases (NPPs) of the NPP family are ubiquitous membrane-associated or secreted enzymes [1,2]. Mammals have five genes that encode NPP isoforms and these have been numbered according to their order of discovery. NPP1–NPP3 (NPP1–3) release 5'-monophosphorylated nucleotides from a variety of nucleotides and nucleotide derivatives [1,2]. In addition, NPP1 [3], NPP2 [4] and NPP3 (R. Gijsbers and M. Bollen, unpublished work) contain intrinsic alkaline phosphatase activity. Moreover, NPP2 also expresses lysophospholipase D activity [5,6]. The enzymic properties of NPP4 and NPP5 have not been studied yet. NPP1–3 are homodimers of polypeptides of about 110 kDa [1,2]. The monomers consist of a short intracellular N-terminal domain (10–80 residues) involved in the targeting to the plasma membrane [7], a single transmembrane domain (≈ 20 residues) and a large extracellular part (≈ 800 residues) encompassing two consecutive somatomedin-B-like (SMB) domains, a catalytic domain and a poorly characterized C-terminal domain. Somatomedin B is a cysteine-rich serum peptide (44 residues) that is proteolytically derived from the multifunctional blood and cell-matrix protein vitronectin [8] and has been demonstrated to interact with both the urokinase receptor [9] and the plasminogen activator inhibitor-1 [10]. The secreted forms of NPP1–3 are believed to be generated by intracellular proteolysis somewhere near the SMB domains of the membrane-associated species [11–13]. Of all three ectodomains of NPP1–3, only the catalytic domain has been studied in some detail. The latter shows structural and catalytic similarities with a superfamily of phospho/sulpho-coordinating enzymes that include alkaline phosphatases [3]. The NPP1–3-

mediated hydrolysis of nucleotides involves a two-step mechanism and occurs via a covalent, nucleotidylated intermediate. With ATP as a substrate, PP_i is released in the first catalytic step and AMP is covalently attached to the catalytic-site threonine. In the second catalytic step, AMP is set free. Since NPP1 also possesses an intrinsic alkaline phosphatase activity, some ATP is converted to ADP and this occurs via a phosphorylated catalytic-site threonine.

NPP1–3 function in a variety of processes. NPP1 is involved in bone matrix mineralization and soft tissue calcification, which has been explained by its ability to generate PP_i, an inhibitor of calcification [14,15]. NPP1 has also been implicated in insulin signalling and in the aetiology of insulin resistance but, surprisingly, this function does not require the catalytic-site threonine and has been suggested to involve a direct interaction between NPP1 and the insulin receptor (IR) [16–18]. NPP2 augments the carcinogenic and metastatic potential of *ras*-transformed cells [19] and these effects have been linked to the lysophospholipase D activity of NPP2, which results in the release of lysophosphatidic acid [5,6]. NPP3 has been demonstrated to promote the differentiation and invasive properties of glial cells [20].

Whereas the functions of the intracellular and catalytic domains of NPP1–3 are fairly well understood, nothing is known about the role of the non-catalytic ectodomains. We have studied the structure and function of these ectodomains and demonstrate here that the SMB domains mediate homodimerization whereas the C-terminal domain is required for the stability, correct subcellular localization and enzymic activity of NPP1–3. Our data do not support a role for the non-catalytic ectodomains (or any other domain) of NPP1 in insulin signalling.

Abbreviations used: BS₃, bis(sulphosuccinimidyl) suberate; EGFP, enhanced green fluorescent protein; HA, haemagglutinin A; IR, insulin receptor; NPP, nucleotide pyrophosphatase/phosphodiesterase; pNP-TMP, *p*-nitrophenyl thymidine 5'-monophosphate; SMB, somatomedin-B-like domain; *ttw*, tip-toe walking.

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A. Conservation of the nuclease-like domain of NPP1–3

B. Model of the nuclease-like domain of NPP1–3

Figure 1 Structural conservation (A) and three-dimensional model (B) of the nuclease-like domain of NPP1–3

(A) A CLUSTAL X-generated multiple sequence alignment of the indicated protein sequences of *Mus musculus* (Mm) and *Serratia marcescens* (Sm) was manually refined. The SWISS-PROT/TrEMBL accession numbers of the listed proteins are P06802 (MmNPP1), Q9R1E6 (MmNPP2), O08600 (MmNuc; where Nuc is nuclease) and P13717 (SmNuc). The protein sequence of MmNPP3 was derived from overlapping EST (expressed sequence tag) sequences (GenBank™ accession numbers BU511904, BU554301, BI152443, BI851049, BE376939 and BE282480). The regular numbers at the beginning and end of the sequences indicate the distance to the beginning and end of each protein, respectively. The numbers in parentheses represent the size of the gaps between the aligned segments. Conserved [small (G, A, S, T), hydrophobic (V, I, L, M), aromatic (F, Y, W) or acidic (D, E)] residues and identical residues among NPPs are boxed in black, whereas residues in endonucleases that are also conserved in NPPs are boxed in grey. The C-terminal end of NPP1-Δ804–905 is indicated by a downwards arrow. Arrowheads indicate residues that are essential for catalysis by the *S. marcescens* nuclease. The predicted secondary structure (ss) of mouse NPP1 (see the Results section) was aligned with the known secondary structure of the *S. marcescens* nuclease (PDB code 1QAE), as determined by the Swiss-Pdb Viewer. The secondary structures are shown above the primary sequence alignment. α -Helices are lettered and shown as cylinders; β -strands are numbered and represented as arrows. (B) A model of the nuclease-like domain of NPP1–3 as deduced from the known structure of the *S. marcescens* nuclease. The α -helices and β -strands are lettered and numbered as in (A). Points in the surface loops where additional protein fragments should be introduced are indicated as breaks.

EXPERIMENTAL

Mutants of NPP1

Mouse NPP1 (residues 35–905) and the indicated mutants were cloned into the pMB001 vector and expressed as haemagglutinin (HA)-tagged fusion proteins in COS-1 cells or HeLa cells [3]. To generate the enhanced green fluorescent protein (EGFP) fusions of NPP1, the respective PCR-amplified cDNAs were subcloned into the pEGFP-N1 vector (Clontech). Myc-tagged wild-type mouse NPP1 (residues 35–905) was constructed using mouse NPP1 in the pEGFP-N1 vector as a template. The cDNA encoding EGFP was cut out with *Bam*HI/*Not*I and replaced with an adaptor encoding the amino acid sequence EQKLISEEDL (residues 410–419) of human c-Myc. Cysteines were mutated using the QuikChange site-directed mutagenesis protocol (Stratagene): cysteines were mutated to serine, except for Cys a and Cys b (see Figure 4A, below), which were mutated into alanine. NPP1-Δ568–905 (tip-toe walking, or *ttw*, mutant) was made by changing codon 568 into a stop codon [21]. The cDNA encoding full-length human NPP1 (923 residues) in the pCI-Neo vector was provided by Dr J. Goding (Monash University, Prahran, Australia). The K173Q-mutated HA-tagged human NPP1 was made by introducing a point mutation in codon 173 (AAG to CAG), using the QuikChange site-directed mutagenesis protocol. It should be pointed out that Lys-173 is often wrongly referred to as Lys-121 [22,22a], based on the usage of an incorrect transcriptional start site. The cDNA encoding rat NPP3 was donated by Dr H. Deissler (University of Ulm, Ulm, Germany) and subcloned into pMB001 using *Xba*I and *Bam*HI, N-terminally fused to an HA tag. All PCR amplifications were conducted using *Pwo* proofreading polymerase (Roche Diagnostics). All mutations and subcloning steps were verified by DNA sequencing.

Cell cultures

COS-1 cells and HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and 100 units/ml of both penicillin and streptomycin. Cells were transfected at 30–40% confluency, using the FuGene™6 reagent (Roche Diagnostics). After 48 h, the subcellular localization of the EGFP fusions in HeLa cells was analysed by fluorescence confocal microscopy. After transfection of the COS-1 cells (48 h), the cells were washed twice in ice-cold PBS and lysed in a buffer containing 20 mM Tris/HCl, pH 7.5, 0.5 mM PMSF, 0.5 mM benzamidine, 0.3 M NaCl and 0.2% Triton X-100. After centrifugation (5 min at 5000 g) the HA-tagged fusion proteins were immunoprecipitated from the supernatant with monoclonal HA-tag antibodies (clone 12CA5) and Protein A-TSK® (Affiland, Liège, Belgium). Immunoprecipitates were washed once with 0.25 M LiCl and twice in Tris-buffered saline, resuspended in 50 mM Hepes (pH 7.5) and assayed for enzymic activity or dimer formation.

Stably transfected NIH-3T3 fibroblast cells (a gift from Dr A. Ullrich, Max Planck Institute for Biochemistry, Martinsried, Germany) overexpressing wild-type human IR were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 4.5 mg/ml glucose, 100 units/ml penicillin, 100 units/ml streptomycin and 1 mg/ml G418.

Northern and Western blot analysis

Total RNA was extracted from transiently transfected COS-1 cells using the GenElute™ Total RNA kit (Sigma). Northern blot analysis was conducted as described previously using 18 S rRNA and mouse NPP1 cDNA as probes [23]. Following

SDS/PAGE on 7.5% tricine gels, proteins were electroblotted on to PVDF membranes (Bio-Rad) at 39 V in 50 mM Tris base plus 50 mM boric acid (pH 8.3). Aspecific binding sites were blocked in PBS supplemented with 5% (w/v) powdered milk and 0.2% (v/v) Triton X-100. Affinity-purified polyclonal antibodies raised against LRLKTHLPFSQED (residues 892–905 of mouse NPP1) were used to visualize NPP1, Myc-tagged fusion proteins were detected by monoclonal antibodies against clone 9E10 (Sigma), HA-tagged proteins were visualized by monoclonal antibodies against clone HA-7 (Sigma) or clone 12CA5, and EGFP-fusion proteins were visualized with polyclonal anti-GFP antibodies (Santa Cruz Biotechnology). The IR was detected by polyclonal rabbit antibodies directed against the β-subunit (Sigma) and autophosphorylation of the IR was evaluated using anti-phosphotyrosine monoclonal antibodies (clone 4G10; Upstate Biotechnology). Peroxidase-labelled secondary antibodies were used for subsequent chemiluminescent detection.

Assay of NPP1 activity

The NPP activity was measured using *p*-nitrophenyl thymidine 5'-monophosphate (*p*NP-TMP) as a substrate [3]. The nucleotidylated intermediate, which is formed during the hydrolysis of 50 μM [α -³²P]ATP by mouse NPP1 enzyme, was trapped as described in [24], with slight modifications [25]. The trapped intermediate was visualized by autoradiography, following SDS/PAGE. The phosphorylated intermediate of mouse NPP1 was obtained following incubation at 30 °C for 60 min in 100 mM Hepes (pH 7.5) and 50 μM [γ -³²P]ATP. 5'-Nucleotidase from *Crotalus atrox* (3 units/ml; Sigma) was added to remove inhibitory AMP [26]. Autophosphorylation was visualized by autoradiography, following SDS/PAGE.

Cross-linking of NPP1

Chemical cross-linking was conducted as described previously [27], with slight modifications. Briefly, transiently transfected cells were washed twice with ice-cold PBS and kept on ice. The cross-linking agent bis(sulphosuccinimidyl) suberate (BS₃) was freshly dissolved in PBS and added to the cells at a final concentration of 1 mM. Cross-linking was quenched after 45 min by the addition of 50 mM glycine at pH 6.5 for 15 min. Subsequently, proteins were extracted as described above and the NPP1 monomers and dimers were visualized by Western blotting.

Interaction of NPP1 with IR (signalling)

NIH-3T3 cells stably expressing wild-type IR were transfected at 30% confluency with different HA-tagged NPP1 constructs using FuGene™6. After 48 h the cells were exposed for 5 min to 10 nM insulin. Subsequently, the cells were washed twice with ice-cold PBS and lysed. After centrifugation (5 min at 5000 g) the cell lysate was used for immunoprecipitation of NPP1 and IR, using monoclonal anti-HA-tag antibodies (clone 12CA5) or monoclonal anti-Ab-3 antibodies (Oncogene), respectively. The co-precipitation of IR and NPP1 in the immunoprecipitate was evaluated by Western blotting.

The NIH-3T3 cells stably overexpressing wild-type IR and transiently expressing HA-tagged NPP1 constructs were exposed to the indicated concentrations of insulin. Subsequently, the phosphorylation level of IR was measured by Western blotting with Ab-3 antibodies (clone 4G10).

Figure 2 Expression and subcellular localization of C-terminally truncated NPP1 mutants

(A) Total RNA was prepared from COS-1 cells transiently transfected with an empty vector or with expression constructs for wild-type NPP1, NPP1-Δ568–905 or NPP1-Δ804–905, each N-terminally fused to an HA tag. Northern blot analysis was performed using the cDNAs for mouse NPP1 and 18 S rRNA as probes. It should be noted that NPP1-Δ568–905 was generated by introducing a stop codon (Gly-568 → stop), which explains why the corresponding transcript was the same size as the wild-type transcript. (B) Lysates from the same cells were used for Western analysis using HA-7 antibodies. The monomers are indicated by open arrowheads and the dimers by closed arrowheads. It should be pointed out that NPP1 monomers are known to migrate as doublets. The minor band at around 120 kDa represents an aspecifically recognized polypeptide. (C) HeLa cells were transiently transfected with either wild-type NPP1 or NPP1-Δ804–905, both with an N-terminal HA tag and a C-terminal EGFP tag. After 48 h the cells were analysed by fluorescence confocal microscopy. Single x - y sections were taken from the middle of the cells. Scale bars, 10 μ m.

RESULTS

NPP1–3 contain a nuclease-like domain

The C-terminal one-third of NPP1, NPP2 and NPP3 are structurally related (Figure 1A). To identify more distantly related sequences, we used residues 630–905 of mouse NPP1 as a query to search the non-redundant protein sequence database at NCBI

with the position-specific iteration (PSI)-BLAST algorithm [28]. Convergence was reached after eight iterations and yielded, besides other NPP family members and some hypothetical proteins, only proteins classified as DNA/RNA non-specific endonucleases. The multiple alignment of the primary structures of the C-terminal domains of NPP1–3 and those of the endonucleases indeed revealed conservation of specific patches throughout the entire sequence (Figure 1A). Therefore, we will henceforth refer to the C-terminal domain of NPP1–3 as the ‘nuclease-like’ domain.

Interestingly, the crystal structure of one of the endonucleases, i.e. the nuclease of the enterobacterium *Serratia marcescens*, has been solved [29]. This information was used to gain more insight into the structure of the nuclease-like domain of NPP1–3. A consensus secondary structure for the nuclease-like domain of mouse NPP1 was compiled (Figure 1A) from the suggested secondary structures on the PHD, PSI-Pred, SamT99, JPREP and Prof prediction servers [30–34], since it is known that the reliability of secondary structure predictions improves by using a combination of several independent prediction methods [35]. The alignment of the predicted secondary structure of the nuclease-like domain of NPP1 and the known secondary structure elements of the *S. marcescens* nuclease disclosed a striking conservation in both the occurrence and the length of α -helices and β -strands (Figure 1A), suggesting that the nuclease-like domain of NPP1–3 and the *S. marcescens* endonuclease adopt a similar fold. Accordingly, the fold-recognition program GenTHREADER [36], which uses threading potentials to evaluate the quality of fold assignments, predicted with the highest level of confidence that the nuclease-like domain of NPP1 adopts the fold of the *S. marcescens* endonuclease. Fitting of the predicted secondary structure elements of the nuclease-like domains of NPP1 on to the corresponding fragments of the *S. marcescens* endonuclease backbone allowed us to build a rough structural model of the nuclease-like domain (Figure 1B). According to this model, the nuclease-like domain consists of an anti-parallel β -sheet flanked by α -helices and two crossed β -strands.

Function of the nuclease-like domain of NPP1–3

NPPs and DNA/RNA non-specific endonucleases both catalyse the hydrolysis of phosphate esters, releasing 5'-phosphorylated nucleotides or oligonucleotides with 3'-OH ends, respectively. Yet, it has been shown unequivocally that the NPP reaction is catalysed by a domain that is N-terminal to the nuclease-like domain (reviewed in [1]). It is unlikely that the nuclease-like domain of NPP1–3 represents a second catalytic domain since none of the residues involved in the catalysis of the endonucleases [37] is conserved in NPP1–3 (arrowheads in Figure 1A).

Although the nuclease-like domain is likely to be catalytically inactive, data from the literature suggest that it is essential for the function of NPP1. Indeed, deletion of the nuclease-like domain, which originates from a nonsense mutation (Gly-568 → stop) in the naturally occurring *ttw* mouse, results in osteoblast-mediated hyperossification, which is a similar phenotype to that of the NPP1-null mouse [21,38]. To further elucidate the role of the nuclease-like domain, we generated C-terminal deletion mutants of mouse NPP1, i.e. NPP1-Δ804–905 and NPP1-Δ568–905. The latter corresponds to the *ttw* mutant, whereas the nuclease-like domain is only truncated by one-third in NPP1-Δ804–905. These C-terminal truncations did not affect the transcription of EGFP-tagged NPP1 in COS-1 cells (Figure 2A), but decreased the steady-state level of the expressed proteins, in particular of NPP1-Δ568–905 (Figure 2B). Furthermore, whereas wild-type NPP1 was preferentially located in the plasma membrane,

Figure 3 Effect of the deletion of residues 804–905 on the enzymic properties of NPP1

COS-1 cells were transiently transfected with the same expression constructs as in Figure 2(C). After 48 h the fusion proteins were immunoprecipitated with anti-HA-tag antibodies. Equal amounts of protein, as assessed by Western blot analysis with GFP antibodies (A), were used to assay the nucleotide phosphodiesterase activity with *p*NP-TMP as a substrate (B). Results are means $\pm$ S.E.M. from six independent experiments. The immunoprecipitates were also used for trapping of the covalent phosphorylated (C) and nucleotidylated (D) intermediates in the presence of [γ - 32 P]ATP and [α - 32 P]ATP, respectively.

NPP1-Δ804–905 remained entirely intracellular and showed a distribution typical for proteins that are associated with the endoplasmic reticulum (Figure 2C). Taken together, these data suggest that the nuclease-like domain is required for the stability of NPP1 and for its translocation to the plasma membrane.

We have also examined whether the truncation of the nuclease-like domain affects the catalytic activity of NPP1. For that purpose wild-type NPP1 and NPP1-Δ804–905 were expressed in COS-1 cells as fusions with EGFP. Following immunoprecipitation, equal amounts of the fusions were assayed for nucleotide phosphodiesterase activity, using *p*NP-TMP as a substrate (Figures 3A and 3B). A partial truncation of the nuclease-like domain, as in NPP1-Δ804–905, resulted in a complete loss of catalytic activity. Since some inactive mutants of NPP1 have been reported to be specifically hampered in the second step of catalysis, resulting in an accumulation of the catalytic intermediate [3], we also explored whether the C-terminal deletion mutants of NPP1 are still able to form the covalent catalytic intermediate. However, in contrast with the wild-type protein, neither a nucleotidylated nor a phosphorylated catalytic intermediate could be detected for NPP1-Δ804–905 in the presence of [γ - 32 P]ATP and [α - 32 P]ATP,

respectively (Figures 3C and 3D). Thus the nuclease-like domain is also required for the expression of catalytic activity by NPP1.

Mapping of the homodimerization domain

NPP1–3 are disulphide-linked homodimers [1,2] and dimers can even be detected after SDS/PAGE in the presence of reducing agents (Figures 2B and 4B). We have found that the deletion of either the nuclease-like domain (Figure 2B) or both the catalytic and the nuclease-like domains, as in NPP1-(35–191), did not hamper the ability of mouse NPP1 to form dimers (Figure 4B). However, no dimers were detected after the additional deletion of the SMB domains and their flanking sequences [NPP1-(35–80) in Figure 4B]. The mere deletion of a single SMB domain did not affect dimerization (results not shown). The SMB domains of NPP1–3 each contain eight conserved cysteines (Figure 4A). In addition, there are two conserved cysteines C-terminal to the SMB domains, denoted as Cys-17 and Cys-18 in Figure 4(A). Neither mutation of the conserved cysteines in the first and/or second SMB domain to serines, nor the mutation of Cys-17 and Cys-18 to serine, abolished the formation of dimers (results not

A. Somatomedin-B-like domains of NPP1-3

B.

C.

Figure 4 The SMB domains mediate the homodimerization of NPP1

(A) Alignment of the transmembrane and SMB domains of mouse (*Mus musculus*, Mm) NPP1-3. Also shown (twice) is the SMB domain (residues 22-58) of mouse vitronectin (AA H18521). Residues conserved among all NPPs are boxed in black. All 18 invariant cysteine residues are boxed in grey and numbered 1-18. The three non-conserved cysteines that only occur in NPP1 are boxed in grey and lettered a-c. The regular numbers preceding and following the sequences denote the distance to the beginning and the end of the each protein, respectively. The numbers in parentheses indicate the sizes of the gaps between the aligned segments. (B) NPP1-(35-191), NPP1-(35-191)-C₁₋₁₈S, NPP1-(35-191)-C_{all}S/A and NPP1-(35-80) were transiently expressed in COS-1 cells as fusions with an HA tag at the N-terminus and EGFP at the C-terminus. C_{all}S/A refers to NPP1-(35-191) where cysteines 1-18 and c (see A) were mutated into serine, and cysteines a and b in the transmembrane domain were mutated into alanine. At 48 h after transfection the cell lysates were used for Western blot analysis and proteins were visualized with HA-7 antibodies. (C) NPP1 and NPP1-C₁₋₁₈S, both with an N-terminal HA tag and a C-terminal EGFP tag, were transiently expressed in COS-1 cells. Prior to the harvesting of the cells, the NPP1 dimers in the indicated conditions were chemically cross-linked with 1 mM BS₃ for 45 min at 4 °C. After SDS/PAGE of the cell lysates, the NPP1 monomers and dimers were visualized with HA-7 antibodies. NPP monomers and dimers are indicated by open and closed arrowheads, respectively.

shown). However, following mutation of all 18 conserved cysteines to serines (C₁₋₁₈S), NPP1-(35-191) no longer formed a dimer (Figure 4B). Strikingly, in this case the monomer migrated as a somewhat larger protein in SDS/PAGE and, in addition to the monomer, an additional band appeared at a position intermediate to that of the monomer and the dimer (Figure 4B). The latter band most likely represents a monomer with intrachain disulphide bonds since this band was absent when the three remaining (unconserved) cysteines in NPP1-(35-191)-C₁₋₁₈S, denoted as cysteines a, b and c in Figure 4(A), were also mutated (Figure 4B).

In accordance with the previous data, the C₁₋₁₈S mutations also abolished the ability of full-length NPP1 to form dimers, as detected after SDS/PAGE (Figure 4C). Likewise, no dimer was detected when COS-1 cells expressing the C₁₋₁₈S mutant were chemically cross-linked with the membrane-impermeable agent

BS₃. In contrast, this cross-linker dramatically increased the detection of dimers in cells expressing wild-type NPP1 (Figure 4C), illustrating for the first time that NPP1 exists as a dimer at the surface of living cells.

Some cell types express multiple NPP isoforms. For example, in hepatocytes and articular chondrocytes, both NPP1 and NPP3 are expressed [39,40]. This prompted us to explore whether NPP1 and NPP3 can form mixed dimers. For that purpose, NPP1 was expressed in COS-1 cells as a fusion with a C-terminal Myc tag while NPP3 was co-expressed as a fusion with an N-terminal HA tag. However, NPP1-Myc could not be precipitated with anti-HA-tag antibodies (Figure 5), indicating that no NPP1/NPP3 heterodimer had formed. On the other hand, following the co-expression of HA-NPP1 and NPP1-Myc, the latter could be immunoprecipitated with anti-HA antibodies, indicating that a mixed NPP1 dimer had been formed.

Figure 5 NPP1 and NPP3 do not form heterodimers

NPP1-Myc, HA-NPP1 and/or HA-NPP3 were transiently (co)-expressed in COS-1 cells, as indicated. The HA-tagged fusion proteins were immunoprecipitated with anti-HA-tag antibodies (clone 12CA5) and checked for co-sedimentation of Myc-tagged NPP-protein. The expression and immunoprecipitation of the fusions was verified by Western analysis using anti-Myc-tag or anti-HA antibodies (HA-7).

NPP1-K173Q and insulin signalling

A single-nucleotide polymorphism located in the second SMB domain of human NPP1, resulting in the K173Q replacement, has been associated with insulin resistance and a faster progression of diabetic nephropathy in type 1 diabetes [22a,42], but the underlying mechanism is still unclear. We found that the K173Q mutation did not hamper the dimerization of NPP1 in COS-1 cells (Figure 6A). Neither did this mutation affect the catalytic activity of NPP1, as determined by the ability to hydrolyse *p*NP-TMP (Figure 6B) or to form a phosphorylated (Figure 6C) or nucleotidylated (Figure 6D) catalytic intermediates with ATP as a substrate. It has been suggested that NPP1 and especially the K173Q mutant impedes insulin signalling via direct interaction with the IR [18,22]. However, these conclusions were based primarily on studies in MCF-7 cells, which contain a very low level of IR and display a low sensitivity to insulin ([43]; R. Gijsbers and M. Bollen, unpublished work). We have therefore re-investigated the reported interaction between NPP1 and IR in NIH-3T3 fibroblasts stably over-expressing IR and transiently transfected with either wild-type NPP1 or the K173Q mutant. Although pull-down of NPP1

Figure 6 Effect of the K173Q mutation on catalytic activity and dimerization of NPP1

COS-1 cells were transiently transfected with expression plasmids encoding either wild-type human NPP1 or the K173Q variant, both with an N-terminal HA tag. At 48 h after transfection, the NPP1 fusions were immunoprecipitated with anti-HA-tag antibodies. Similar amounts of precipitated proteins, as judged from Western blot analysis with anti-NPP1 antibodies (A), were used to determine the nucleotide phosphodiesterase activity with *p*NP-TMP as a substrate (B). These results represent the means $\pm$ S.E.M. from 10 independent experiments. The immunoprecipitates were also used to trap the covalent phosphorylated (C) or nucleotidylated (D) intermediates in the presence of [γ - 32 P]ATP and [α - 32 P]ATP, respectively. NPP monomers and dimers are indicated by open and closed arrowheads, respectively.

Figure 7 NPP1 and IR do not interact in NIH-3T3 cells

(A) NIH-3T3 cells stably overexpressing wild-type human IR were transiently transfected with an empty vector (mock) or with expression plasmids encoding human HA-NPP1 (NPP1) or the K173Q-mutant (NPP1-K173Q). Expression of the fusions was verified by Western analysis (results not shown). NPP1 was immunoprecipitated (IP) with anti-HA-tag antibodies (HA-Ab) and the IR with Ab-3 antibodies. The immunoprecipitated proteins were detected by Western analysis using IR antibodies (left-hand panel) or NPP1 antibodies (right-hand panel). As a negative control, the same cell-lysates were incubated without primary antibodies. (B) The effect of the overexpression of human NPP1 or the K173Q mutant on the insulin-induced autophosphorylation of the IR in the same NIH-3T3 cells. The autophosphorylation of IR was measured in cell lysates with phosphotyrosine (pY) antibodies (top panel), the overexpression of IR was demonstrated with IR antibodies (middle panel) and the overexpression of NPP1 and the K173Q mutant was verified by NPP1 antibodies (bottom panel). NPP monomers and dimers are indicated by open and closed arrowheads, respectively.

(wild-type or mutant) or human IR by their respective antibodies could be demonstrated, we failed to detect co-precipitation of these proteins (Figure 7A). Moreover, we were unable to detect any effect of an overexpression of wild-type NPP1 or NPP1-K173Q on the insulin-dependent autophosphorylation of the human IR (Figure 7B). Thus our data do not provide any evidence for an interaction between human NPP1 and IR.

DISCUSSION

The nuclease-like domain

We have shown here that the C-terminal one-third of NPP1-3 displays structural similarities with DNA/RNA non-specific endonucleases and is likely to adopt a nuclease-like fold (Figure 1). Yet, the residues that are essential for catalysis in the DNA/RNA non-specific endonucleases are not conserved in NPP1-3, which is a strong indication that the nuclease-like domain has not retained catalytic activity. This conclusion is in agreement with numerous reports showing that the hydrolysis of nucleotides is mediated by the central domain of NPP1-3, which

shares structural and catalytic features with a superfamily of phospho-/sulpho-coordinating metalloenzymes [3]. On the other hand, we found that the nuclease-like domain of NPP1 is required for catalysis by NPP1 (Figure 3). Possibly, the nuclease-like domain is involved in the binding of substrates and/or functions as a substrate-specifying region. Anyway, combined with our observations that NPP1 lacking the nuclease-like domain is less stable and not properly targeted to the plasma membrane (Figure 2), these data can account for the similar phenotype of the *ttw* and the NPP1-null mouse [15,21,38].

SMB domains

Somatomedin B is a serum peptide that is proteolytically derived from vitronectin (reviewed in [8]) and that has been shown to be the only region in vitronectin involved in the binding of plasminogen activator inhibitor-1 (reviewed in [10]) and of the urokinase receptor [9]. However, the SMB domains of NPP1-3 do not have any affinity for plasminogen activator inhibitor-1 or the urokinase receptor [9,44]. We have demonstrated here by

site-directed mutagenesis that the conserved cysteines in both SMB domains of NPP1 mediate homodimerization (Figure 4). This was unexpected, since vitronectin is predominantly found in the plasma as a monomer and the cysteine residues of the SMB domain are not involved in the disulphide-linked oligomerization of vitronectin in the extracellular matrix [8]. Since SMB domains are also present in a variety of other proteins, such as a macrophage-specific scavenger receptor in flies [45], a secreted proteoglycan known as superficial zone protein [46] and some amphibian DNases [47], it is possible that they have a more general role as dimerization domains. Surprisingly, NPP1 and NPP3 did not form heterodimers when co-expressed in cultured cells (Figure 5), even though all cysteines that are involved in the homodimerization of NPP1 are conserved in NPP3 (Figure 4A).

NPP1 and insulin signalling

Although various studies suggest a role for NPP1 in the aetiology of insulin resistance and diabetes, the available data are inconsistent and sometimes even contradictory. Firstly, NPP1 has been described as an inhibitor of the IR tyrosine kinase activity [17], but later investigations revealed that this inhibition is aspecific and stems from the hydrolysis of the substrate ATP [48]. Secondly, it has been reported that NPP1 binds directly to the IR and that the overexpression of NPP1 is associated with an impaired insulin signalling [18,22]. However, an NPP1–IR interaction with purified components has not been demonstrated and the effects on insulin signalling were mainly obtained in MCF-7 cells, a cultured breast cancer cell line which contains very low levels of IR. We (Figure 7) and others [43,49] failed to detect a NPP1–IR interaction and did not observe any effect of the expression of NPP1 on the autophosphorylation of the IR. Neither have we been able to reconstitute a complex between purified IR from human placenta and NPP1 from rat liver (R. Gijssbers and M. Bollen, unpublished work). Thirdly, increased NPP1 levels have been associated with whole-body insulin resistance and reduced IR tyrosine kinase activity [16,17,50], but other studies did not find this correlation [43,51] or even reported decreased NPP1 levels in specific cases of insulin resistance [52]. Finally, some reports indicate that the K173Q mutation of NPP1 is correlated with an increased incidence of the occurrence of insulin resistance, the development of type II diabetes and the progression of nephropathy in type I diabetes [22a,42], but this also has not been a consistent finding [53–55]. In addition, we have not been able to confirm data [22,22a] indicating that the K173Q mutation endows NPP1 with an (increased) affinity for the IR. In conclusion, our data do not support a link between NPP1 and insulin signalling. That NPP1 is not important for insulin signalling is also in accordance with recent findings that the NPP1-null mouse has normal blood glucose levels [38].

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