[Frontiers in Bioscience 13, 6716-6729, May 1, 2008]

Mammalian ADP-ribosyltransferases and ADP-ribosylhydrolases

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TABLE OF CONTENTS

1. ABSTRACT

ADP-ribosyltransferases (ARTs) and ADP-ribosylhydrolases (ARHs) catalyze opposing reactions, which are termed ADP-ribosylation and de-ADP-ribosylation. ARTs transfer the ADP-ribose unit from NAD (nicotinamide adenine dinucleotide) onto an acceptor, while ARHs release the ADP-ribose from the target. Like protein phosphorylation, ADP-ribosylation is a posttranslational modification regulating protein function. In many cases, ADP-ribosylation inactivates the target protein. Numerous bacterial toxins intoxicate cells by attaching an ADP-ribose moiety to a functionally important amino acid residue, thereby blocking the interaction of the target protein with other proteins. In other cases, ADP-ribosylation activates protein function. On the surface of T cells, ART2.2 ADP-ribosylates the P2X7 purinoceptor on arginine 125, thereby gating the P2X7 ion channel by presenting a ligand to its nucleotide-binding site. ADP-ribosylation is not limited to protein targets and ARTs have been described that ADP-ribosylate DNA, RNA, and small molecules. Mammalian cells express distinct families of ARTs and ARHs. Recently, molecular cloning, site directed mutagenesis and three-dimensional structural analyses of prototype mammalian ARTs and ARHs have shed fresh insight into the structure and function of these intriguing enzymes.

2. INTRODUCTION

ADP-ribosyltransferases (ARTs) and ADP-ribosylhydrolases (ARHs) catalyze opposing reactions (Figure 1) (1-8). ARTs transfer the ADP-ribose moiety from NAD onto an acceptor, utilizing the high energy bond between nicotinamide and ADP-ribose. Figure 1 illustrates this reaction for a protein target. This reaction is called ADP-ribosylation and generates an ADP-ribosylated acceptor, while releasing nicotinamide. ARHs hydrolyze the linkage between ADP-ribose and the acceptor (X). This reaction, in turn, is called de-ADP-ribosylation or ADP-ribosyl-X hydrolysis. De-ADP-ribosylation regenerates the original state of the acceptor, while releasing ADP-ribose. The net reaction of ADP-ribosylation and de-ADP-ribosylation is the hydrolysis of NAD into ADP-ribose and nicotinamide. In the absence of a physiological target, many ARTs can utilize water as an acceptor, resulting in the direct hydrolysis of NAD to ADP-ribose and nicotinamide. This reaction is termed NAD-hydrolysis or NAD-glycohydrolysis.

ADP-ribosylation was originally discovered as a posttranslational modification (PTM) affecting protein function. Akin to protein-phosphorylation, the attachment of the bulky negatively charged ADP-ribose moiety onto a specific amino acid residue serves to modify protein

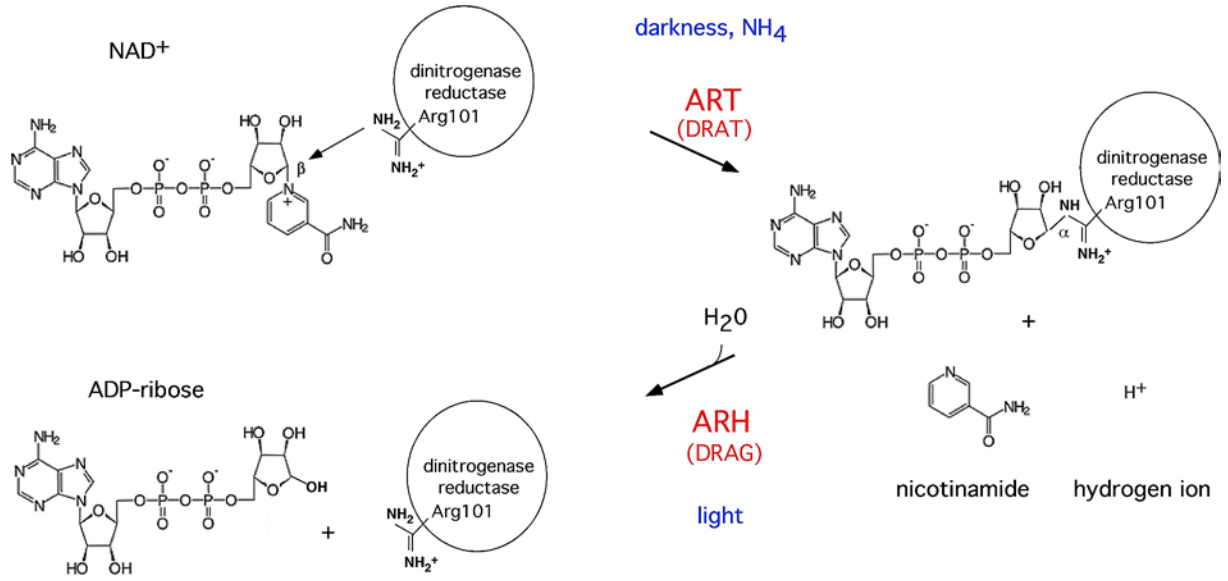

Figure 1. ARTs and ARHs catalyze ADP-ribosylation and de-ADP-ribosylation of targets such as arginine 101 in dinitrogenase reductase. ARTs transfer the ADP-ribose moiety from β-NAD onto specific sites on targets. ARHs hydrolyze the α-glycosidic linkage generated by ARTs resulting in the release of ADP-ribose and native target. In phototrophic bacteria, the key enzyme of nitrogen fixation, dinitrogenase reductase, is ADP-ribosylated on Arginine at position 101 in the polypeptide chain by an arginine-specific ART designated DRAT (dinitrogenase reductase ADP-ribosyltransferase). DRAT is activated by darkness and by ammonium. Light activates an ADP-ribosyl-arginine-specific ARH designated DRAG (dinitrogenase reductase ADP-ribosylglycohydrolase). DRAG regenerates active DR by de-ADP-ribosylation. The net reaction is the hydrolysis of NAD to ADP-ribose and nicotinamide. Note that during ADP-ribosylation, the glycosidic linkage is converted from the β-conformation in NAD to the α-conformation in the ADP-ribosylated target.

function. Today, we know that numerous non-proteinaceous substances can serve as ART targets, including nucleic acids, sugars, amino acids, and other small molecules. During bond formation between ADP-ribose and the acceptor, the configuration of the bond shifts from the beta-conformation in NAD to the alpha-conformation in the ADP-ribosylated target (Figure 1). ADP-ribosylation reactions are inhibited by high concentrations of nicotinamide, as occupation of the binding pocket by nicotinamide competes with the binding of NAD.

3. AN ART AND ARH ENZYME PAIR NAMED DRAT AND DRAG REGULATES NITROGEN FIXATION IN PHOTOTROPHIC BACTERIA

ADP-ribosylation regulates nitrogen fixation (i.e. the conversion of nitrogen to ammonium) in *Rhodospirillum rubrum* and other free-living photosynthetic bacteria (Figure 1) (9). Following exposure to darkness or high concentrations of ammonium, the rate limiting enzyme dinitrogenase reductase (DR) is ADP-ribosylated on Arg101 by DRAT (dinitrogenase reductase ADP-ribosyl-transferase) (10, 11). ADP-ribosylation inactivates DR, thereby shutting-off nitrogen fixation. Light illumination or exhaustion of ammonium in turn induces de-ADP-ribosylation of DR by a distinct enzyme designated DRAG (dinitrogenase reductase-activating glycohydrolase), reactivating DR and resulting in the resumption of nitrogen fixation (12).

4. PURIFICATION, CLONING, AND STRUCTURE DETERMINATION OF ARTS AND ARHS FROM MAMMALS

A large body of biochemical evidence indicated that mammalian cells produce endogenous enzymes related to DRAT and DRAG that catalyze ADP-ribosylation and de-ADP-ribosylation reactions (Table 1). A number of such enzymes were successfully purified to homogeneity from tissue extracts and then identified by partial amino acid sequence analyses and molecular cloning (Table 1A). ART1 is a promiscuous arginine-specific ART, originally purified from rabbit skeletal muscle (13). ART1 is an ecto-enzyme composed of an isolated catalytic domain that is attached to the cell membrane via covalent linkage of the C-terminal amino acid residue to a membrane glycolipid, glycosylphosphatidylinositol (GPI). Poly-ADP-ribose-polymerase-1 (PARP-1), one of the most abundant nuclear proteins, was originally purified from human placenta (14). PARP-1 catalyzes ADP-ribosylation of itself and of other proteins on glutamate residues as well as on ADP-ribose units themselves (15-17). This results in the attachment of polymers of ADP-ribose (PAR) onto PARP-1 and other proteins. PARP-1 is a multi-domain protein. The C-terminal catalytic domain is linked to a DNA-binding regulatory domain that mediates activation of PARP-1 by single stranded DNA-breaks. tRNA phosphotransferase 1(TRPT1) is an enzyme originally purified from yeast which catalyzes the dephosphorylation of an RNA- during splicing of t-RNA (18). This reaction requires NAD,

Table 1. Milestones in the structural characterization of mammalian ARTs and ARHs

A) purification and molecular cloning of the prototype family member			
	source		references
ART1	rabbit skeletal muscle		13, 93
PARP1	human placenta		14
TRPT1	yeast		18
ARH1	rat brain		20
PARG	bovine thymus		21
B) X-ray crystallography of the prototype family member			
	source	pdb	references
ART2	rat	1GXY	53
PARP1	chicken	1PAX	94
TRPT1	*Aeropyrum pernix*	1WFX	95
ARH3	human	2FOZ	71
PARG	--	--	--
C) related gene family members in the human and mouse genomes			
	human	mouse	references
ARTs	4	6	31
PARPs	17	16	24, 96
TRPT1	1	1	97
ARHs	3	3	31
PARG	1	1	21

intermediate and TRPT1 initially catalyzes the ADP-ribosylation of the terminal phosphate residue in RNA (19). An intramolecular rearrangement of bonds results in the release of 1"-2" cyclic phosphodiester ADP-ribose. ARH1 was originally purified from rat brain as an enzyme that specifically cleaves ADP-ribosylarginine linkages (20). PARG was purified from bovine thymus as an enzyme that cleaves poly-ADP-ribose polymers generated by PARP (21) (Table 1).

Prototype members of ADP-ribosylating and de-ADP-ribosylating enzyme families have been crystallized and their 3D-structures have been elucidated (Table 1B). The core of the ART catalytic domain contains two conserved beta-sheets that form the upper and lower jaws of a deep NAD-binding crevice. The structure is reminiscent of a pacman and this is the symbol we use here to indicate the ART catalytic domain (Figure 2). The core of the ARH fold is composed entirely of alpha-helices. Two magnesium ions are located at the bottom of the active site crevice. The structure is reminiscent of a pumpkin and this is the symbol we use to indicate the ARH catalytic domain (see below, Figures 5 and 7). The crystal structures indicate that the known mammalian ARTs are more closely related to subfamilies of bacterial toxin ARTs than they are to each other. ART2.2 shows closer structural similarity to DRAT and to the VIP2 family of toxin-ARTs than to PARP-1 or TRPT1, while the latter more closely resemble diphtheria toxin (DT) and rifampin-ADP-ribosyltransferase (RART) than the mammalian ecto-ARTs (22-24) (see also note added in proof).

5. PROTEIN-ADP-RIBOSYLATION BY MAMMALIAN ENZYMES UNRELATED TO THE ART FAMILY

Two other NAD-dependent mammalian enzymes have been reported to catalyze ADP-ribosylation of proteins under certain circumstances (25, 26) (Figure 2C). ADP-ribosylcyclases are multifunctional enzymes that can catalyze the conversion of NAD to the calcium-mobilizing second messenger cyclic-ADP-ribose as well as the hydrolysis of NAD and cyclic-ADP-ribose to ADP-ribose (27). CD38 is a membrane-anchored ecto-enzyme of the ecto-ADP-ribosylcyclase family that has been shown to hydrolyze NAD to ADP-ribose, which then reacts non-enzymatically with reactive cysteine residues of secretory proteins (25). Sirtuins are NAD-dependent enzymes that catalyze the removal of acetyl groups from acetylated lysine residues in proteins. Mitochondrial SIRT4 has recently been shown to catalyze ADP-ribosylation of glutamate dehydrogenase on a cysteine residue. The crystal structures of CD38 and of SIRT4-related Sir2 from yeast, however, clearly show that these enzymes do not share the ART-fold and constitute distinct evolutionary inventions of NAD-catabolizing proteins (28-30).

6. GENOME DATABASE AND 3D-STRUCTURE ANALYSES DELINEATE DISTINCT SUBFAMILIES OF MAMMALIAN ARTS AND ARHS

In silico analyses of the fully sequenced human and mouse genomes revealed the presence of several additional genes encoding ART1-like and PARP-like putative catalytic domains, but only a single gene encoding a TRPT1-like protein (Table 1C) (24, 31). Similarly, the human and mouse genomes each contain three genes encoding ARH1-like putative catalytic domains, but only a single copy PARG gene (21, 31). Many of these proteins have hence been cloned, produced as recombinant proteins and crystallized. Below, the distinct subfamilies of mammalian ARTs and ARHs will be described in separate sections.

6.1. DRAT-related mammalian ARTs

The human genome contains four functional genes known or predicted to encode DRAT-like ARTs, whereas the mouse genome contains six functional ART-encoding genes (Table 2). This difference is accounted for by a regional duplication in the murine lineage *vs.* gene silencing in the human lineage affecting ART2. The human *ART2* gene, like its primate counterparts carries three premature stop codons and no *ART2*-specific transcripts can be detected in human cells (32). In contrast, the mouse genome contains two functional *Art2* gene copies, derived from a regional duplication event (33). The gene products, designated ART2.1 and ART2.2, are expressed as GPI-anchored proteins on the cell surface of macrophages and T cells (34, 35). ART2.1 is unusual in that it carries an extra pair of cysteine residues not found in any of its orthologs. Its enzymatic activity is dependent on reducing agents (35, 36).

Mouse and human ART1, mouse ART2.1, mouse ART2.2, and human ART5, display promiscuous Arg-specific ART-activity (31). ART1 and ART2 can ADP-ribosylate the same targets when overexpressed in cell culture (37), but it is not known to what extent they do so *in vivo*. Rat ART2 and mouse ART5 display potent NAD-hydrolase but little if any arginine-specific ART

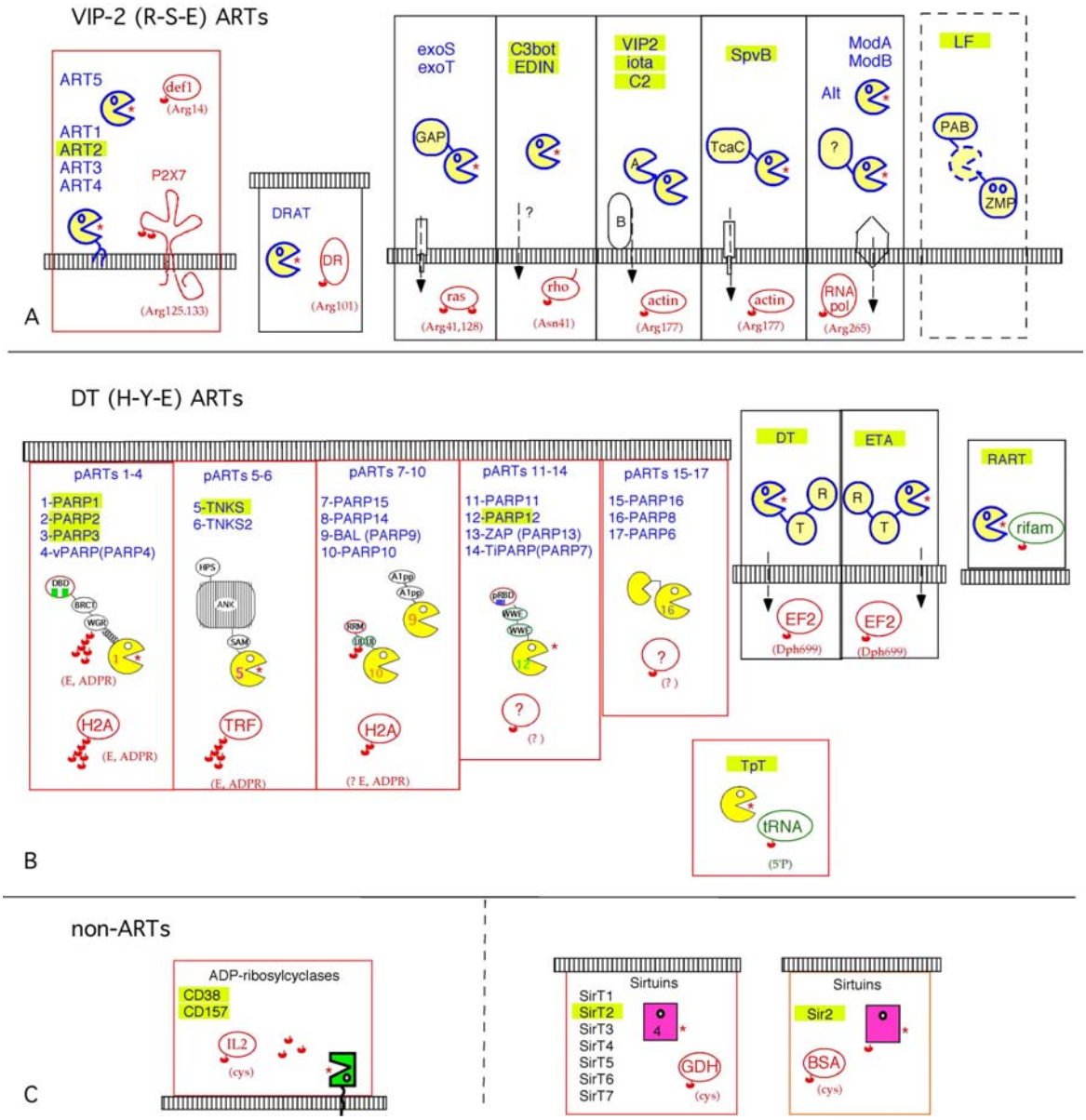

Figure 2. Gallery of mammalian ecto-ARTs and related enzymes. DRAT-related mammalian ecto-ARTs and other ADP-ribosylating enzymes are grouped on the basis of structural and sequence similarities. Names of proteins with known 3D-structures are highlighted in yellow. The ART catalytic domain is symbolized by a pacman in reference to two core β-sheets that form the upper and lower jaws of a deep NAD-binding crevice (see Figure 3). NAD is symbolized by a star. Other genetically linked domains are indicated by ovals. Major target proteins are in red with the modified amino acid, where known, given in parentheses. DNA, RNA and rifampin targets are indicated in green. exoS and exoT: exoenzymes S and T from *Pseudomonas aeruginosa*; C3bot: C3 exoenzyme form *Clostridium botulinum* phage; EDIN: Epidermal cell differentiation inhibitor from *Staphylococcus aureus*; VIP2: vegetative insecticidal protein from *Bacillus cereus*; iota: iota toxin from *Clostridium perfringens*; C2: C2 toxin from *Clostridium botulinum*; SpvB: Salmonella plasmid virulence protein B from *Salmonella enterica*; Mod A, B and Alt proteins from the T4 bacteriophage; LF: lethal factor from *Bacillus anthrax*; DT: Diphtheria toxin from *Corynebacterium diphtheriae* phage, ETA: exotoxin A from *Pseudomonas aeruginosa*, RART: rifampin ADP-riboslytransferase from *Mycobacterium smegmatis*, TpT: t-RNA-phosphotransferase from *Aeropyrum pernix*. (A) Mammalian ecto-ARTs are related to DRAT and to the VIP2-family of bacterial toxins. The NAD-binding crevice of this subfamily carries a characteristic R-S-E triad of amino acids and a characteristic core of conserved secondary structure units (see Figure 6). (B) Diphtheria toxin and related mammalian pARTs share a distinct set of conserved secondary structure units and carry a characteristic H-Y-E triad of amino acids within the NAD-binding crevice. The RART and TpT ART subfamilies show closer structural similarity to DT and to PARP than to the DRAT/VIP2 subfamily. (C) ADP-ribosylcyclases and sirtuins constitute two structurally distinct enzyme families, some members of which have been reported to catalyze protein ADP-ribosylations.

activity. The most prominent target of ART1 on skeletal muscle cells is the α7β1 integrin (38, 39). This protein is ADP-ribosylated on two distinct residues, which influences its binding to the extracellular matrix protein laminin (7). ART1 has also been shown to ADP-ribosylate and thereby modulate the function of soluble proteins, including the platelet derived growth factor PDGF-BB and the antimicrobial peptide defensin 1 (40, 41). The expression of ART1 on lung epithelial cells is up-regulated by pathogen associated molecular patterns (42).

A prominent target of ART2.2 on murine T cells is the integrin LFA-1 (37, 43) (Figure 3). ADP-ribosylation of LFA-1 inhibits homotypic aggregation of LFA-1 expressing cells (43). A second prominent target of ART2.2 is the P2X7 purinoceptor (44). ADP-ribosylation of P2X7 occurs on two amino acid residues (45). ADP-ribosylation of Arg125 activates P2X7 by presenting a covalent ligand to its nucleotide binding site. ADP-ribosylation of the second site (Arg133) does not activate P2X7, presumably because the ADP-ribose moiety at this position is out of reach of the ligand-binding site. However, ADP-ribosylation at this site may modulate the sensitivity of P2X7 to activation by the soluble ligand ATP. KO-mice have been described for the two *Art2* genes (46). ART2KO mice are healthy and fertile and do not show any overt phenotype under standard breeding conditions. *In vitro*, T cells from ART2KO mice are resistant to the anti-proliferative effects of extracellular NAD. In the Con-A hepatitis model for experimentally induced autoimmune hepatitis, ART2KO mice show a milder form of the disease (47). In the NOD mouse model of genetically determined autoimmune diabetes, ART2 deficiency by itself does not effect disease progression. However ART2-deficiency does retard an aggressive version of the disease observed in mice made genetically deficient for the major ecto-NADase CD38 (48).

Mammalian ARTs 1-4 are anchored to the cell membrane by covalent linkage of the C-terminal amino acid residue to the membrane glycolipid GPI (31) (Figure 3). The GPI-anchor likely mediates association of these ARTs with lipid rafts, specialized membrane microdomains enriched in cholesterol (49). Rafts play important roles in signal transduction, endocytosis, and synapse formation between immune cells (50, 51). In case of mouse ART2.2, it has been shown that the sequestration in lipid rafts focuses the target specificity of this enzyme onto other raft-associated proteins (49). Two distinct mechanisms have been identified by which ART2.2 can be released from the cell membrane as an active soluble ecto-enzyme: following T cell activation, the membrane-bound metalloprotease TNF-alpha converting enzyme (TACE) proteolytically cleaves ART2.2 in proximity to its juxtamembrane stalk (52). Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) and mammalian phospholipase D can release ART2.2 by cleavage within the GPI-moiety (31). Soluble ART2.2 efficiently ADP-ribosylates other soluble extracellular proteins including certain cytokines. The 3D-structure of a catalytically inactive mutant of rat ART2 in complex with NAD revealed a fold more closely related to that of the VIP2/SpvB2/C2/C3 subfamily of toxin ARTs than to PARPs (53, 54) (Figure 4). The members of the ART2 and VIP2-subfamily of toxins share a characteristic R-S-E triad of amino acids in the active site as well several secondary structure elements neither found in PARPs nor in diphtheria toxin.

Little is known about ART3 and ART4, which are apparently inactive members of the ecto-ART family (Table 2). ART3 is most prominently expressed in testes (55, 56). The expression of ART3 has recently been shown to be strongly upregulated in cardiomyocytes during corticosteroid treatment (57). ART4 is most prominently expressed on erythrocytes, monocytes and endothelial cells (58, 59). Its expression in strongly upregulated by Toll-receptor ligands (42, 59). On human erythrocytes ART4 has been identified as the carrier of the Dombrock alloantigens (58, 60, 61). Several amino acid substitutions as well as gene-inactivating mutations in human ART4 have been shown to underlie anti-ART4 antibody responses following blood transfusions. A few cases of homozygous carriers of ART4 inactivating mutants have been described that do not show any overt phenotype related to ART4 deficiency (62, 63).

6.2. Diphtheria toxin-related mammalian ARTs

The human genome contains two gene-families known or predicted to encode proteins harbouring a catalytic domain resembling diphtheria toxin (Table 1C, Figure 2B). TRPT1 is a single copy gene in humans and most other mammals and catalyzes the ADP-ribosylation of tRNA during removal of the terminal phosphate residue (19). We have recently proposed a classification of the family of PARP-1-related poly-ADP-ribosyltransferases (pARTs 1-17), based on structural considerations (24). The human genome encodes 17 PARP-1-related proteins, and the mouse genome 16 (17, 24). This difference in gene numbers is accounted for by a regional duplication in the human genome in the region encoding pARTs 7-9 (PARPs 15, 14, 9) (24). The PARP-like pARTs can be subdivided into five main subgroups on the basis of structural similarities and conserved intron positions (Figure 2B) (24). Subgroups 1 and 2 contain PARPs 1-3, vault PARP, and the two tankyrases. All of these harbour the H-Y-E motif present in diphtheria toxin and can catalyze poly ADP-ribosylation of target proteins and of themselves. The catalytic Glu residue is substituted by other amino acids (Leu, Thr, Ile, or Tyr) in members of subgroups 3-5 (24). It is tempting to speculate that some of these ARTs may function as mono-ADP-ribosyltransferase rather than poly-ADP-ribosyltransferases.

PARP-1 is one of the most abundant nuclear proteins and is activated by breaks in DNA (15-17). PARP-1 activation can have quite dramatic consequences, e.g. PARP activation after treatment of cells with high concentrations of DNA-damaging reagents can deplete the cellular NAD pool faster than it can be replenished. Moreover, the accumulation of PAR polymers induces mitochondria to release apoptosis inducing factor, indicating that PAR polymers may act as death signals (64). Interruption of oxygen supply to tissues following the clotting of arteries in stroke or heart attack is followed by

Table 2. Mammalian DRAT-related ARTs and DRAG-related ARHs

A) DRAT-related ecto-ARTs		
	expression	target/function
ART1	muscle, lung, neutrophils	arginine on integrins, defensin, platelet derived growth factor
ART2.1	macrophage mouse only	arginine, activated by reduction of allosteric disulfide
ART2.2	T cells rodents	arginine on integrins, P2X7 purinoceptor, shed as active enzyme by metalloprotease
ART3	testis	developmentally regulated, no enzyme activity yet detected
ART4	erythrocytes	carrier of the Dombrock blood group alloantigen, no enzyme activity yet detected
ART5	heart, lymphoma,	arginine, targets unknown, secretory enzyme associated non-covalently with other membrane proteins on lymphoma cells
B) DRAG-related ADP-ribosylhydrolases		
ARH1	ubiquitous, cytosolic	arginine-ADP-ribose
ARH2	muscle, cytosolic	unknown
ARH3	ubiquitous, mitochondrial, nuclear	poly-ADP-ribose, O-acetyl-ADP-ribose

reperfusion, causing massive cell death. Some of this collateral damage can be prevented by pharmacological inhibition or genetic deletion of PARP-1 (65, 66).

6.3. DRAG-related mammalian ARHs

The human genome encodes three proteins related to the ADP-ribosylarginine hydrolase of *Rhodospirillum rubrum,* DRAG (31) (Table 1C, Table 2B, Figure 5). ARH1 displays an enzyme activity similar to that of DRAG and can hydrolyze arginine-ADP-ribosyl linkages on most proteins and can even hydrolyze ADP-ribosyl-agmatine (20, 67). ARH1KO mice are healthy and fertile. Cells derived from these mice show enhanced sensitivity to the cytotoxic effects of cholera toxin (CT), an Arg-specific ART that ADP-ribosylates the α-subunit of heterotrimeric G proteins, suggesting that ARH1 may counteract the toxic effects of Arg- specific ARTs (68). ARH2 is the closest relative of ARH1, with which it shares 45% sequence identity, but it does not exhibit any detectable activity when expressed as a soluble recombinant protein (69). When expressed as a recombinant protein, ARH3 displayed unexpected enzyme activities, i.e. it could not de-ADP-ribosylate proteins mono-ADP-ribosylated on arginine, asparagine, cysteine or diphthamide residues, but it was well capable of hydrolyzing both ADP-ribosyl polymers as well as O-acetylated-ADP-ribose, a metabolite of the sirtuin family of NAD-dependent lysine deacetylases (70, 71). ARH3 has recently been shown to localize in nuclei and in mitochondria, where it could de-ADP-ribosylate PAR generated by a genetically engineered mitochondrial PARP (72).

The 3D-structure of human ARH3 shows an all α-helical protein fold with pseudo two fold symmetry. Two magnesium ions are bound at the bottom of the shallow putative active site crevice (Figure 6) (71). These magnesium ions are coordinated by acidic residues at the tip of four central, highly conserved α-helices. The structure is reminiscent of a pumpkin and this is the symbol we use to illustrate the ARHs here. The 3D-structure of mouse ARH3 (2QTY) is very similar to that of its human ortholog (see note added in proof). Two procaryotic proteins whose structures have been determined in structural proteomic projects show a similar fold: ttARH from the gram-negative eubacterium *Thermus thermophilus (*pdb code 2CWC) and mjARH from the archaeon *Methanococcus janaschii* (pdb code 1T5J) (Figure 5). The predicted active site residues of ARH3 are conserved in these proteins, suggesting that these proteins may be active enzymes.

An alignment of ARH3 and PARG amino acid sequences has been proposed to indicate a structural relationship (69). However, it is rather unlikely that the structure of ARH3 can serve as model for PARG, since secondary structure prediction programs indicate that PARG is likely to be a mixed αβ-protein (71). In the human and mouse, PARG is encoded by a single copy gene (73).

7. ART AND ARH FAMILY MEMBERS THAT LACK DETECTABLE ENZYME ACTIVITY

No enzymatic activities have yet been detected upon incubation of recombinantly produced ART3 or ART4 with NAD and mixtures of potential targets (31). In comparison to their promiscuous Arg-specific orthologs, ART3 and ART4 show conspicuous substitutions of key amino acids lining the active site crevice. It is conceivable that ART3 and ART4 have acquired novel target specificities or that they may have lost ART-enzyme activity. Similarly, conspicuous deviations from the canonical active site residues have also been observed for several members of the PARP subfamily (24). Eleven pARTs/PARPs lack the catalytic Glu and some of these do not show detectable ART activity *in vitro*.

One member of the ARH family also seems to lack enzyme activity. ARH2 hydrolyzes neither ADP-ribosyl-amino-acid nor ADP-ribosyl-ADP-ribose linkages (69). ARH2 shows conspicuous substitutions of several residues that coordinate the magnesium ions in ARH3 and it is not clear whether ARH2 binds metal ions. Moreover, in the jelly fish *Tripedalia crystophora*, members of the ARH family (J1-proteins) evidently have adopted a new structural function as lens crystallins (74) (Figure 5). Another member of the ARH-family (SelJ) from zebrafish has recently been shown to contain a selenocysteine residue. The expression of this protein is restricted to the eye lens and has been suggested to play a structural role (75).

Examples from other enzyme families have been reported for members that have lost activity while retaining the capacity to bind to ligands resembling the original substrate. Some of the secreted pattern recognition receptors of invertebrate immune systems seem to derive from enzyme domains. Similarly, domains related to NUDIX hydrolases (Nucleoside-diphosphate linked to some other moiety) and A1pp (ADP-ribose-1"-monophosphatase) with little if any residual enzyme

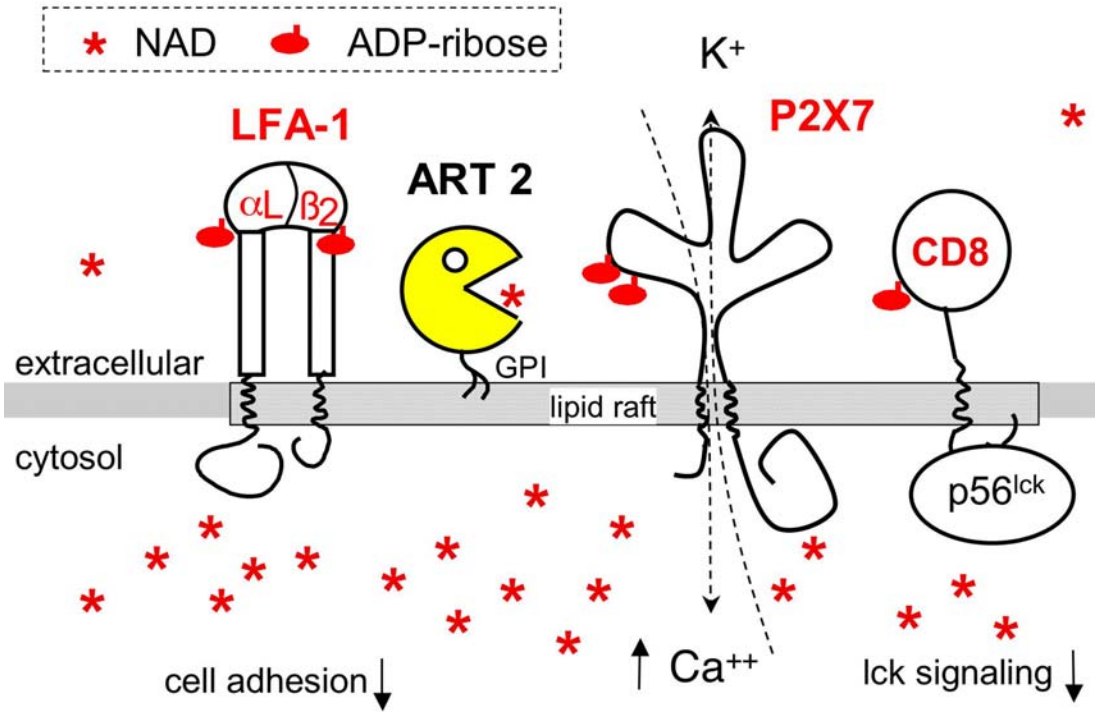

Figure 3. On T cells, raft associated ART2.2 ADP-ribosylates LFA-1, the P2X7 purinoceptor, and other proteins. The ART-substrate NAD is released from cells by lytic and non-lytic mechanisms. ART2.2 consists of an isolated catalytic domain, that is attached to the cell membrane by a covalent linkage of the C-terminal amino acid to the membrane glycolipid, glycosylphosphatidylinositol (GPI). The GPI-anchor restricts the local distribution of ART2.2 in the plasma membrane to cholesterol-rich microdomains, designated lipid rafts. On the T cell surface, ART2.2 is focused onto specific targets by virtue of its association with lipid rafts. ADP-ribosylation of LFA-1 blocks the interactions of LFA-1 with its ligands, ADP-ribosylation of CD8 blocks lck-mediated signal transduction, and ADP-ribosylation of P2X7 activates its cation channel function. Chronic activation of P2X7 by ADP-ribosylation induces the formation of a membrane pore, and ultimately cell death.

activity have been found in transmembrane receptors and nuclear proteins, and some of these may have acquired a new function as allosteric ADP-ribose binding domains (76, 77) .

8. THE "TOPOLOGY PARADOX" OF ARTS AND ARHS

DRAT and DRAG regulate nitrogen fixation by catalyzing ADP-ribosylation and de-ADP-ribosylation of dinitrogenase reductase at Arg101 (Figure1) (9). The detection and purification of similar enzyme activities from mammalian tissues (Figure 3, Figure 4) led to the prediction that reversible ADP-ribosylation cycles control physiological functions also in mammalian cells (4, 78). The successful purification, molecular cloning and structure determination of DRAT- and DRAG-related enzymes from human and other mammalian cells has lent support to this idea (13, 20). However, the results obtained to date also yielded an unexpected topological paradox that still needs to be resolved: the subcellular localization of ARTs as ecto-enzymes and of ARHs as cytosolic and mitochondrial enzymes seems to preclude their action on the same targets (Figure 7). Four DRAT-related ARTs (ART1-ART4) are expressed as GPI-anchored membrane proteins, the fifth (ART5) is a secreted enzyme, i.e. the entire protein component of these enzymes is extracellular (31). Conversely, two of the three DRAG-related ARHs are expressed as soluble cytosolic proteins, while the third is most prominently expressed in mitochondria (72). Indeed, PARP-1 and PARG is the only pair of enzymes for which convincing evidence has been obtained that they act on a common target. An apparent paradox for the pART-subfamily, on the other hand, is that there are 16-17 PARP-related proteins in mammals but only a single PARG, albeit with different isoforms (17). It is still unclear whether any of these PARG isoforms or the ARHs can de-ADP-ribosylate the targets of the other PARP-like ARTs (17).

These startling findings raise the question whether all mammalian enzymes capable of ADP-ribosylating and de-ADP-ribosylating proteins have already been identified or whether other mammalian ARTs and/or ARHs are yet to be discovered. Several lines of arguments can be put forth to suggest that such hitherto undiscovered enzymes do indeed exist. Firstly, a wealth of biochemical data points to the presence of ART and ARH enzyme activities in mammalian cells, for which none of the identified ARTs and ARHs are plausible candidates, such as the ADP-ribosylation of myelin basic protein (79), neurogranin (80), actin (81-83), glutamate dehydrogenase (84), the β-subunit of heterotrimeric G proteins (85),

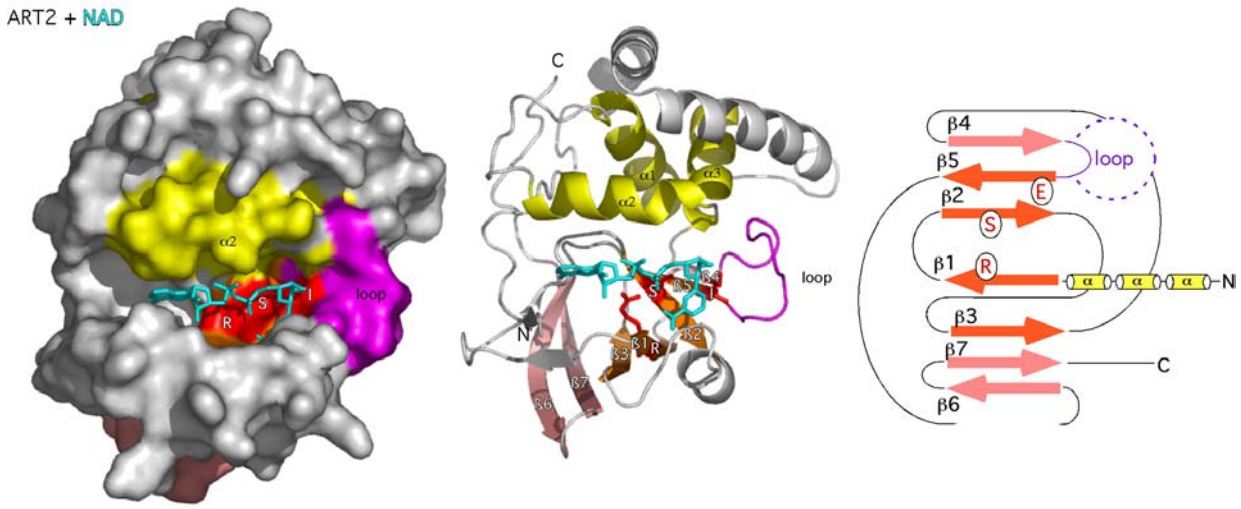

Figure 4. 3D-structure of a prototype mammalian ecto-ART. The Pacman-like fold of rat ART2 is similar to that of toxin ARTs and PARPs, consistent with the concept that these enzymes are derived from a common ancestor. The 3D-fold of ART2 with bound NAD more closely resembles that of VIP2 than that of PARP or DT. In ART2, as in all other ARTs that have been co-crystallized with NAD, the nicotinamide moiety is buried in a deep hydrophobic pocket while the rest of the molecule is stretched out along a wide crevice. Four anti-parallel β-strands form the core of this crevice (β1, β2, β3, and β5, highlighted in orange). Outside of this core, rat ART2 has greater structurally similarity to the VIP2/SpvB subfamily of toxin ARTs than to PARPs, i.e. the β-strands depicted in pink and the α-helices shown in yellow are found also in VIP2 and SpvB but not in CT or PARP. The side chains of a characteristic pair of amino acid residues in the two central β-strands (Arg in β1Ser in β2indicated in red flank the NAD-binding site and help to coordinate the nicotinamide-proximal ribose and phosphate groups. This pair of amino acids is one of the distinguishing features of the major ART subfamilies, i.e. these are Arg and Ser in the VIP2 and CT subfamilies, His and Tyr in the DT/PARP and TRPT1 subfamilies. Most but not all ARTs carry a characteristic Glu residue (E, in red) at the beginning of β5. The side chain of this residue is positioned close to the scissile glycosidic bond in NAD and likely coordinates the nucleophilic attack of the target on this linkage. A non-conserved surface loop (violet) just before this Glu may contribute to target specificity.

elongation factor 2 (86, 87), rho (88), BiP (89) and erythrocyte proteins (90).

Secondly, proteins of similar structure and function can have amino acid sequences that are too divergent to be recognized by sequence similarity searches. Indeed, even the powerful position sensitive interative search program PSI-BLAST fails to connect more distantly related subgroups of the ART-family (24, 31). Sometimes, the function of a protein can be predicted from its 3D-structure. To this end, structural genomics projects strive to uncover the structures of prototype members of protein families of unknown functions. And, indeed, the structure of one such protein - CC0527 of *Caulobacter crescentus* - (pdb code 2O0P) reveals striking similarity to the ART fold (our own unpublished observations). CC0527 is a member of the pfam protein family DUF952, which consists of several hypothetical bacterial and plant proteins of unknown function. Similarly, it is conceivable that some mammalian proteins of unknown function may turn out to harbour an unrecognized ART-fold.

Thirdly, proteins of different structures can acquire similar enzyme activities. Indeed, ART-activity has been discovered fortuitously as a side reaction for members of at least one completely different enzyme family - the sirtuin NAD-dependent lysine de-acetylases. SIRT4, catalyzes mono-ADP-ribosylation of a cysteine residue in glutamate dehydrogenase, the net reaction resembles the cysteine-specific ADP-ribosylation catalyzed by pertussis toxin (26). Further, rat ART2 and human ART5 share potent NAD-glycohydrolase activity with the structurally unrelated CD38 and ADP-ribose generated by these enzymes can attach covalently to free cysteine residues in proteins (25, 31).

Similarly, it is conceivable that enzymes structurally unrelated to DRAG/ARHs1-3 may catalyze de-ADP-ribosylation of proteins. For example, it has been shown that the ADP-ribose group on membrane proteins can be at least partially removed by extracellular phosphodiesterases (PDE) (e.g. of the PC-1/CD203 family of enzymes) (43, 91, 92). PDE-catalyzed cleavage of the phosphodiester bond in ADP-ribose would leave a phospho-ribosyl moiety attached to the target, which could be processed further by a phospho-ribosyl-hydrolase and/or by the combined action of an alkaline phosphatase (AP/CD) and a ribosyl-hydrolase. Finally, the structure of PARG is not yet known but is predicted to be distinct from that of ARH/DRAG (71). Thus, it is possible that the structure of PARG will provide a new lead toward other proteins capable of reversing ADP-ribosylation.

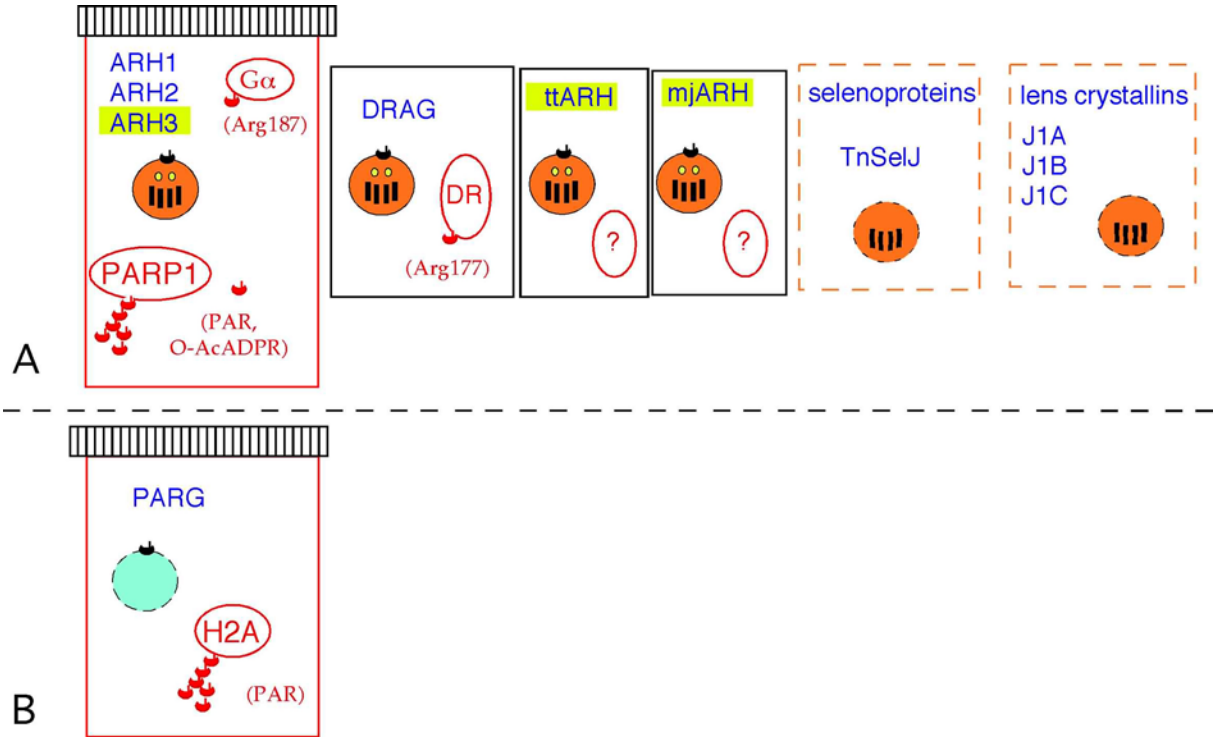

Figure 5. Gallery of DRAG-related mammalian ecto-ARHs and related enzymes. ARHs are grouped on the basis of structural and sequence similarities. The names of proteins with known 3D-structures are highlighted in yellow. The ARH catalytic domain is symbolized by a pumpkin in reference to four central α-helices that coordinate two magnesium ions at the bottom of a shallow active site crevice. ADP-ribose is indicated by a crescent (see Figure 6). Known targets are in red with the modified amino acids or ADP-ribosyl-linkages indicated in parentheses. (A) Mammalian ARHs are closely related to DRAG and to two predicted procaryotic proteins whose structures have been determined in structural proteomic projects: ttARH, a putative ARH from the gram-negative eubacterium *Thermus thermophilus (*pdb code 2CWC) and mjARH, a putative ARH from the archaeon *Methanococcus janaschii* (pdb code 1T5J). Pufferfish selenoproteins (TnSelJ) and jellyfish lens crystallins (J1A-J1C) show significant sequence similarity to the ARHs and are therefore predicted to exhibit a similar fold. However, these proteins show deviations in key active site residues and are predicted to exhibit little if any enzymatic activity. (B) The 3D-structure of PARG is not yet known, but secondary structure prediction programs indicate that its catalytic domain is a mixed alphabeta structure distinct from the all-α-helical ARH fold.

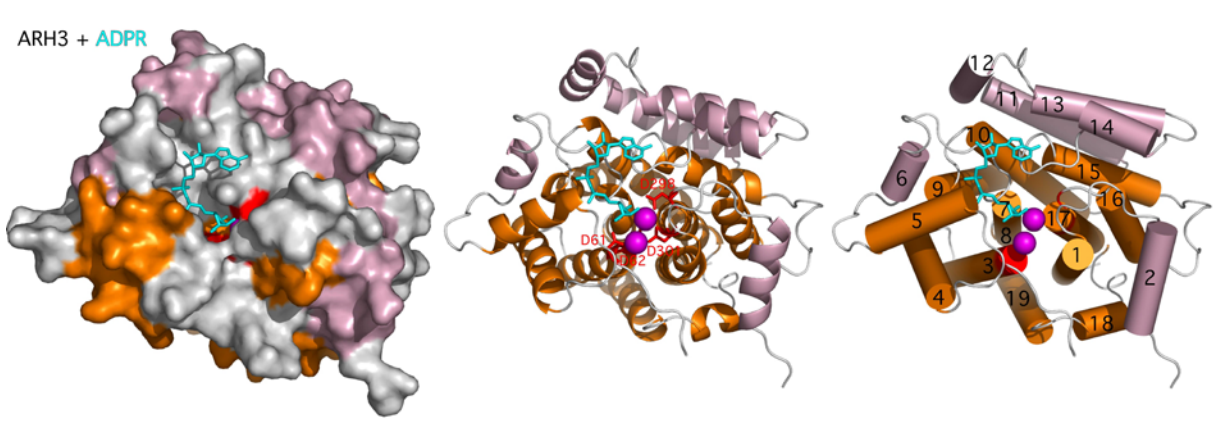

Figure 6. 3D-structure of a prototype human ARH. The pumpkin-like fold of ARH3 is composed only of α-helices. Four conserved anti-parallel core helices (1, 3, 7, 17 in orange) bear four acidic and two Thr residues that coordinate two magnesium ions (magenta). The core helices are surrounded by eight conserved (orange) and several non-conserved (pink) helices. Docking experiments indicate a possible mode for the binding of ADP-ribose in a shallow groove, suggesting that one of the magnesium ions may act as an electron sink to increase the lability of the scissile glycosidic linkage to the nucleophilic attack by a water molecule.

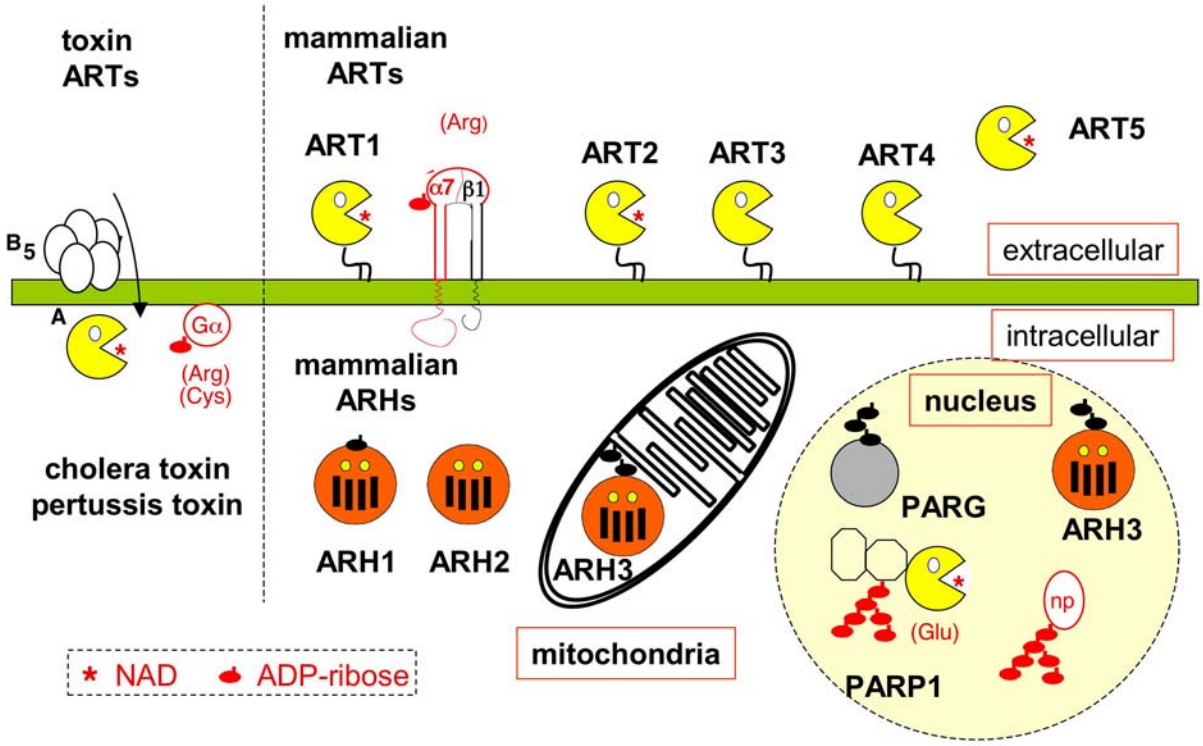

Figure 7. The topological paradox of mammalian ARTs and ARHs The DRAT-related ARTs and DRAG-related ARHs identified in mammals to date pose a topological paradox. The catalytic domains of these enzymes are localized in different cellular compartments. This would preclude them from acting on common targets - unless one of these enzymes can translocate across the cell membrane akin to the toxin ARTs. PARP-1 and the major full length isoform of PARG co-localize in the same cellular compartment and have been shown to poly-ADP-ribosylate and de-ADP-ribosylate common targets in the nucleus. Some family members, e.g. ART3 and ART4 (depicted without NAD) and ARH2 (depicted without ADP-ribose) do not exhibit any detectable enzyme activities, suggesting that they may have acquired novel functions.

9. PERSPECTIVES

Recent years have witnessed many exciting discoveries regarding mammalian ADP-ribosyltransferases, ADP-ribosylhydrolases, and related enzymes. The results have raised many new questions so that equally exciting and perhaps surprising discoveries on these intriguing enzymes can be expected in the future. An important endeavour will be to determine which of the molecularly characterized ARTs and ARHs are enzymatically active and to determine their target specificities. To this end, the crystal structures of more ART and ARH family members will provide leads for functional studies, e.g. site directed mutagenesis experiments. Moreover, crystal structures of ADP-ribosylated target proteins as well as co-crystals of ARHs and PARGs with ADP-ribose will pave the way for structure-based design of ARH and PARG inhibitors. New knock-out and transgenic mouse models may provide clues on the *in vivo* function of other ART and ARH family members. Last not least, several biochemically characterized enzyme activities, including cytosolic ARTs and the ADP-ribosyl-glutamate lyase, yet await identification on the molecular level. Classical protein purification and sequencing may uncover hitherto unrecognized enzyme subfamilies.

10. ACKNOWLEDGEMENTS

This review is dedicated to Helmut Hilz and Heinz-Günther Thiele, two Hamburgian pioneers of ADP-ribosylation research.This work was supported by grants from the Deutsche Forschungsgemeinschaft (to FK-N, FH, and MSW) and the Studienstiftung des Deutschen Volkes (to SK).

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Note added in proof: After completion of this manuscript, two studies appeared describing the 3D-structure of mouse ARH3 (2QTY) and its relationship to ttARH (2CWC) and mjARH (1T5J) (98) and the 3D-structure of the DT/PARP-related Rifampin ADP-riboslytransferase from *Mycobacterium smegmatis* (2HW2) (99).

Key Words: ADP-Ribosylation, Posttranslational Protein Modification, 3D-structures, Enzyme Design, Review

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