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Nucleotide Pyrophosphatases/ Phosphodiesterases on the Move

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ABSTRACT: Nucleotide pyrophosphatases/phosphodiesterases (NPPs) release nucleoside 5′-monophosphates from nucleotides and their derivatives. They exist both as membrane proteins, with an extracellular active site, and as soluble proteins in body fluids. The only well-characterized NPPs are the mammalian ecto-enzymes NPP1 (PC-1), NPP2 (autotaxin) and NPP3 (B10; gp130^{RB13-6}). These are modular proteins consisting of a short N-terminal intracellular domain, a single transmembrane domain, two somatomedin-B-like domains, a catalytic domain, and a C-terminal nuclease-like domain. The catalytic domain of NPPs is conserved from prokaryotes to mammals and shows remarkable structural and catalytic similarities with the catalytic domain of other phospho-/sulfo-coordinating enzymes such as alkaline phosphatases. Hydrolysis of pyrophosphate/phosphodiester bonds by NPPs occurs via a nucleotidylated threonine. NPPs are also known to auto(de)phosphorylate this active-site threonine, a process accounted for by an intrinsic phosphatase activity, with the phosphorylated enzyme representing the catalytic intermediate of the phosphatase reaction. NPP1-3 have been implicated in various processes, including bone mineralization, signaling by insulin and by nucleotides, and the differentiation and motility of cells. While it has been established that most of these biological effects of NPPs require a functional catalytic site, their physiological substrates remain to be identified.

KEY WORDS: NPP, nucleotide pyrophosphatase/phosphodiesterase; phosphodiesterase-I, alkaline phosphodiesterase, PC-1; B10, autotaxin; ecto-enzyme.

I. INTRODUCTION

Phosphodiesterases hydrolyze diesters of phosphoric acid into phosphomonoesters (Razzell, 1963). They can be classified, according to the nature of their substrate, into nucleotide and lipid phosphodiesterases (Stefan, 1999). The latter enzymes comprise phosphoglyceride phosphodiesterases (e.g., phospholipases C and D) and sphingomyelinases. The nucleotide phosphodiesterases consist of cyclic-nucleotide phosphodiesterases, nucleic-acid phosphodiesterases (ribonucleases and deoxyribonucleases), and nucleotide pyrophosphatases/ phosphodiesterases or NPPs.

NPPs represent a group of widely distributed and phylogenetically conserved proteins. *In vitro*, these enzymes can hydro-

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lyze both pyrophosphate bonds (in, e.g., ATP) and phosphodiester bonds (in, e.g., oligonucleotides) and thereby produce nucleoside 5′-monophosphates. It has been our aim to provide an in-depth and critical overview of the enzymology, regulation, and function of NPPs. Additional information on NPPs can be found in other recent reviews (Stracke et al., 1997; Goding et al., 1998; Zimmermann, 1999; Zimmermann and Brown, 1999; Goding, 2000).

II. NOMENCLATURE

The nomenclature of NPPs has always been a matter of great confusion (Table 1). All NPP isozymes have been (re)discovered independently, and for none was the enzymic function clear from the beginning, which explains their inconsistent nomenclature. In addition, it has not always been recognized that the pyrophosphatase and phosphodiesterase activities are catalyzed by the same catalytic site (Decker and Bischoff, 1972; Rebbe et al., 1993), and the ability of NPPs to auto(de)phosphorylate (see Section VI.B) has led some investigators to classify NPPs as ATPases (Heilmann et al., 1994; Kreisel et al., 1996; Clair et al., 1997b).

According to the recommendations of the IUBMB nomenclature committee (Webb, 1992), NPPs could, in principle, be categorized as type I phosphodiesterases (EC 3.1.4.1) or nucleotide pyrophosphatases (EC 3.6.1.9) or 5′-exo(deoxy)ribonucleases (EC 3.1.11, EC 3.1.13, EC 3.1.15). The designation of NPPs as 5′-nucleotide phosphodiesterases or type-I phosphodiesterases makes no longer sense, however, because these terms were initially introduced to differentiate these enzymes from 3′-nucleotide phosphodiesterases or type II phosphodiesterases, which are now classified as 3′-exo(deoxy)ribonucleases (EC 3.1.16). Moreover, the name ‘phosphodiesterase-II’ is currently also used for a structurally unrelated subgroup of cyclic-nucleotide phosphodiesterases (Delporte et al., 1996; Perry and Higgs, 1998). NPPs should not be classified as exonucleases either because, in contrast to canonical exonucleases, they act much better on oligonucleotides than on polynucleotides. In principle, NPPs could be cataloged as ATPases (EC 3.6.1.3), because autophosphorylation and autodephosphorylation result in net conversion of ATP into ADP and P_i. However, as discussed in Section VI.B, the ability of NPPs to produce ADP from ATP rather stems from an intrinsic phosphatase activity that is associated with the nucleotide pyrophosphatase/phosphodiesterase catalytic site.

In an effort to bring an end to the confusing and illogical nomenclature, the name ‘nucleotide pyrophosphatases/phosphodiesterases’ (NPP) was recently proposed and adopted at an international workshop on ‘Ecto-ATPases and related ectonucleotidases’ (Zimmermann et al., 2000). In accordance with the nomenclature of other enzymes, this name includes the substrate (nucleotides and their derivatives) followed by the reaction that is catalyzed (hydrolysis of either pyrophosphate or phosphodiester bonds). As all well-characterized NPPs appear to be ecto-enzymes, it was also proposed to denote this family of enzymes as ecto-NPPs or E-NPPs (Zimmermann et al., 2000). While this prefix emphasizes an important property of these enzymes and may also prevent a mix up with the ‘Natriuretic Peptide Precursor’ proteins, the use of this prefix may eventually have to be reconsidered if structurally related intracellular NPPs are identified.

NPP proteins are numbered according to their order of discovery (Zimmermann et al., 2000; Table 1), and the splice variants are differentiated by Greek suffixes. In accordance with the rules for the nomencla-

TABLE 1
Nomenclature of Mammalian Members of the E-NPP Family

Proteins		Genes	
Current name[a]	**Previous names**[b]	**Current name**[c]	**Previous names**[b]
NPP1	PC-1 (Takahashi et al., 1970) MAFP (Oda et al., 1991) NPPase (Funakoshi et al., 1992) NPPγ (Stefan et al., 1999a)	**ENPP1** (6q22-23)	*PDNP1* (Accession number AB032016) *NPPS* (Nakamura et al., 1999) *Pca-1* (Buckley and Goding, 1992) *Npps* (Okawa et al., 1998a
NPP2	Autotaxin (Murata et al., 1992) PD-Iα (Narita et al., 1994) NPPα (Stefan et al., 1999a)	**ENPP2** (8q24.1)	*PDNP2* (Kawagoe et al., 1995) *Pdnp2* (Piao et al., 1999)
NPP3	gp130^{RB13-6} (Deissler et al., 1995) B10 (Scott et al., 1997) PD-Iβ (Jin-Hua et al., 1997) NPPβ (Stefan et al., 1999a)	**ENPP3** (6q22)	*PDNP3* (Jin-Hua et al., 1997) *Pdnpno* (Koelsch et al., 1996)
NPP4 (putative)	None (Gijsbers et al., 2000a)	**ENPP4** (6p11.2-21.1)	KIAA0879 (Nagase et al., 1998)
NPP5 (putative)	None (Gijsbers et al., 2000a)	**ENPP5** (6p11.2-21.1)	None

[a] As proposed by Zimmermann et al. (2000).
[b] Key references in parentheses.
[c] The localization of the human genes is indicated in parentheses.

ture of human genes, the NPP encoding genes are denoted by the four-letter code ENPP, for EctoNucleotide Pyrophosphatase/ Phosphodiesterase, and receive the same suffix number as the encoded protein (Table 1, see also http://www.gene.ucl.ac.uk/nomenclature/).

III. DIVERSITY OF NPPs

A. Mammalian NPPs

Currently, there are five human genes known to encode established or putative NPPs (Table 1). ENPP1 and ENPP3 have both been mapped to chromosome 6q22-23 (Buckley et al., 1990; Funakoshi et al., 1992; Jin-Hua et al., 1997), suggesting that these two genes originated by duplication of a common ancestor gene. Consistent with this view, NPP1 and NPP3 are more closely related to each other (50% identity at the protein level) than to NPP2 (39 to 41% identity). Likewise, ENPP4 and ENPP5 have probably evolved by the duplication of a common ancestor because they are both localized on chromosome 6p11.2-21.1 and are more closely related to each other (51% identity) than to the corresponding domain of NPP1-3 (29 to 37% identity). ENPP4 and ENPP5 contain only two introns, but these correspond to introns 13 and 15 in ENPP1-3, providing additional evidence for their common ancestry.

1. NPP1

This is by far the best characterized NPP-isozyme. NPP1 was initially discovered as a surface marker of antibody-secreting B-lymphocytes, hence the name Plasma-Cell differentiation antigen-1 or PC-1 (Takahashi et al., 1970). It is now known, however, that NPP1 is expressed in a large variety of tissues (see Section IV). NPP1 from bovine liver was also independently identified as a protein that co-purifies with the aFGF receptor and binds aFGF, hence the name MAFP, for Major aFGF-stimulated Protein (Oda et al., 1991).

2. NPP2

NPP2α or autotaxin was discovered as a glycoprotein that is secreted by the human melanoma cell line A2058 and stimulates the motility of these cells at subnanomolar concentrations (Stracke et al., 1992). More recently, a splice-variant, now termed NPP2β, was cloned from human teratocarcinoma (Lee et al., 1996b) and human retina cDNA libraries (Kawagoe et al., 1995). NPP2β differs from NPP2α by the absence of 52 residues in the central domain. A rat brain isoform of NPP2 has been cloned that is likely to represent still another splice variant (NPP2γ), because it differs from NPP2β by the presence of an additional 25 residues in the C-terminal third of the polypeptide (Narita et al., 1994).

3. NPP3

NPP3 was initially described as gp130^{RB13-6}, that is, a plasma-membrane associated glycoprotein of 130 kDa that is recognized by the monoclonal antibody RB13-6. This antibody visualizes a subpopulation of neural precursor cells in prenatal rat brain that are sensitive to malignant conversion by *N*-ethyl-*N*-nitrosourea (Deissler et al., 1995). More recently, it has been reported that the latter protein is identical to B10, a membrane protein on the apical surface of rat hepatocytes (Scott et al., 1997). Likewise, the rat intestinal NPP (Scott et al., 1997) and a human prostate NPP (Jin-Hua et al., 1997) were identified as NPP3.

4. Novel Mammalian NPP Isozymes

NPP4 and NPP5 have been described as putative nucleotide pyrophosphatases/phosphodiesterases based on their homology with the catalytic domain of NPP1-3 (Gijsbers et al., 2000a; Figure 1). All residues that are known to be essential for the catalytic activity of NPP1-3 are conserved in NPP4-5, but whether the latter display a true NPP activity remains to be established.

NPP1-5 are known or expected to be integral membrane proteins (see Section IV). In some tissues, however, a fraction of the NPP activity can be released by incubation with phosphatidylinositol phospholipase C (Nabakayashi and Ikezawa, 1986; Nakabayashi et al., 1994). Unless this release is accounted for by proteases that contaminate the phospholipase C preparation, these data indicate that the released NPP activity stems from enzyme(s) that are anchored to the membrane via glycosylphosphatidylinositol. Thus, it is possible that there also exist ecto-NPPs without a transmembrane domain. The latter may represent either novel NPPs or enzymes that are generated by alternative processing of the NPP1-5 transcripts or proteins. Evidence has also been presented for the existence of cytosolic species of NPP in tumor cells (Fukazawa et al., 1988) and in liver (Canales et al., 1995), but the nature of these enzymes has not been explored.

B. NPPs in Plants, Lower Eucaryotes, and Prokaryotes

Homology searches revealed the existence of proteins, with an origin ranging from bacteria to plants, that share a very significant structural similarity with the catalytic domain of mammalian NPPs (Gijsbers et al., 2000a; Figure 2). This similarity includes both the primary structure and the predicted fold. Also, the catalytic-site residue and all residues known to be involved in the binding of metals in NPP1-3 are conserved in these putative NPPs (Figure 2). Recently, it has also been reported that the fowlpox-virus genome encodes a protein that is similar to NPP1 (Laidlaw et al., 1998). RNA encoding the viral homolog of NPP1 was shown to be strongly expressed early and late in infection. Yet, the virally encoded NPP1 was catalytically inactive (Anwar and Skinner, 2000), in agreement with the lack of conservation of three out of six residues that are required for the coordination of metals in the catalytic site of NPP1 (Figure 2).

IV. TISSUE AND SUBCELLULAR DISTRIBUTION

NPP1-5 show a distinct but partially overlapping tissue distribution, as revealed by various immunological approaches, by northern analysis, and by EST analysis (Table 2). The expression pattern of the NPP isozymes is also remarkably species-dependent (not shown), which is in agreement with the striking lack of conservation of the 5′-flanking promotor sequences of ENPPs (Andoh et al., 1999). Judged from the relative abundance of NPP-related ESTs, NPP4-5 seem to be expressed at rather low levels, which may account for their late discovery (Gijsbers et al., 2000a).

NPP1-3 are integral proteins of the plasma membrane. In polarized cells their presence is restricted to specialized areas of the plasma membrane. Thus, in rat hepatocytes NPP1 is mainly located on the basolateral surface, while NPP3 is primarily confined to the apical surface (Scott et al., 1997). A fraction of

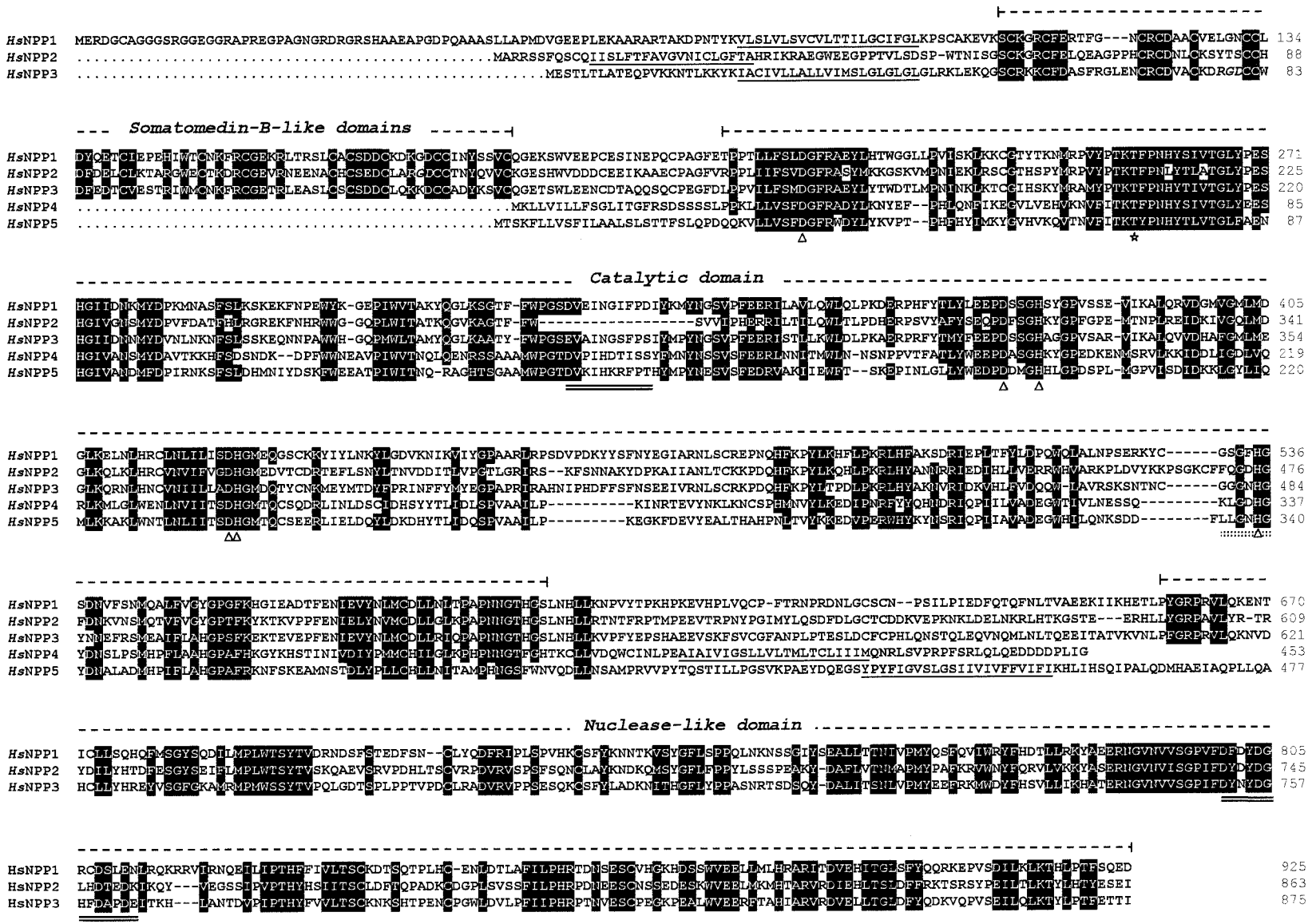

FIGURE 1. Aligment of human NPP1-5. The protein sequences were initially aligned using CLUSTAL X (Gijsbers et al., 2000a), and the alignments were subsequently refined manually. Conserved and identical residues are boxed. The dashed lines on top of the sequence delineate the functional domains that are discussed in Section V. The double dotted line denotes the GXGXXG-sequence described in Section V.C. The single-underlined sequences indicate the transmembrane segments. The double-underlined sequences represent the putative (variant) EF hand motifs discussed in Section V.D. Italic RGD motifs refer to putative integrin-binding sites. The numbers indicate the position of amino acids in the protein. ☆, catalytic-site residue. ∆, putative metal binding residues (see also Figure 5). For NPP1, the first AUG of the transcript was used as start codon (Belli and Goding, 1994a). The accession numbers of the listed proteins are AAF36094 (*Hs*NPP1), BAA08260 (*Hs*NPP2), AAC51813 (*Hs*NPP3), BAA74902 (*Hs*NPP4) and CAB56566 (*Hs*NPP5).

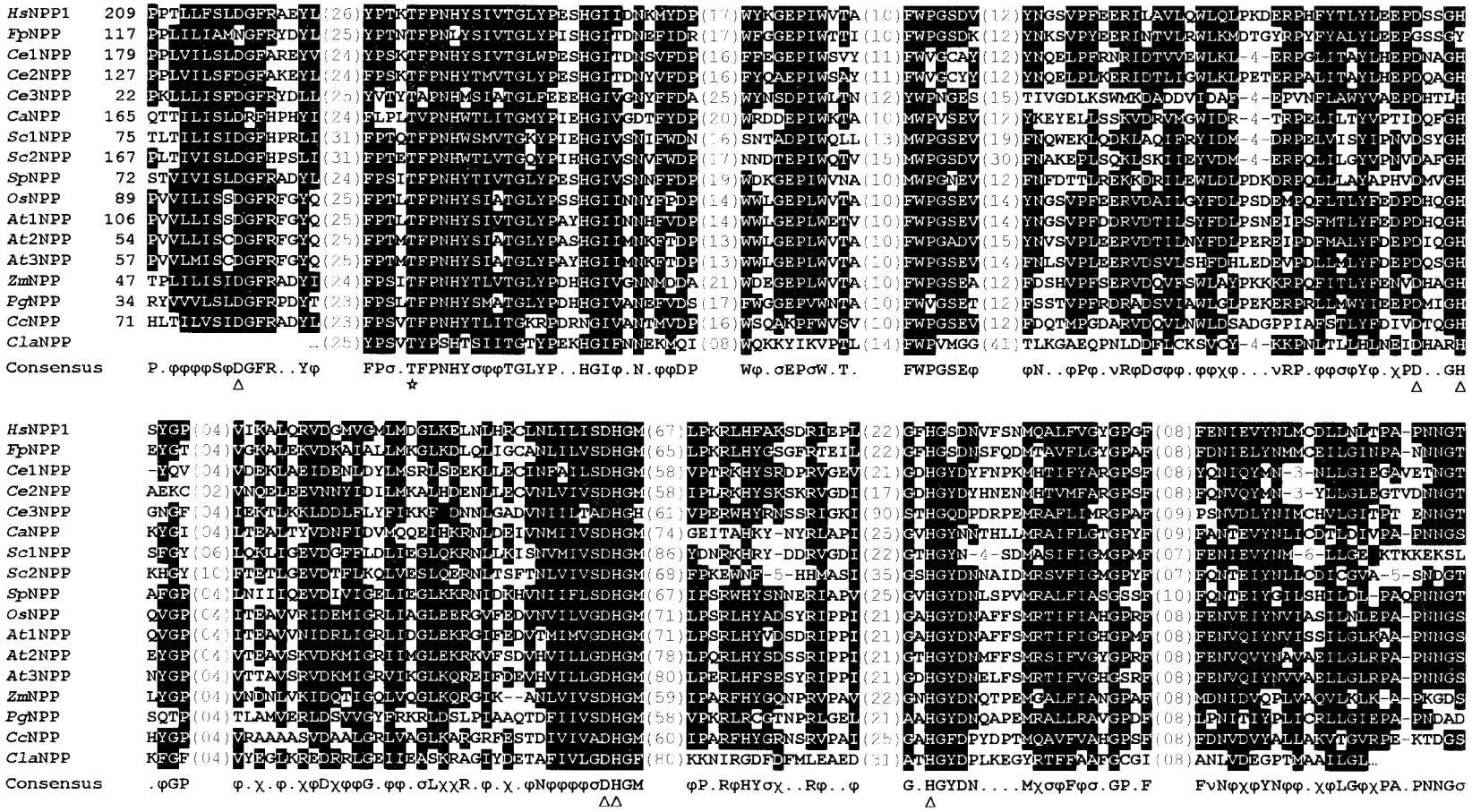

FIGURE 2. Phylogenetic conservation of the catalytic domain of NPPs. The alignments were prepared as indicated in the legend of Figure 1. The regular numbers indicate the distances to the beginning and the end of each protein, and the numbers in parenthesis indicate the sizes of the gaps between the aligned segments. The figure also shows an NPP 'consensus sequence': χ, charged residue (D, E, K, R, N, Q); φ, bulky hydrophobic residue (I, L, V, M, F, Y); σ, small residue (G, A, S, T); ν, acidic residues (D, E); ., no particular residue. ☆, catalytic-site threonine. Δ, putative metal binding sites. The Genbank accession numbers for the enlisted cDNA's or protein sequences are *Hs*NPP1, *Homo sapiens* NPP1 (D12485); *At*1NPP, *Arabidopsis thaliana* 1 NPP (CAB45328); *At*2NPP (CAB45329); *At*3NPP (CAB45330); *Ce*1NPP, *Caenorhabditis elegans* NPP (CAB02784); *Ce*2NPP (CAB02785); *Ce*3NPP (AAC47919); *Fp*NPP, Fowlpox virus (CAA07014); *Os*NPP, *Oryza sativa* NPP (U25430); *Sp*NPP, *Schizosaccharomyces pombe* NPP (CAA22177); *Sc*1NPP, *Saccharomyces cerevisiae* NPP (AAB64493); *Sc*2NPP (P25353); *Zm*NPP, *Zymomonas mobilis* NPP (AAC70363). The sequence of *Ca*NPP (*Candida albicans* NPP), *Cc*NPP, (*Caulobacter crescentus* NPP), *Cla*NPP (*Clostridium acetobutylicum* NPP) and *Pg*NPP (*Porphyromonas gingivalis* NPP) were derived from preliminary sequence data (http://genome-www.stanford.edu and http://www.tigr.org).

TABLE 2
Tissue Distribution of Human NPPs

Tissues	*Hs*NPP1	*Hs*NPP2	*Hs*NPP3	*Hs*NPP4	*Hs*NPP5
Aorta	·	+	·	+	+
Bone	·	+	+	+	·
Brain	+	+	+	+	·
Cartilage	+	·	+	·	·
Colon	+	+	+	+	·
Heart	+	+	·	+	+
Kidney	+	+	+	+	+
Liver	+	·	+	+	·
Lung	·	+	+	+	·
Ovary	·	+	·	+	·
Pancreas	+	+	+	+	·
Parathyroid	+	·	·	+	+
Placenta	+	+	+	·	·
Prostate	+	+	+	+	+
Skeletal Muscle	+	·	·	+	·
Skin	+	·	·	+	·
Small Intestine	+	+	+	·	·
Spleen	+	+	·	·	·
Stomach	·	+	+	·	·
Testis	+	+	+	+	+
Thymus	+	·	+	+	·
Uterus	+	+	+	+	·

Note: The table was constructed using data mainly obtained by Northern blotting, Western analysis and/or RT-PCR (Morley et al., 1987; Yano et al., 1987; Buckley et al., 1990; Huang et al., 1993; Kawagoe et al., 1995; Frittitta et al., 1996; Lee et al., 1996b; Jin-Hua et al., 1997; Nagase et al., 1998; Andoh et al., 1999; Johnson et al., 1999; Frittitta et al., 2000). In other cases the expression pattern was derived from EST-database analysis (Unigene clusters Hs.11951; Hs.174185; Hs.264750; Hs.54037; Hs.247901). +, indicates expression, ., indicates no expression has been reported (yet).

NPP1 is associated with the endoplasmic reticulum (Hickman et al., 1985; Huang et al., 1993; Rebbe et al., 1993; Stefan et al., 1998), which may simply represent the pool of NPP1 that is on its way to the cell surface. In addition, there is evidence for the existence of active, secreted forms of NPP1-3. Soluble forms of NPP1 have been identified in mouse serum, in the medium of cells that had been transfected with an expression vector for the membrane form of NPP1 (Belli et al., 1993; Frittitta et al., 1999; Hosoda et al., 1999), and in snake venom (Culp and Butler, 1986). As surface radio-iodinated NPP1 was not released into the medium, it was proposed that soluble NPP1 had been generated by the intracellular proteolytic cleavage of the membrane form (Belli et al., 1993). NPP2 was first identified as a soluble, secreted protein (Stracke et al., 1992) that originates from proteolytic cleavage of the membrane form (Murata et al., 1994; Hosoda et al., 1999). A soluble form of NPP3 accounts for 13% of the total NPPase in rat serum, and this level increases substantially following bile duct ligation and during the development of cholangiocarcinoma (Meerson et al., 1997).

V. NPPs ARE MODULAR PROTEINS

The membrane forms of NPP1-3 are disulfide-linked homodimers with a proven type-II orientation, that is, they consist of a

short N-terminal intracellular domain, a single transmembrane domain, and a large extracellular C-terminal domain (Figure 3). By contrast, NPP4-5 are predicted by the MEMSAT2 (Jones et al., 1994) and TMHMM programs (Sonnhammer et al., 1998) to have a C-terminal transmembrane domain and to display a type I orientation, which would imply that they are also ectoenzymes (Figure 3).

The extracellular part of NPP1-3 consists of two consecutive somatomedin-B-like domains, a catalytic domain, and an hitherto unrecognized C-terminal nuclease-like domain (Figures 1 and 3). NPP4-5, as well as the putative NPPs from lower eukaryotes and prokaryotes, lack somatomedin-B-like and nuclease-like domains, which accounts for their much smaller size (Figures 1 and 3).

NPP1-5 contain 4 to 10 consensus sequences (Asn-X-Ser/Thr) for *N*-linked glycosylation (Hofmann et al., 1999; Gijsbers et al., 2000b). In accordance with the occurrence of *N*-glycosylation *in vivo*, the electrophoretic mobility of NPP1-3 is lower than expected from their calculated mass, and a treatment with glycosidases increases their electrophoretic mobility (Rebbe et al., 1991; Uriarte et al, 1993; Belli and Goding, 1994; Stracke et al., 1995; Scott et al, 1997; Hosoda et al., 1999). The biological role of the *N*-linked glycosylations of NPPs remains to be established. It has been reported though that the removal of the oligosaccharide side chains does not interfere with the function of NPP2 as a motility factor (Stracke et al., 1995).

A. The Cytoplasmic Domain

The intracellular domain of NPPs, which is N-terminal for NPP1-3 and presumably C-terminal for NPP4-5 (Figure 3), is rather short (11 to 77 residues) and is different for each isozyme. Bello et al. (2000) showed that the basolateral expression of mouse NPP1 in MDCK kidney cells was lost by the deletion of the first 34 residues, suggesting that the cytoplasmic domain is involved in its polarized targeting. The deleted fragment comprised a conserved leucine-leucine dipeptide motif, which may represent a consensus sequence for targeting to the basolateral surface of polarized cells (Pond et al., 1995; El Nemer et al., 1999). Bello et al. (2000) also noted that fusions of various proteins with the cytoplasmic plus transmembrane domains of NPP3 were all apically expressed, indicating that these domains of rat NPP3 mediate its targeted expression. Interestingly, the polarized expression of rat NPP3 may be regulated by phosphorylation of Ser17, a consensus phosphorylation site for protein kinase A, as the S17A mutant was expressed both on apical and on basolateral surfaces, while the S17D mutant and the wild type were mainly apical (Meerson et al., 2000).

In addition to a targeting role, it is also tempting to speculate on a role of the cytoplasmic domain in 'inside-out' signaling. For example, the cytoplasmic domain may bind intracellular (macro)molecules, which affect the activity of the ecto-enzyme.

B. The Somatomedin-B-Like Domains

Somatomedin-B is a serum peptide that was initially believed to be a growth factor. However, it is now known that its mitogenic activity was derived from contaminating epidermal growth factor (Heldin et al., 1981). Somatomedin-B is proteolytically derived from vitronectin, a serum and extracellular-matrix protein that is involved in cell adhesion (reviewed by Preissner, 1991;

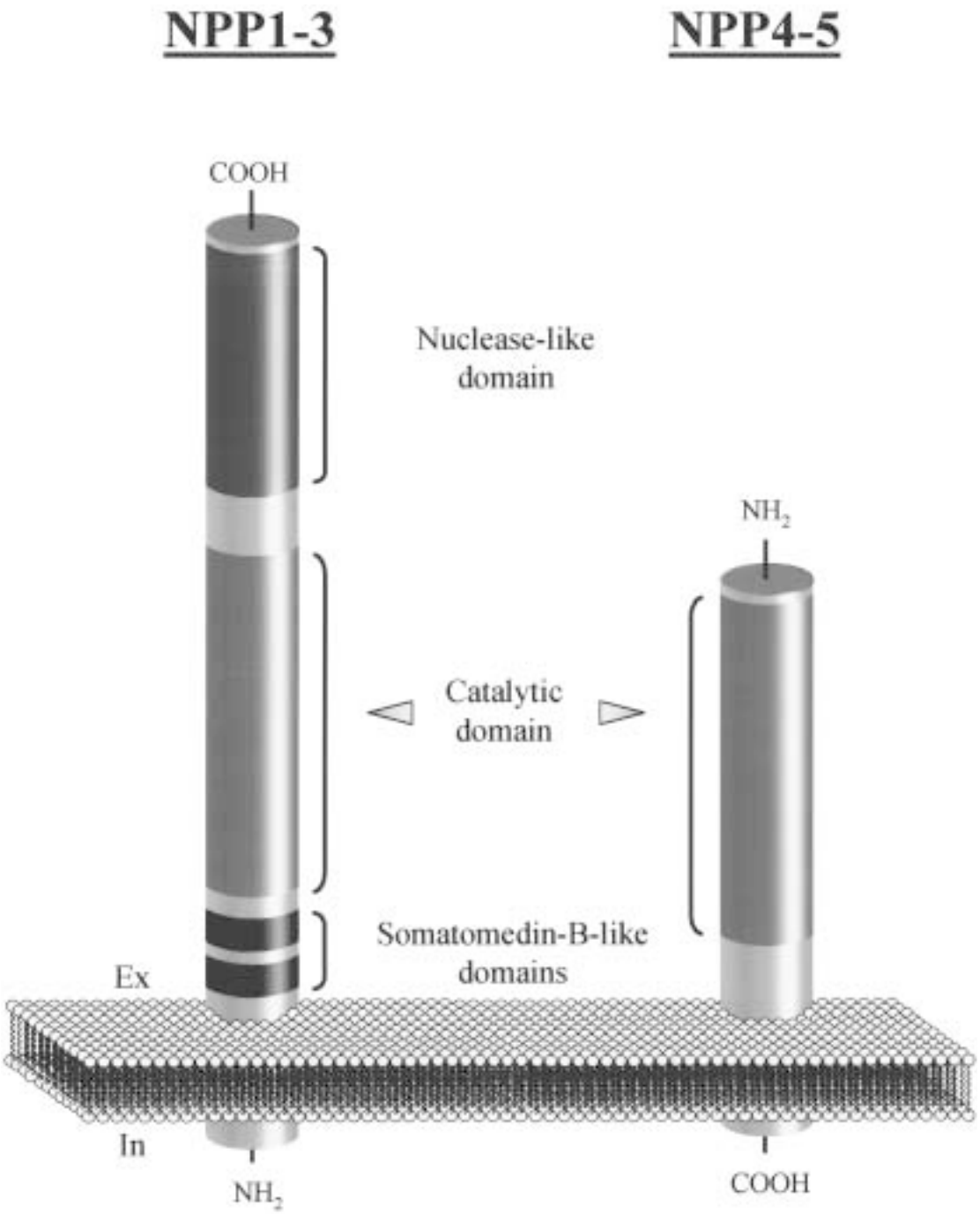

FIGURE 3. Domain structure and membrane orientation of NPP1-5. In, intracellular; Ex, extracellular. For simplicity it is not indicated that NPP1-3 are actually homodimers.

Schvartz et al., 1999). In contrast to somatomedin-B itself, the somatomedin-B-like domains in NPP1-3 do not display any affinity for the plasminogen-activator inhibitor-1 (Deng et al., 1996). Vitronectin contains close to its somatomedin-B domain an RGD-tripeptide motif that mediates the interaction with integrin receptors (Preissner, 1991; Schvartz et al., 1999). Similarly, NPP2 and NPP3 contain an RGD motif in the second and the first somatomedin-B-like domain, respectively (Figure 1), which may mediate an interaction with integrins. It is also worthy of note that the somatomedin-B-like domains are cysteine rich and therefore may mediate the dimerization of the NPPs via disulfide bonds. Cysteine-rich regions close to the extracellular surface are indeed often involved in the dimerization of membrane proteins (Geisow, 1986).

C. The Catalytic Domain

Using various bioinformatics tools, it recently emerged that the catalytic domain of NPPs is structurally related to that of a superfamily of phospho-/sulfo-coordinating metalloenzymes that include arylsulfatases, phosphopentomutases, 2,3-bisphosphoglycerate-independent phosphoglycerate mutases, and alkaline phosphatases (Galperin et al., 1998; Gijsbers et al, 2000a). Within this superfamily, the E-NPP family was positioned intermediate between arylsulfatases and alkaline phosphatases (Gijsbers et al., 2000a). Because the catalytic domain of the latter enzymes assumes a basically identical fold, that is, a β-sheet sandwiched between α-helices, it seems likely that the catalytic domain of NPPs would adopt the same fold (Figure 4A). Strikingly, a rough structural model of the catalytic site of NPPs suggested that the metal-binding and active-site residues are superposed onto those of alkaline phosphatases and the phosphoglycerate mutases (Gijsbers et al., 2000a). Thus, the six residues that are known to coordinate the two metals in the catalytic site of alkaline phosphatases and phosphoglycerate mutases are conserved in NPPs (Figure 4A) and were predicted to display a similar spatial arrangement relative to the residue that forms the catalytic intermediate, which is a Thr in NPPs (Gijsbers et al., 2000a). As predicted, a mutation of each of these residues in mouse NPP1 indeed (nearly) abolished the enzymic activity but the activity could be partially or largely restored by the addition of a large excess of a divalent cation like Zn^{2+}.

NPP1 and NPP3 contain in their catalytic domain the motif GXGXXG (Figure 1), which represents a consensus sequence for the binding of dinucleotides (Bossemeyer, 1994). The importance of this motif for catalysis is underscored by findings that mutation of these three glycines into alanine abolished the nucleotide phosphodiesterase activity of NPP1 (Gijsbers et al., 2000b). The GXGXXG motif is not conserved in NPP2. However, photoaffinity labeling and peptide sequencing has revealed that NPP2 contains, in addition to the catalytic site, an isozyme-specific sequence that can be crosslinked to nucleotides (Clair et al., 1997a).

D. The Nuclease-Like Domain

A structural analysis also disclosed striking similarities between the carboxyterminal domain of NPP1-3 (ca. 250 residues) and a family of DNA/RNA non-specific endonucleases (Gijsbers et al., 2000b). The similarities include both the primary structure and the predicted fold (Figure 4B). According to this model the nuclease-like domain of NPP1-3 consists of a β-sheet that is sand-

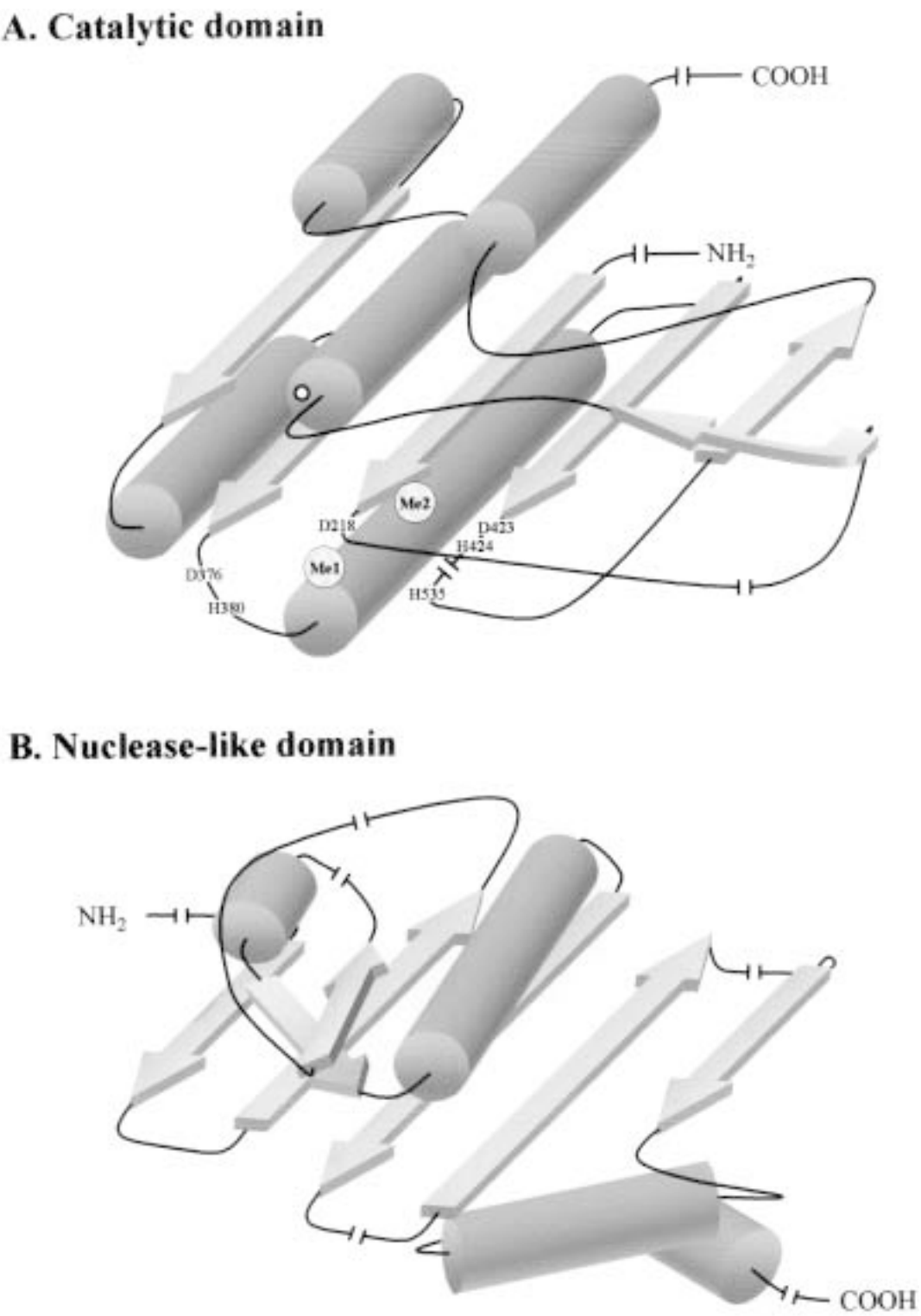

FIGURE 4. Model of the catalytic and nuclease-like domains of human NPP1. The primary structure of the catalytic domain of NPP1 was threaded onto the known 3D-structure of the catalytic domain of alkaline phosphatase (PDB code: 1ALK), taking into consideration the conserved secondary structure elements. Panel A shows the predicted constellation of the metals (Me), the metal-binding residues (numbers) and the catalytic-site threonine (O) of NPP1 (see also Gijsbers et al., 2000a). Likewise, a model of the nuclease-like domain of NPP1 was obtained (panel B) by threading the primary structure of the nuclease-like domain of NPP1 onto the 3D-structure of the *Serratia marcescens* nuclease (PDB code: 1SMN).

wiched between α-helices and two crossed β-strands.

It is not really surprising that NPPs and nonspecific endonucleases have common structural features because they both catalyze a similar reaction.* It is rather unexpected, however, that the nuclease-like domain lies outside the catalytic domain of NPPs. It is highly improbable that NPP1-3 contains a second catalytic site in their nuclease-like domain because none of the residues that are essential for catalysis by endonucleases are conserved in NPPs (not illustrated). We speculate that the nuclease-like domain of NPP1-3 functions as a substrate-binding or substrate-specifying region.

The nuclease-like domain of NPP1-3 contains the sequence DXD/NXDGXXD (Figure 1), which has been proposed to represent an EF hand (Belli et al., 1994; Andoh et al., 1999), a Ca^{2+}-binding motif that is also present in proteins such as calmodulin and parvalbumin. However, while the DXD/NXDGXXD sequence appears to be essential for the enzymic activity of NPP1 (Gijsbers et al., 2000b) and NPP3 (Andoh et al., 1999), it is very unlikely that this motif acts as an EF hand. Indeed, a classic EF hand represents an helix-loop-helix structure, and the DXD/NXDGXXD sequence only corresponds to the loop-region, which is not sufficient for the binding of Ca^{2+} (Strynadka and James, 1989). Also, the sequence immediately N-terminal to the DXD/NXDGXXD-motif was predicted to assume a β-strand conformation (Gijsbers et al., 2000b). Another candidate region for the binding of Ca^{2+} resides in the catalytic domain of NPP1 (Figure 1) and closely matches some of the critical residues of the 'variant' EF hand of the bovine intestinal Ca^{2+}-binding protein (Belli et al., 1994). The importance of this variant EF hand motif can be questioned, however, because it is not conserved in NPP2-5. The conformational stabilization of NPP1 by Ca^{2+} has been presented as evidence for the presence of a functional (variant) EF hand in NPP1 (Belli et al., 1994). However, a similar stabilization was also offered by Zn^{2+} and Mg^{2+} (Belli et al., 1994), which do not bind to an EF hand. An alternative explanation for the stabilization of NPP1 by divalent cations is saturation of the two metal-binding sites near the catalytic site (Figure 4A; Section V.C).

VI. ENZYMIC PROPERTIES

A. The Nucleotide Pyrophosphatase/ Phosphodiesterase Reaction

1. Catalytic Mechanism

Inasmuch as the catalytic sites of NPPs, alkaline phosphatases and phosphoglycerate mutases appear to have a very similar structural arrangement (Section V.C), this also suggests a common mechanism of catalysis. Transposition of the well-understood catalytic mechanism of alkaline phosphatases and phosphoglycerate mutases to NPPs yields a fairly detailed picture of the NPP reaction (Gijsbers et al., 2000a; Figure 5). It is proposed that the catalytic site of NPPs contains two essential divalent metals (Me) and that catalysis occurs by a two-step mechanism. In the first step, the Me2-activated hydroxyl of the catalytic-site threonine (T256 in human NPP1) attacks the phosphate of the incoming substrate, resulting in the generation of a covalent, nucleotidylated intermediate. In the second

* DNA/RNA nonspecific endonucleases produce 5′-phosphorylated oligonucleotides (Friedhoff et al., 1994), while NPPs generate 5′-phosphorylated mononucleotides (see Section VI.A).

catalytic step, a Me1-activated water molecule attacks this E-NMP intermediate, regenerating the catalytic site threonine and releasing a nucleoside 5'-monophosphate.

Several lines of experimental evidence support the reaction scheme proposed in Figure 5. First, it has been demonstrated that NPPs are metalloenzymes as their activity is blocked by metal chelators but can be restored by the addition of divalent cations such as Mg^{2+}, Mn^{2+}, $Ca^{2+,}$ or Zn^{2+} (Yano et al., 1985; Rebbe et al., 1991; Oda et al., 1993; Belli et al., 1994b; Gijsbers et al., 2000a). Second, the catalytic site threonine has been shown to form a nucleotidylated intermediate (Culp et al., 1985; Clair et al., 1997b; Stefan et al., 1996). Third, mutation of the catalytic site threonine or of either of the six residues predicted to be involved in the binding of Me1 or Me2 (Figure 5) essentially abolished the NPPase activity of NPP1 (Gijsbers et al., 2000a). Moreover, the effect of mutation of residues involved in the coordination of metals could be partially reversed by the addition of metals. Fourth, mutation of residues predicted to be involved in the second step of catalysis (corresponding to D376 and H380 in human NPP1) resulted in an enzyme that could still form the covalent intermediate. By contrast, the catalytic intermediate could not be trapped following mutation of residues (D218, D423, H424) that were predicted to be essential for the first catalytic step. Finally, the proposed reaction mechanism (Figure 5) provides an explanation for the alkaline pH optimimum of NPPs. By analogy with what is known for alkaline phosphatases (Holtz and Kantrowitz, 1999), it is suggested that an alkaline pH promotes the ionization of the Me1-coordinated H_2O, which acts as a nucleophile in the hydrolysis of the nucleotidylated intermediate (Figure 5). At acidic pH values this second step of catalysis becomes rate-limiting, resulting in a trapping of the catalytic intermediate (Landt and Butler, 1978).

2. Substrate Specificity

Members of the E-NPP family generally have a much broader substrate specificity than intracellular pyrophosphatases and phosphodiesterases. They hydrolyze various phosphodiester bonds (e.g., in oligonucleotides and artificial substrates like the *p*-nitrophenyl ester of TMP) and pyrophosphate bonds (e.g., in (d)NTP, (d)NDP, NAD, FAD, di-adenosine polyphosphates and UDP sugars) and thereby generate nucleoside 5'-monophosphates (Figure 6). NPPs are also able to hydrolyze the sulfate-phosphate bond in 3'-phosphoadenosine 5'-phosphosulfate. The latter as well as the *p*-nitrophenyl ester of TMP (Figure 6) are used routinely for the *in vitro* assay of NPPs. The NPP reaction is characterized by a K_m of 50 to 500 μ*M* and a V_{max} of 5 to 300 μmol/min/mg enzyme (Kelly et al., 1975; Hosoda et al., 1999). A notable exception are diadenosine polyphosphates, which have been reported to be hydrolyzed by NPP1 with a K_m of only 2 μ*M* and a V_{max} that is some 50-fold lower than that for hydrolysis of the *p*-nitrophenyl ester of TMP (Gasmi et al., 1998). Conversely, Kelly et al. (1975) showed that NPPs efficiently hydrolyze phosphonate esters (V_{max} = ca. 700 μmol/min/mg enzyme), but they have a very low affinity for these substrates (K_m = ca. 10 μ*M*). The high K_m for the hydrolysis of phosphonate esters can possibly be accounted for by the lack of a nucleoside phosphate moiety, which has been proposed to increase the affinity of NPPs for their substrates (Gijsbers et al., 2000a).

3',5'-cAMP has been reported to be either no substrate (Bischoff et al., 1975; Picher and Boucher, 2000) or a poor sub-

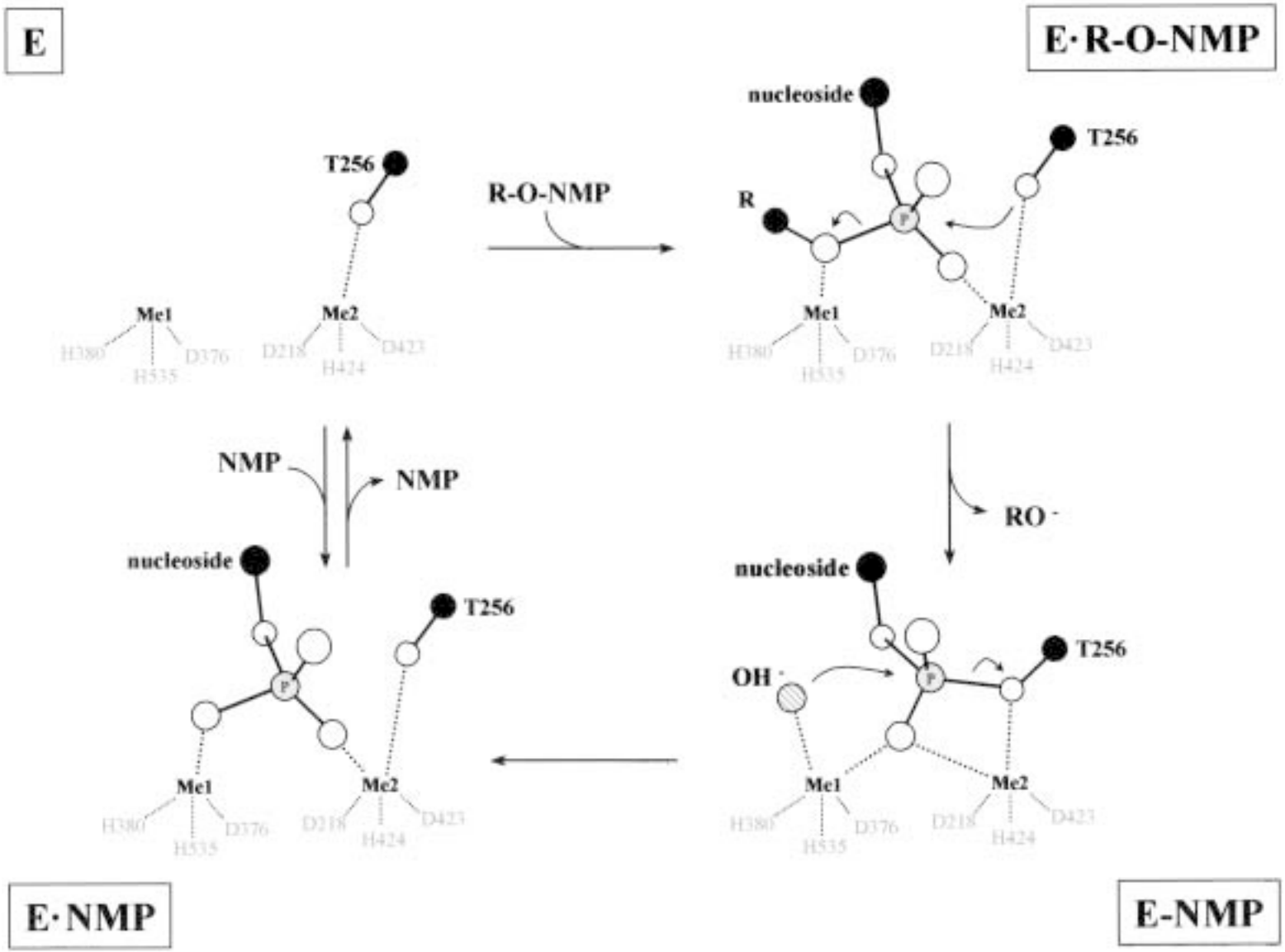

FIGURE 5. Mechanism of the nucleotide pyrophosphatase/phosphodiesterase reaction. The open circles represent OH or O groups. R refers to various substrate components such as a nucleoside monophosphate or a (pyro)phosphate moiety. In the free enzyme (**E**) the hydroxyl group of the catalytic-site threonine (T256 in human NPP1) is stabilized by Me2 in its nucleophilic state. The enzyme-substrate complex (**E·R-O-NMP**) is formed through coordination of the NMP-ester oxygen atoms by Me1 and additional interaction of one of the non-bridging oxygen atoms of the substrate with Me2. T256 holds a position opposite to the leaving group of the NMP-ester substrate. A nucleophilic attack of the hydroxyl group of T256 results in the formation of the covalent, nucleotidylated intermediate (**E-NMP**), departure of the alcohol leaving-group and inversion of the phosphorus center. At alkaline pH a water molecule coordinated by Me1 attacks the phosphorus apically, resulting in the hydrolysis of the nucleotidylated intermediate, inversion of the phosphorus center and the formation of a non-covalent enzyme-NMP complex (**E·NMP**). The free enzyme is regenerated by dissociation of NMP (nucleoside monophosphate). Also shown are the residues that coordinate Me1 and Me2 (see also Figures 1 and 2). (Modified after Gijsbers et al. [2000a].)

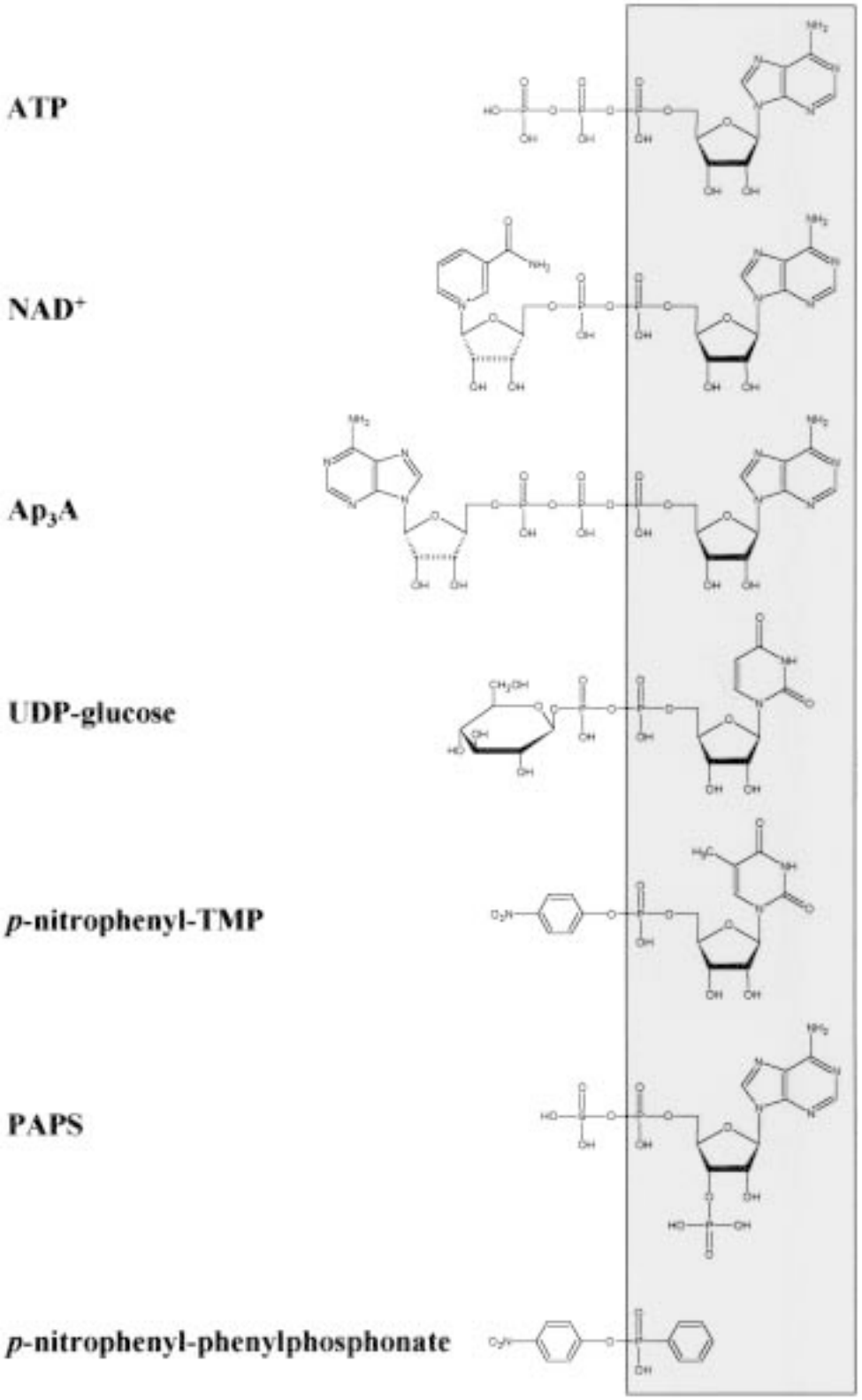

FIGURE 6. Some substrates of NPPs. The grey box highlights the moieties (usually a nucleoside 5′-phosphate) that are covalently attached to the catalytic-site threonine. Based on data from Razell, 1963; Kelly et al., 1975; Blytt et al., 1985; Yano et al., 1985 and 1987; Byrd et al., 1985; Oda et al., 1991 and 1993; Funakoshi et al., 1992; Belli et al., 1993; Rebbe et al., 1993; Lee et al., 1996a; Gasmi et al., 1998. PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

strate of NPPs (Kelly et al., 1975), which may be explained by sterical constraints at the catalytic site caused by the cyclic phosphodiester bond. AMP is not a substrate for NPPs (Bischoff et al., 1975; Yano et al., 1985; Uriarte et al., 1995), as expected from the absence of phosphodiester or pyrophosphate bonds. Reports indicating that NPPs can convert AMP into adenosine and P_i (Uriarte et al., 1993; Clair et al., 1997b) may be accounted for by contamination of the NPP preparations with 5'-nucleotidase, as was shown for NPP1 purified from rat liver (Uriarte et al., 1995). On the other hand, AMP competitively inhibits the NPP reaction (Landt and Butler, 1978; Gijsbers et al., 2000b) and AMP-Sepharose has been used to purify NPPs (Bischoff et al., 1975; Yano et al., 1985), suggesting that NPPs contain an AMP-binding site. Probably AMP occupies the catalytic site as a product of the NPP reaction, in agreement with kinetic evidence that NPPs bind their products more strongly that they bind their substrates (Moe and Butler, 1983).

3. Effectors

NPPs are competitively inhibited by substrates and products of the NPP reaction (Decker and Bishoff, 1972; Moe and Butler, 1983) as well as by the glycosaminoglycans heparin and heparin sulfate (Hosoda et al., 1999). Reducing agents such as dithiothreitol or 2-mercaptoethanol are also inhibitory to NPP1 (Yano et al., 1985; Uriarte et al., 1995). As the NPP monomers are catalytically active (Hosoda et al., 1999), the inhibition by reducing agents must be due to a disruption of disulfide bridges within the NPP monomers. The acidic fibroblast growth factor was reported to be a potent inhibitor of NPP1 (Uriarte et al., 1995), but later investigations revealed that this inhibition was caused by contaminating EDTA (Stefan et al., 1997; López-Gómez et al., 1998). Imidazole is an uncompetitive inhibitor of the NPP reaction, implicating that it only binds to the substrate-bound enzyme (Moe and Butler, 1983). Accordingly, imidazole has been used to trap the covalent intermediate of NPP1 (Blytt et al., 1985; Stefan et al., 1996), suggesting that it specifically blocks the second step of catalysis. Possibly, imidazole acts as an histidine analog and specifically chelates Me1 (Figure 5). The histidine acylator diethylpyrocarbonate was also shown to block NPP1 (Moe and Butler, 1983; Gijsbers et al., 2000a), in keeping with the proposed roles of histidines in coordinating Me1 and Me2 (Figure 5).

B. Auto(de)phosphorylation

1. Initial Observations

An incubation of purified NPP1 (Oda et al., 1993; Uriarte et al., 1995) or NPP2 (Lee et al., 1996a; Clair et al., 1997b) with [γ-^{32}P]-labeled ATP results in a substoichiometric autophosphorylation of the catalytic-site threonine. An autophosphorylation of NPP1 has also been demonstrated in COS cells transfected with an expression vector for this isozyme (Belli et al., 1995). Autophosphorylation of a calf intestinal NPP, which presumably represents NPP3, could only be observed at very high enzyme concentrations (Gijsbers et al., 2000b), which may explain previous failures to detect an autophosphorylation of this preparation (Stefan et al., 1996a).

The autophosphorylation of NPP1 only occurs at low, (sub)micromolar ATP concentrations (Uriarte et al., 1995; Stefan et al., 1996a). At higher ATP concentrations

an autophosphorylation of NPP1 is only seen after a latency during which most of the ATP is hydrolyzed into adenosine and P_i by the combined action of NPP1 and contaminating or exogenously added 5′-nucleotidase. The autophosphorylation of NPP1 is transient and is followed by an autodephosphorylation. Inasmuch as the autodephosphorylation is stimulated by nucleotides, the lag period before phosphorylation at higher nucleotide concentrations has been explained by simultaneous autocatalytic phosphorylation and dephosphorylation, which prevents a net phosphorylation as long as the nucleotide concentration is sufficiently high to enable the phosphatase activity to nullify the kinase activity (Uriarte et al., 1995). In agreement with this interpretation, the length of the latency before phosphorylation was found to increase with the initial ATP concentration and with the dilution of NPP1 (Uriarte et al., 1993 and 1995).

Clair et al. (1997b) suggested that the autophosphorylation of NPP2 actually represents the catalytic intermediate of an NPP2-associated ATPase activity. Inspired by the ion-translocating 'P'-type ATPases, which form covalent phosphorylated intermediates by the transfer of the γ-phosphate from ATP to an aspartic acid in the catalytic site (Pedersen and Carafoli, 1987), Clair et al. (1997b) proposed that NPP2 was the first member of a novel class of ATPases that they termed the 'T'-type ATPases, where 'T' refers to the phosphorylated catalytic-site threonine.

2. Does Auto(de)phosphorylation Result from An Intrinsic Phosphatase Activity?

Recently, Gijsbers et al. (2000a) demonstrated that the autophosphorylation of NPP1 can also be observed with [β-^{32}P]ADP as substrate. This proves that the autophosphorylation cannot be due to a classic kinase or ATPase reaction, which use ATP as substrate. Based on the similarities between the catalytic site of NPPs and alkaline phosphatases (see Section V.C), it was proposed that the ability of NPPs to auto(de)phosphorylate is a reflection of an intrinsic alkaline phosphatase activity, with the autophosphorylated enzyme representing the covalent phospho-intermediate (Gijsbers et al., 2000a). Alkaline phosphatases are indeed known to act via a phosphorylated intermediate (Holtz and Kantrowitz, 1999) and they can also use ADP and ATP as substrates (Gijsbers et al., 2000a). Moreover, the mutation in mouse NPP1 of residues that are essential for the binding of Me1 and Me2 (Figure 5) abolished both the NPP and the autophosphorylation reaction, suggesting that both activities are catalyzed by the same catalytic site (Gijsbers et al., 2000a). The proposal that phosphorylated NPPs represent catalytic intermediates of a phosphatase reaction also accounts for (1) the low metal requirement of the 'autophosphorylation' reaction (Oda et al., 1993; Uriarte et al., 1995), (2) the low, substoichiometric level of 'autophosphorylation' (Stefan et al., 1996a), and (3) the inhibition of the autophosphorylation and stimulation of autodephosphorylation by vanadate (Uriarte et al., 1995), a phosphate analog and established inhibitor of enzymes that form covalent phospho-intermediates (Holtz et al., 1999).

In retrospect it is not really surprising that NPPs may also display a phosphatase activity because alkaline phosphatases have also been shown to display a nucleotide pyrophosphatase/phosphodiesterase activity (Rezende et al., 1994; O'Brien and Herschlag, 1998). Actually, phosphatases and phosphodiesterases catalyze similar reactions, that is, the hydrolysis of phosphate or nucleotidyl esters, respectively. For NPPs

or alkaline phosphatases to display both enzymic activities, one only has to imply that (some) substrates can be bound in two different ways. For example, with ATP as a substrate for NPPs it is either the α- or the γ-phosphate that will become covalently bound to the catalytic site threonine (Figure 7). The two modes of substrate binding can also explain why the accumulation of the phospho-intermediate, but not the nucleotidiylated intermediate, is blocked by physiological ionic strength (Clair et al., 1997a; Gijsbers et al., 2000b). Indeed, for the phosphatase reaction to take place, the substrate needs to be bound via its phosphate group, initially just via hydrogen bonds and ionic interactions, which are broken by salt. On the other hand, the substrate binding for the phosphodiesterase reaction may also involve the binding of the nucleoside moiety via hydrophobic bonding (Gijsbers et al., 2000a), which is enhanced by salt.

Based on the relative production rates of ADP, AMP, PP_i, and P_i from ATP, it can be calculated that the NPP and phosphatase activities of purified NPP1 and NPP2 are of a similar order of magnitude (Uriarte et al., 1995; Clair et al., 1997b). If anything, the contribution of the phosphatase activity was underestimated in these experiments because ADP, a product of the phosphatase reaction, is also a substrate of the NPP reaction. On the other hand, it is also obvious that these activity ratios are substrate dependent. For example, *p*-nitrophenyl phosphate, which is a model substrate for alkaline phosphatases, is not measurably hydrolyzed by NPP1 (Gijsbers et al., 2000a).

3. Remaining Questions

There are still some observations concerning the multifunctional role of the catalytic site of NPPs that are currently difficult to explain at the molecular level. Thus, it is not clear why only the catalytic intermediate of the NPP reaction is trapped by imidazole (Stefan et al., 1996a). Conversely, nucleotides only promote the hydrolysis of the covalent intermediate of the phosphatase reaction. It is also intriguing that, despite all the structural and catalytic similarities between NPPs and alkaline phosphatases, they have a different catalytic site residue. Moreover, mutation of the catalytic site threonine of NPP1 or NPP2 into a serine, which corresponds to the catalytic site residue in alkaline phosphatases, largely blocks both enzymic activities of these NPPs (Belli et al., 1995; Lee et al., 1996a; Gijsbers et al., 2000a). Further investigations have revealed that the latter mutation in NPP1 does not affect the formation of the phosphorylated or nucleotidylated intermediate but inhibits the subsequent hydrolysis of the intermediate (Gijsbers et al., 2000a). However, the same mutation in NPP2 also blocked the autophosphorylation reaction (Lee et al., 1996a). This discrepancy may possibly be explained by differences in the experimental conditions, for example, an incubation by Lee et al. (1996a) at 0°C, which may have prevented the (less active) mutated NPP2 to degrade ATP to concentrations below the threshold that inhibits autophosphorylation (see above).

NPP1 has been reported to phosphorylate exogenous substrates such as histone IIa or myelin basic protein (Oda et al., 1991; Uriarte et al., 1995). However, this has not been a consistent finding (Belli et al., 1995), and the phosphorylation of the exogenous substrates occurred with rather poor kinetics (Uriarte et al., 1995). We suggest that the phosphorylation of proteins by NPP1 represents a transfer of the phosphate from the covalent intermediate of the phosphatase reaction to specific serine/threonine side chains of these proteins rather than to water. Similarly, it has been demonstrated that the

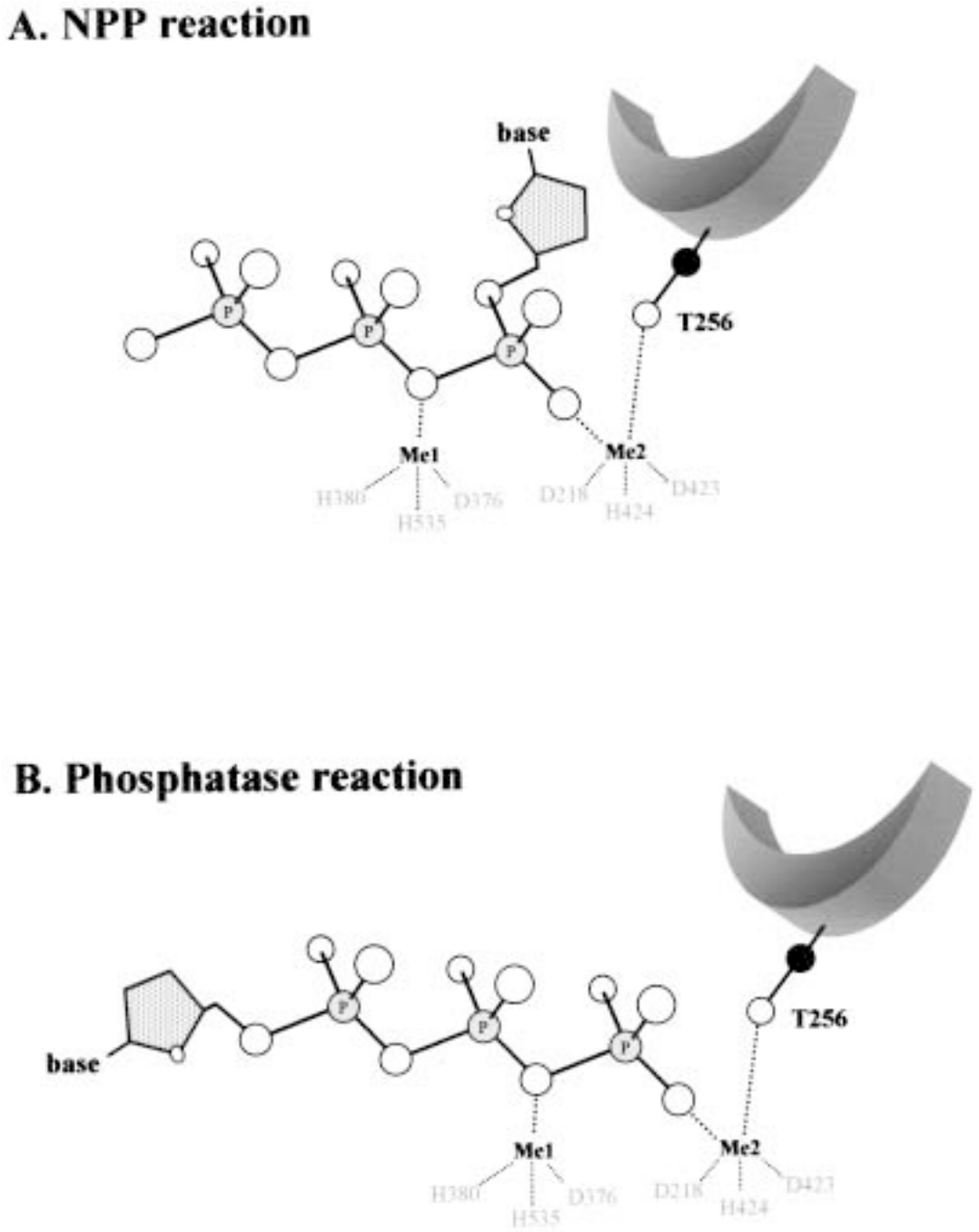

FIGURE 7. Binding of nucleotides to human NPP1 as substrates for the NPP (A) and phosphatase (B) reaction. It is suggested that NPPs function as either nucleotide pyrophosphatases/phosphodiesterases or as phosphatases, depending on the binding mode of the substrate. In both cases it is the phosphate group that is coordinated by both Me1 and Me2 that will be covalently bound to the catalytic-site threonine (T256 for human NPP1), resulting in the formation of either a nucleotidylated (A) or phosphorylated (B) intermediate.

nucleotidyl group of the covalent intermediate of the NPP reaction can also be transferred to alcohols (Ribeiro et al., 2000).

VI. PROCESSES THAT MAY BE CONTROLLED BY NPPs

NPPs have been implicated in the regulation of various intra- and extracellular processes, including cell differentiation and motility, bone and cartilage mineralization, and signaling by nucleotides and insulin.

A. Extracellular Nucleotide Metabolism

Generally, nucleotides occur extracellularly only at micromolar concentrations, which is about three orders of magnitude lower than their intracellular concentration (El-Moatassim et al., 1992; Dubyak and El-Moatassim, 1993; Lazarowski et al., 2000). However, appreciable extracellular nucleotide levels (up to 1 m*M*) may accumulate locally, for example, near nerve endings. Nucleotides can end up extracellularly by exocytosis, by release via intrinsic plasma membrane channels or pores, or by cell damage. Ecto-nucleotides (or their metabolites) mediate their effects by interacting with specific receptors in the plasma membrane, by serving as substrates for putative ecto-protein kinases, or by permeabilizing cell membranes. NPPs have been implicated in the generation, breakdown, and recycling of extracellular nucleotides.

1. Nucleotide Signaling

NPPs, in association with other nucleotide-hydrolyzing ecto-enzymes, may modulate nucleotide signaling. For example, NPP1 and 5′-nucleotidase have been implicated in purinoceptor-mediated signaling in various cell lines because their combined action converts a P2-purinoceptor signaling by ATP into a P1-purinoceptor signaling by adenosine (Grobben et al., 1999 and 2000; Lazarowski et al., 2000). Likewise, in choroid-plexus epithelial cells NPP2 and 5′-nucleotidase have been proposed to generate extracellular adenosine (Fuss et al., 1997), a modulator of cerebrospinal fluid production and cerebral blood flow (Faraci et al., 1988). For many cell types, including hepatocytes and cells of the immune system, ATP is apoptotic (Che et al., 1997; Chow et al., 1997). The destruction of extracellular ATP, possibly by NPPs, thus may represent an essential mechanism to protect cells from the damaging effect of ATP.

2. Recycling of Extracellular Nucleotides

Actively dividing cells like lymphoid cells meet their high purine and pyrimidine requirements (e.g., for the synthesis of nucleic acids, ATP, NAD^+, FAD, and nucleotide sugars), both by *de novo* synthesis and the salvage of extracellular nucleotides that are released from damaged cells. Nucleotides are first converted to nucleosides that are taken up by cells via specific transporters (Che et al., 1997). Therefore, NPPs may be part of cascade system(s) that enable extracellular nucleotides to be converted to nucleosides. The importance of such a cascade system(s) is illustrated by observations that T-cell proliferation in the presence of azaserine, a potent inhibitor of *de novo* purinergic nucleotide synthesis, is dependent on the presence of NAD^+, ADP-ribose, or AMP in the incubation medium (Deterre et al., 1996). In the same study it was also

shown that treatment of human T cells with activators of protein kinases A or C results in an increased expression of the cell-surface enzymes CD38 (an ecto-NAD^+-glycohydrolase), NPP1, and a 5′-nucleotidase. These findings led the authors to suggest that the coordinated expression of the latter enzymes allows activated T-cells to use NAD^+ from dying cells as a source of adenosine (Figure 8).

Nucleotide metabolism is particularly intense in hepatocytes. The liver actually secretes nucleotides into the blood for use by extrahepatic cells like erythrocytes that are incapable of sufficient *de novo* nucleotide synthesis (Murray, 1971). The liver also releases nucleotides (ATP, ADP, and AMP) into the bile as autocrine or paracrine modulators of bile secretion (Chari et al., 1996; Che et al., 1997; Schlenker et al., 1997). The nucleotides are salvaged from the bile in a process that is likely to require NPP3, 5′-nucleotidase(s), and the adenosine transporter, which are all present in the canalicular, bile-facing domain of the hepatocyte plasma membrane (Che et al., 1997; Scott et al., 1997; Meerson et al., 1997).

Intestinal NPP3 is likely to be involved in the hydrolysis of food-derived nucleotides, as an essential step in their uptake by enterocytes (Byrd et al., 1985; Scott et al., 1997).

3. Bone and Cartilage Mineralization

The matrix calcification by osteoblasts and chrondrocytes is initiated by the release of vesicles from the plasma membrane. The interior of these ‘matrix vesicles’ serves as a sheltered environment for hydroxyapatite crystal formation. The matrix vesicles contain several proteins, including NPP1, that are implicated in the ability of the vesicles to form calcium phosphate depositions (Hashimoto et al., 1998; Johnson et al., 1999 and 2000). The PP_i formed by the hydrolysis of NTPs by NPP1 may serve both as a source of P_i (Caswell et al., 1991) and as an (inhibitory) modulator of calcification (Meyer, 1984).

There is now substantial evidence that NPP1 is required to prevent excessive calcification. Thus, the naturally occurring tiptoe walking mouse (*ttw/ttw* mouse) exhibits ossification of the spinal ligaments. The *ttw* phenotype results from a nonsense mutation in ENPP1 (glycine 568 to stop), resulting in the generation of truncated NPP1 (Okawa et al., 1998a and 1998b; Nakamura et al., 1999). The targeted disruption of ENPP1 in the mouse was reported to cause a very similar phenotype (Goding, 2000). Moreover, the results of a large-scale screening of human patients with ossification of the posterior longitudinal ligament of the spine support the view that a dysfunction of NPP1 plays a role in the pathogenesis of this disease (Nakamura et al., 1999). The results of overexpression experiments are also in accordance with an inhibitory role of NPP1 in calcification. Thus, osteoblastic MC3T3 cells that were made to overexpress NPP1 displayed an increased matrix-vesicle content of PP_i and deposited 80 to 90% less hydroxyapatite (Johnson et al., 1999).

NPPs have also been implicated in the deposition of calcium pyrophosphate dihydrate crystals in cartilage, resulting in chrondrocalcinosis and a form of arthritis known as pseudogout (Rosenthal et al., 1991). Furthermore, NPP1 was shown to be present in chondrocyte-derived apoptotic bodies that, similar to the matrix vesicles, are able to form calcium phosphate depositions and appear to contribute to the pathological calcification in aging and osteoarthritis (Hashimoto et al., 1998).

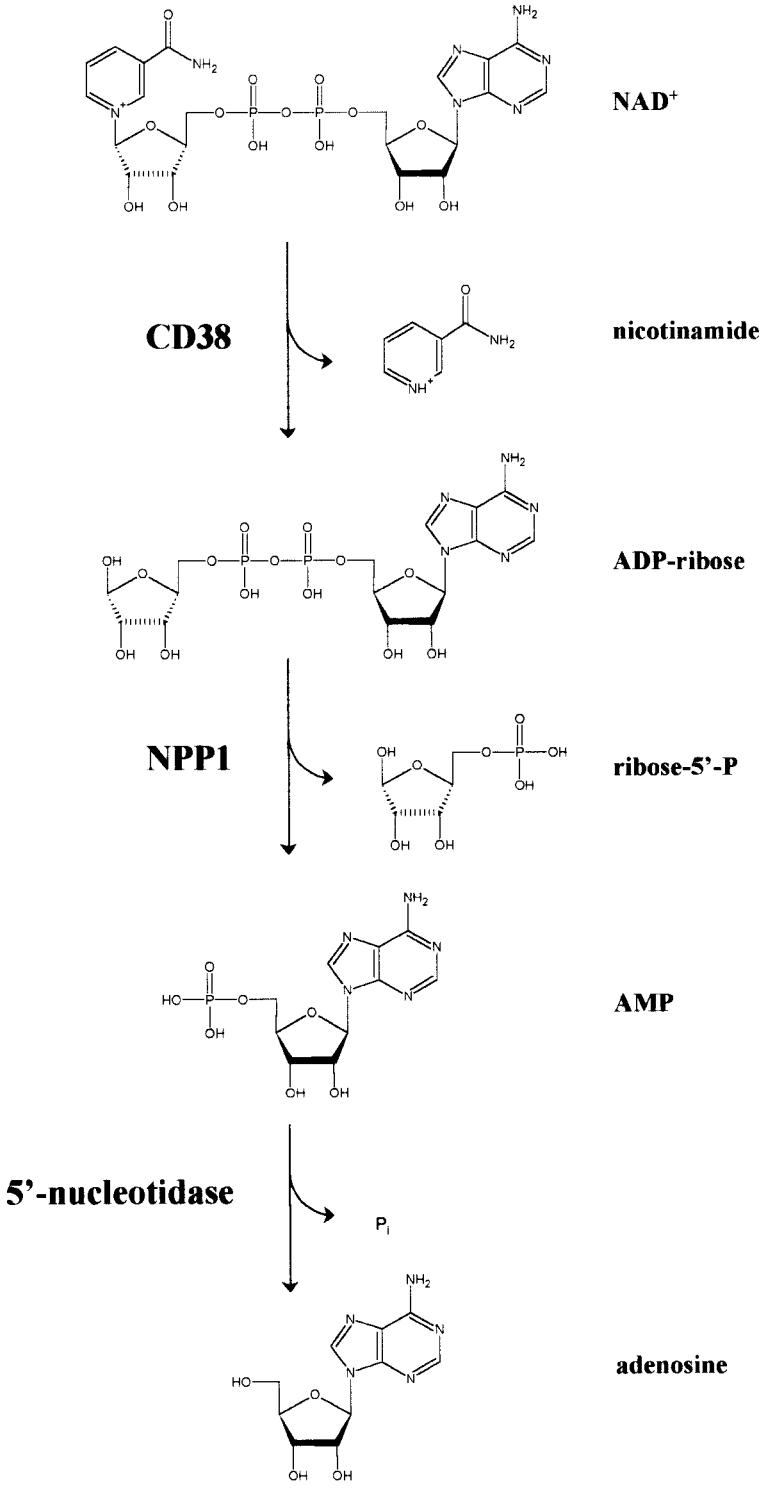

FIGURE 8. A role for NPP1 in the recycling of nucleotides by T-cells. (Based on data from Deterre et al. [1996].)

B. Cell-Cell and Cell-Matrix Communication

NPP2 was first described as a soluble motility-stimulating factor (Stracke et al., 1992) and has been implicated in tumor metastasis (Kawagoe et al., 1997). Recently, Nam et al. (2000) showed that autotaxin also augments the invasive and metastatic potential of *ras*-transformed cells. These effects of NPP2 require a functional catalytic site (Lee et al., 1996b; Nam et al., 2000) and are probably mediated by receptor(s) that are coupled to G-proteins, because they were blocked by pertussis toxin (Stracke et al., 1992). NPP2 signaling may be mediated by its binding to adhesion receptors, such as integrins, via its RGD-tripeptide motif in the second somatomedin-B-like domain (Sheetz et al., 1998; Figure 1).

NPP3 is expressed around birth by a specific subset of brain cells that are targeted by the carcinogen *N*-ethyl-*N*-nitrosourea (Deissler et al., 1995; Blass-Kampmann et al., 1997). A role for NPP3 in neuro-oncogenesis is also indicated by observations that fibroblasts and glioma cells that were made to express NPP3 displayed an altered morphoplogy and invasive properties (Deissler et al., 1999). The latter changes were associated with the expression of astroglial proteins, suggesting a role for NPP3 in the astrocyte differentiation program, which is known to depend on cell-cell interactions.

C. NPP1 and Insulin Signaling

Several lines of evidence have been presented, mainly by Goldfine and co-workers, for a role of NPP1 in insulin resistance. However, the data are not conclusive, and the molecular basis of the potential anti-insulin effect of NPP1 remains elusive.

1. NPP1 Binds to and Inhibits the Insulin Receptor

NPP1 has been shown to co-immunoprecipitate with the insulin receptor (Belfiore et al., 1996; Maddux and Goldfine, 2000) and to co-purify with the insulin receptor on insulin-Sepharose (Belfiori et al., 1996; Goldfine et al., 1998). However, NPP1 did not bind to an insulin receptor that lacked a fragment of the α-subunit that is essential for activation of the β-subunit by insulin, which led Maddux and Goldfine (2000) to suggest that NPP1 interacts with the α-subunit. At variance with the above data, Sakoda et al. (1999) did not find any interaction between baculovirus-expressed insulin receptor and NPP1.

NPP1 has been isolated from fibroblasts of patients with insulin resistance and non-insulin-dependent diabetes as an inhibitor of the insulin-receptor tyrosine kinase activity *in vitro* (Sbraccia et al., 1991; Maddux et al., 1993; Maddux et al., 1995). Stefan et al. (1996b) have shown, however, that purified NPP1 is an aspecific inhibitor of all protein kinases, simply because it hydrolyzes the substrate ATP. When care was taken to prevent a complete hydrolysis of ATP, purified NPP1 had no effect on the autophosphorylation of the purified insulin receptor. On the other hand, Maddux and Goldfine (2000) reported that the insulin receptor, immunocaptured from MCF-7 cells overexpressing NPP1, showed a reduced autophosphorylation.

Collectively, the above data suggest that the interaction between insulin receptor and NPP1 can only be demonstrated with crude cell fractions. This may indicate that the binding and inhibition of the insulin receptor by NPP1 is mediated by a third component that is present in the cell lysates but is lost during purification of the insulin receptor or NPP1.

2. The Overexpression of NPP1 is Associated with Insulin Resistance

A 16-fold overexpression of NPP1 in 3T3-L1 cells did not have any effect on insulin signaling (Sakoda et al., 1999). By contrast, an overexpression of NPP1 in MCF-7 cells and in Chinese hamster ovary cells has been associated with insulin resistance, as deduced from both insulin receptor autophosphorylation and the phosphorylation of the insulin receptor substrate IRS-1 (Grupe et al., 1995; Maddux et al., 1995; Kumakura et al., 1998). Several biological effects of insulin were also attenuated in cells overexpressing NPP1. On the other hand, the overexpression of NPP1 had no effect on the tyrosine kinase activity of the IGF-I or EGF receptors (Kumakura et al., 1998; Maddux and Goldfine, 2000). It has also been demonstrated that insulin resistance also results from overexpression of catalytically inactive NPP1, as shown by site-directed mutagenesis of the catalytic site threonine (Grupe et al., 1995). Likewise, the decreased activity of the insulin receptor that was immunocaptured from cells that overexpress NPP1 was also observed with this inactive NPP1 mutant (Maddux and Goldfine, 2000). Thus, the anti-insulin action represents the first biological effect of NPP1 that does not appear to depend on its enzymatic activity.

Remarkably, no effects on insulin receptor autophosphorylation and phosphorylation of IRS-1 were seen when both the insulin receptor and NPP1 were overexpressed, even though some biological effects of insulin (glucose and amino acid uptake, activation of p70 S6 kinase) were attenuated (Kumakura et al., 1998). This suggests that NPP1 also interferes with downstream effector(s) of the insulin signaling pathway. Perhaps NPP1 plays a role in insulin receptor internalization because an NPP activity was found to be enriched in the vesicles that contained internalized insulin (Smith and Peters, 1982).

3. The Level of NPP1 In Vivo Is Inversely Correlated with Insulin Sensitivity

In further agreement with an inhibitory role for NPP1 in insulin signaling, it was reported that the level of NPP1 was inversely correlated with insulin sensitivity in skeletal muscle (Maddux et al., 1995; Frittitta et al., 1996; Youngren et al., 1996; Shao et al., 2000), adipocytes (Frittitta et al., 1997) and fibroblasts (Maddux et al., 1995; Frittitta et al., 1998; Teno et al., 1999). In contrast, the level of soluble NPP1 in plasma was decreased in patients with insulin resistance (Frittitta et al., 1999). The cause of the increased cellular level of NPP1 in type-2 diabetes remains unknown but does not appear to result from the increased insulin levels per se, as shown by the normal levels of NPP1 in skeletal muscle of patients with insulinoma (Frittitta et al., 2000).

Apart from the fact that correlations cannot be used as evidence for a causal relationship, it is not always clear from the previously mentioned studies to which NPP(s) the correlation applied, as the adopted assays were not proven to be isoform specific. Moreover, in some cases of insulin resistance the level of tissue NPP1 was either not affected or even decreased. Thus, the development of insulin resistance with high-fat feeding or with the administration of streptozotocin in rats did not result in an increased NPP1 activity in skeletal muscle (Özel et al., 1996; Sakoda et al., 1999; Stefan, 1999). Dermal fibroblasts from pseudo-acromegalic patients with a severe form of insulin resistance did not show any change

in the level of NPP1 mRNA or protein either (Whitehead et al., 1997). In fibroblasts from patients with mutations in the insulin receptor or IRS-1, the concentration of NPP1 mRNA and protein was reported to be markedly decreased (Whitehead et al., 1997). Finally, in two established models of hepatic insulin resistance, that is, the neonatal rat and the diabetic *db/db* mouse, the level of NPP1 was severely decreased (Stefan, 1999; Stefan et al., 1999).

Recently, Stefanovic et al. (1999) demonstrated that the level of lymphocyte-associated NPP1 was severely increased in patients with type-2 diabetes, but was restored to control levels by the administration of the antihyperglycemic agent dimethylbiguanide (metformin). The authors speculated that the improved insulin sensitivity after the administration of metformin stems from a direct interaction of this compound with NPP1.

A frequently occurring polymorphism (K121Q) in the NPP1 gene (Nakamura et al., 1999) recently was reported to be strongly associated with insulin resistance in Caucasians from Sicily (Pizzuti et al., 1999). Skin fibroblasts derived from heterozygote carriers of this polymorphism also had a reduced insulin receptor tyrosine kinase activity when compared with the homozygote KK carriers (Pizzuti et al., 1999). However, the Q-allele frequency was not increased in type-2 diabetics from Scandinavia (Gu et al., 2000; Rasmussen et al., 2000) but among the type-2 diabetic patients carriers of the Q allele had a higher fasting blood glucose and insulin concentration (Gu et al., 2000). Also, the Q-allele in patients with type-1 diabetes was associated with a faster progression of nephropathy, as indicated by their faster decline in the glomerular filtration rate (De Cosmo et al., 2000). The effect of the K121Q mutation has not been explored yet at the molecular level, but it seems unlikely that this mutant has grossly perturbed enzymic properties because K121 (which is actually K173 when the correct transcriptional start site is used, see Figure 1) is not conserved in rodent NPP1. Because the latter residue is mapped to the end of the second somatomedin-B like domains, it is possible that the K121Q mutation interferes with the homodimerization of NPP1.

D. An Intracellular Role of NPPs?

Because part of the cellular pool of NPP1 is integrated in the membrane of the endoplasmic reticulum, this isozyme may also have an intracellular role. Hickman and coworkers proposed that NPP1 controls the availability of nucleotide sugars that serve as precursors for oligosaccharide synthesis in the endoplasmic reticulum (Hickman et al., 1985; Rebbe et al., 1991). The major evidence was that the capacity for glycosylation, as derived from the incorporation of radioactively labeled mannose into the larger lipid-linked oligosaccharides, was inversely correlated with the cellular level of NPP1 (Hickman et al., 1985).

3′-phosphoadenosine 5′-phosphosulphate is the donor for sulphation of glycosaminoglycans in the endoplasmic reticulum (Uhlin-Hansen and Yanagishita, 1993) and is also a substrate of NPPs (Figure 6). It has been suggested that NPPs limit the availability of this sulfate donor (Yamashina et al., 1983); indeed, the activity of NPPs is severalfold increased in fibroblasts from patients with Lowe's oculocerebrorenal syndrome, an X-linked genetic disease that is reportedly associated with undersulfation of glycosaminoglycans (Yamashina et al., 1983. However, others have found that fibroblasts from patients with Lowe's syndrome do not show any deficiency in the

sulfation glycosaminoglycans, despite an elevated NPP activity (Harper et al., 1987). More recently, it has been shown that Lowe's syndrome is caused by a deficiency of a phosphatidylinositol 4,5-bisphosphate 5-phosphatase (Suchy et al., 1995).

VII. REGULATION OF NPPS BY HORMONES AND GROWTH FACTORS

The expression of NPP1 in FAO hepatoma cells, osteosarcoma cells, and chondrocytes was reported to be stimulated by TGF-β_1 and, in chondrocytes, this effect was antagonized by interleukin-1β (Huang et al., 1994; Lotz et al., 1994; Stefan et al., 1999). In osteosarcoma cells and in T-lymphocytes, NPP1 is also induced by agents that activate protein kinases A or C (Deterre et al., 1996; Solan et al., 1996). Corticosteroids have also been reported to stimulate the expression of NPP1 in mouse plasmacytoma cells (Rebbe and Hickman, 1991). NPP2 was shown to be induced during osteo-/chondrogenic differentiation by the Bone-Morphogenic-Protein-2 (Bächer et al., 1999). On the other hand, NPP2 is downregulated by interferon-γ in fibroblasts (Santos et al., 1996). In smooth muscle cells, the NPP3 transcript level is decreased by PDGF and angiotensin-II (Kettenhofen et al., 1998).

Various lines of evidence suggest that the expression of NPPs is growth related. Thus, the activity of ill-defined NPPs was reported to be increased in contact-inhibited cells and in solid tumors (Harshman et al., 1979; Clark and Goodlad, 1993). In FAO-hepatoma cells the level of NPP1 increases with the cell density (Stefan et al., 1998). Also, the level of NPP1-3 increases many-fold during the hepatic growth phase in the first weeks after birth, albeit with a distinct time course (Stefan et al., 1998 and 1999). Furthermore, NPP1 and NPP3 disappear from rat liver within 24 h after a partial hepatectomy, but reappear in the ensuing regeneration period. This transient loss of NPP1 and NPP3 was accounted for by a deficient processing and/or increased turnover of their transcripts. The translational inhibitor cycloheximide was also reported to induce a rapid decrease of the NPP1 and NPP2 transcript level, suggesting that the stability and processing of these transcripts is controlled by proteins with a high turnover (Stefan et al., 1999).

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