Minireview

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Mammalian 5′-Nucleotidases*

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Nucleoside monophosphate phosphohydrolases or 5′-nucleotidases (members of EC 3.1.3.5 and EC 3.1.3.6) dephosphorylate non-cyclic nucleoside monophosphates to nucleosides and inorganic phosphate. Seven human 5′-nucleotidases with different subcellular localization have been cloned (Table I). Sequence comparisons show high homology only between cytosolic 5′-nucleotidase IA (cN-IA)[1] and B and between cytosolic 5′(3′)-deoxynucleotidase (cdN) and mitochondrial 5′(3′)-deoxynucleotidase (mdN). However, the existence of common motifs suggests a common catalytic mechanism for all intracellular 5′-nucleotidases. Some 5′-nucleotidases are ubiquitous (ecto-5′-nucleotidase (eN), cN-II, cdN, and mdN); others display tissue-specific expression (cN-I and cN-III).

Here we summarize recent advances on the structure and cellular functions of the cloned 5′-nucleotidases. We also propose a revised nomenclature, agreed upon with other colleagues active in the nucleotidase field.

Catalytic Mechanism

Crystal structures are known for human mdN (10) and cdN[2] and for *Escherichia coli* periplasmic 5′-nucleotidase (11), a homologue of eN. All intracellular nucleotidases share a D*X*-D*X*(V/T) motif critical for catalysis and show structural similarity to the haloacid dehalogenase superfamily of enzymes (10). eN belongs to a separate family that includes also 2′,3′-cyclic phosphodiesterases and apyrases (11).

The crystal structure of mdN and work on the active site of cN-II form the basis for a reaction mechanism of intracellular 5′-nucleotidases (10, 12). The reaction creates a phosphoenzyme intermediate involving the first aspartate in the D*X*-D*X*(V/T) motif (12). A detailed scheme of the catalytic process derived from the crystal structure of mdN (10) involved both aspartates in the above motif (Fig. 1). The first (Asp-41) generates a pentacovalent phosphorus intermediate with similar basic organization as the intermediate detected in the structure of β-phosphoglucomutase (13). The x-ray structure of cdN suggests a catalytic mechanism identical with that of mdN. Differences within the active sites account for differences in substrate specificity (10).[2] Using the structurally important residues the best alignment was between the two deoxynucleotidases and cN-III (10). Two 5′-nucleotidases, cN-II and cN-III, exhibit phosphotransferase activity (for reviews see Refs. 14 and 15) possibly because of higher stability of the phosphoenzyme intermediate or faster exchange of the nucleoside product with the nucleoside acceptor.

The active site of *E. coli* 5′-nucleotidase, the paradigm for eN, contains two zinc ions and the catalytic dyad Asp-His (11). No phosphoenzyme intermediate is formed during catalysis, but a water molecule performs the nucleophilic attack on the phosphate (16).

Properties, Detection, and Inhibition of 5′-Nucleotidases

All 5′-nucleotidases have relatively broad substrate specificities. In agreement with the structural information on the active sites (10, 11), all family members except eN are absolutely dependent on magnesium for activity. Table II summarizes some distinctive properties of 5′-nucleotidases. Detection of individual nucleotidases by enzymatic assays in cell lysates is problematic because different nucleotidases are co-expressed in the same tissue or cell type. The problem was earlier addressed by immunotitration (for review, see Ref. 14) and more recently by a strategy that exploits differences in optimal conditions for the ubiquitous nucleotidases (17). Differential assays can take advantage of inhibitors of individual nucleotidases (8, 17–20). The most active inhibitors described so far are pyrimidine nucleotide and nucleoside analogs inhibiting cN-I at nanomolar or low micromolar concentrations with up to 1000-fold selectivity for cN-I relative to cN-II or eN (18). Two pyrimidine phosphonates inhibit cdN and mdN (8, 17) with weaker inhibition of cN-I (17). Specific properties of individual 5′-nucleotidases are discussed below.

Ecto-5′-nucleotidase—eN, also known as CD73, is a glycosylated protein bound to the outer surface of the plasma membrane by a glycosylphosphatidylinositol anchor (1) and co-localizes with detergent-resistant and glycolipid-rich membrane subdomains called lipid rafts. Up to 50% of the enzyme may be associated to intracellular membranes (for review, see Ref. 20) and be released during homogenization. Early reports of a soluble low K_m nucleotidase (for review, see Ref. 20) were because of this phenomenon (21). Although eN has broad substrate specificity, AMP is considered to be the major physiological substrate (for review, see Ref. 20) (22–24). Independently of the enzymatic function, the protein acts as co-receptor in T cell activation (for review, see Ref. 23) and as cell adhesion molecule (for review, see Ref. 24). eN is variably expressed in a wide number of cell types under physiological and pathological conditions (for review, see Refs. 20, 23, and 24). In neuronal cells eN expression is linked to developing or plastic states (for review, see Ref. 24). The proximal promoter region of the gene contains a number of tissue-specific elements (25, 26).

Cytosolic 5′-Nucleotidase IA—cN-IA was named AMP-specific 5′-nucleotidase for its high specific activity with AMP at millimolar concentrations. Subsequent detailed kinetic studies revealed high affinities toward deoxypyrimidine monophosphates (18). It is highly expressed in skeletal and heart muscle where it has a physiological function in the generation of signaling adenosine during ischemia (2, 27). The high affinity for deoxynucleoside monophosphates suggests a role in the regulation of deoxyribonucleotide pools. A homologous sequence

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[1] The abbreviations used are: cN, cytosolic 5′-nucleotidase; cdN, cytosolic 5′(3′)-deoxynucleotidase; eN, ecto-5′-nucleotidase; mdN, mitochondrial 5′(3′)-deoxynucleotidase; NT, nucleotidase.

[2] A. Rinaldo-Matthis and P. Nordlund, manuscript in preparation.

TABLE I
Classification of 5′-nucleotidases

Revised protein nomenclature	Full name and gene symbol	UniGene cluster no.	Aliases	Refs.
eN	Ecto-5′-nucleotidase, *NT5E*	Hs.153952	Ecto-5′-NT; low K_m 5′-NT; eNT; CD73	1
cN-IA	Cytosolic 5′-nucleotidase IA, *NT5C1A*	Hs.307006	AMP-specific 5′-NT; cN-I	2, 3
cN-IB	Cytosolic 5′-nucleotidase IB, *NT5C1B*	Hs.120319	cN-IA homolog; AIRP	4
cN-II	Cytosolic 5′-nucleotidase II, *NT5C2*	Hs.138593	High K_m 5′-NT; purine 5′-NT; GMP,IMP-specific 5′-NT	5
cN-III	Cytosolic 5′-nucleotidase III, *NT5C3*	Hs.55189	PN-I; P5′N-1; UMPH	6
cdN	Cytosolic 5′(3′)-deoxynucleotidase, *NT5C*	Hs.67201	dNT-1; PN-II	7, 8
mdN	Mitochondrial 5′(3′)- deoxynucleotidase, *NT5M*	Hs.16614	dNT-2	9

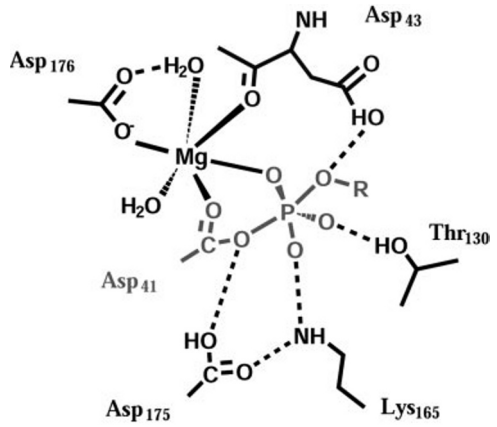

FIG. 1. **Structure of the active site of mdN with the pentavalent phosphorous intermediate produced by nucleophilic attack of Asp-41 on the phosphate (10).** Asp-43 first promotes the protonation of the leaving deoxyribonucleoside (*R*) and then activates the water nucleophile that releases the phosphate. Asp-41 and Asp-43 are the two aspartates in the motif conserved in intracellular 5′-nucleotidases.

related to human autoimmune infertility gene (*AIRP*) and with highest expression in testis has been cloned and designated cN-IB (4).

Cytosolic 5′-Nucleotidase II—cN-II is a 6-hydroxypurine-specific nucleotidase, most active with (d)IMP (for review, see Ref. 14) and critically positioned to regulate ATP and GTP pools. This tetrameric protein is stimulated by (d)ATP and GTP and regulated by substrate and phosphate in a complex manner (for review, see Ref. 14) (27–29), possibly involving subunit association and dissociation (30). Under physiological conditions cN-II can catalyze phosphotransfer from a purine nucleotide donor to inosine or guanosine (31, 32). This reaction is responsible for the activation of several anti-viral and anti-cancer nucleoside analogs that are not substrates for cellular nucleoside kinases (33, 34).

Cytosolic 5′-Nucleotidase III—cN-III is highly expressed in red blood cells where it participates in the degradation of RNA during erythrocyte maturation (for review, see Ref. 15). It prefers pyrimidine ribo- over deoxyribonucleotides with CMP being the best substrate. It is inactive with purine nucleotides. The enzyme has a phosphotransferase activity (35) less efficient than cN-II (32). The sequence of cN-III coincides with that of p36, an interferon α-induced protein of unknown function (6). Alternative splicing of exon 2 gives rise to two proteins that are 286 and 297 amino acids long (36), with the shorter form corresponding to cN-III.

Cytosolic 5′(3′)-Deoxynucleotidase—cdN is a ubiquitous enzyme, first purified to homogeneity from human placenta (37). It is the major deoxynucleotidase activity in cultured human cells (17, 38). In contrast to cN-III, human cdN is not strictly pyrimidine-specific and works efficiently with dIMP and dGMP. dAMP is a poor substrate and dCMP is inactive (8, 37). The enzyme is very active on 2′- and 3′-phosphates (7, 37). Neither the highly purified human placental cdN nor the recombinant mouse and human enzymes showed phosphotransferase activity (7, 37), in contrast to what was reported for cdN purified from human red blood cells (35).

Mitochondrial 5′(3′)-Deoxynucleotidase—mdN is highly homologous to cytosolic cdN (52% amino acid identity). The two enzymes are coded by nuclear genes with identical structure, probably derived by a gene duplication event (9). With its high preference for dUMP and dTMP, mdN shows remarkably narrow substrate specificity. Similarly to cdN, mdN prefers deoxy- over ribonucleotides and accepts 2′- and 3′-nucleoside monophosphates (9, 38). Its enzymatic features suggest that mdN regulates mitochondrial dTTP and prevents accumulation of mutagenic dUTP within mitochondria.

Physiological Role of 5′-Nucleotidases: Insights from Overexpressing Cell Lines

By opposing the phosphorylation of nucleosides by kinases, intracellular 5′-nucleotidases participate in substrate cycles that regulate the cellular levels of ribo- and deoxyribonucleoside monophosphates and thereby all ribo- and deoxyribonucleotide pools (for review, see Ref. 39) (40). Intracellular 5′-nucleotidases have relatively high K_m values and operate on substrates generally present at (very) low concentrations. Thus they are exquisitely sensitive to oscillations of substrate concentration. Given their overlapping substrate specificities, it is difficult to tie a given enzyme to the maintenance of a specific nucleotide pool. Important information has been obtained with cell lines engineered to overexpress individual nucleotidases. Involvement of a 5′-nucleotidase in a specific substrate cycle is signaled by the increased excretion of the nucleoside produced by that cycle (40). In such experiments it is important to analyze the turnover of individual pools during brief time windows. Changes in nucleotide pool sizes only show the final end point of complex metabolic adaptations and may be a consequence of reduced ATP availability (29, 41). By this strategy cN-IA was shown to operate on AMP (27, 42) and cN-II on IMP and GMP (27, 40), and murine cdN was shown to regulate all pyrimidine deoxyribonucleotide pools (40). In human cells dCMP should be dephosphorylated by a different enzyme, as human cdN is inactive on dCMP. A potential candidate is cN-IA that has high affinity for all deoxyribonucleotides (3, 18), although it is still not clear whether the expression of this enzyme outside skeletal and heart muscle is sufficient to perform this function (3). Strategies such as knockout mice and small interfering RNA may help solve these issues. Indeed, down-regulation of mdN in cultured human cells by small interfering RNA showed that mdN participates in a mitochondrial substrate cycle with the mitochondrial thymidine kinase.[3]

[3] C. Rampazzo and V. Bianchi, unpublished data.

TABLE II
Distinctive features of 5′-nucleotidases

Enzyme	Protein structure (monomer kDa[a])	Substrate affinity (K_m)	Effect of ATP	pH optimum
eN	Dimer (63)	μM	−	7.5
cN-IA[b]	Tetramer (41)	μM-mM[c]	+ (ADP ++)[d]	7
cN-II	Tetramer (65)	sub mM	++	6.5
cN-III	Monomer (33)	sub mM	none	7.5
cdN	Dimer (23)	mM	none	6–6.5
mdN	Dimer (26)	sub mM	none	5.5

[a] Predicted from cDNA sequence and not including posttranslational modifications.
[b] cN-IB not yet characterized.
[c] Micromolar K_m values for pyrimidine deoxynucleotides; millimolar or submillimolar for purine substrates (3, 18).
[d] ADP and dADP are best activators (S. A. Hunsucker and Y. Spychala, manuscript in preparation).

Clinical Implications of 5′-Nucleotidases

The only known genetic syndrome due to 5′-nucleotidase deficiency is the hereditary hemolytic anemia caused by mutation of cN-III (for review, see Ref. 15). Accumulation of normally undetectable pyrimidine nucleotides in erythrocytes of affected subjects highlights the important role of cN-III during maturation of red blood cells.

Anti-viral and anti-blastic nucleoside analogs must be activated by phosphorylation to exert their therapeutic effect. Intracellular 5′-nucleotidases influence the metabolism of the analogs by reversing the activation step and thereby decreasing their pharmacological efficacy. Several of the *in vitro* models of nucleoside analog resistance were linked to high expression of cN-II and cN-IA (3, 43) (for review, see Ref. 44). Consistent with the substrate cycle model, the relative ratio of nucleoside kinase to 5′-nucleotidase may have predictive clinical value with 5′-nucleotidases contributing to drug resistance (45). Development of compounds that inhibit 5′-nucleotidase activity may reverse drug resistance and increase the efficacy of existing analogs. New nucleoside analogs that are poor substrates for 5′-nucleotidases may lead to more effective therapies. Several anti-viral drugs show striking mitochondrial toxicities that pose a serious limitation on their use (46). One way to address this problem is to develop compounds that are differentially metabolized by cytosolic and mitochondrial 5′-nucleotidases, thus allowing for cytoplasm-specific accumulation of pharmacologically active metabolites. Recent development of cdN and mdN inhibitors that differentially target these enzymes suggests that such strategy is possible (17).

Conclusions

The presence in the human genome of at least seven genes for 5′-nucleotidases suggests that these enzymes perform important metabolic functions. With the enzymes available in recombinant form it will soon be possible to complete their biochemical characterization. Gene regulation remains instead an unchartered field. We do not know how strict is the tissue-specific expression of cN-III, how the variable expression levels of the ubiquitous nucleotidases revealed by multiple-tissue Northern blots are obtained, if expression of individual enzymes can be induced or repressed in specific circumstances, and if patterns of expression are altered in specific tumors.

The new RNA technologies and microarray analyses of overall expression profiles in cells will contribute to clarify the role of 5′-nucleotidases in the regulation of nucleotide pools. We are looking forward to the new information to come.

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