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State of the art of protein mono-ADP-ribosylation: biological role and therapeutic potential

Gaia Fabrizio[1], Emanuele Salvatore Scarpa[1], Maria Di Girolamo[1]

[1]G-Protein-Mediated Signalling Laboratory, Department of Cellular and Translational Pharmacology, Mario Negri Sud Foundation, Via Nazionale, 8/A, 66030 Santa Maria Imbaro (CH), Italy

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1. ABSTRACT

Mono-ADP-ribosylation is a post-translational modification that was discovered more than five decades ago, and it consists of the enzymatic transfer of ADP-ribose from NAD^+ to acceptor proteins. In viruses and prokaryotes, mono-ADP-ribosylation is mainly, but not exclusively, a mechanism used to take control of the host cell. In mammals, mono-ADP-ribosylation serves to regulate protein functions, and it is catalysed by two families of toxin-related cellular ADP-ribosyltransferases: ecto-enzymes that modify various cell-surface proteins, like integrins and receptors, and intracellular enzymes that act on a variety of nuclear and cytosolic proteins. These two families have been recently renamed the ARTCs (clostridia toxin like) and ARTDs (diphtheria toxin like), depending on their conserved structural features, and in terms of their relationships to the bacterial toxins. In addition, two members of the structurally non-related sirtuin family can also modify cellular proteins by mono-ADP-ribosylation. Recently, new examples of ADP-ribosylation of proteins involved in signal transduction and intracellular trafficking have been discovered, thus opening the route to the better molecular understanding of this reaction and of its role in human cell physiology and pathology.

2. INTRODUCTION

Cells and organisms regulate protein functions through a variety of post-translational modifications that generally consist of the covalent binding of small molecules to specific amino-acid residues, which leads to target-protein activation or inactivation, and which often changes the three-dimensional structure of the target protein (reviewed by (1)). Through reversible post-translational modifications of proteins, cells can react quickly to changes that occur in their internal or external environments. The number of protein post-translational modifications that have been described to have fundamental biological roles is

now approaching three hundred. These various, reversible, post-translational modifications regulate enzyme activities, proteins–protein interactions, subcellular location of proteins, and protein degradation, thus ultimately regulating the full plethora of protein activities in the cell.

The most intensively studied modification is protein phosphorylation, which involves the attachment of phosphate moieties to serine, threonine or tyrosine residues by protein kinases. Beyond this, together with poly-ADP-ribosylation, mono-ADP-ribosylation is among the more and more studied post-translational modifications. Mono-ADP-ribosylation involves the attachment of one unit of ADP-ribose to an arginine, lysine, cysteine or glutamate residue of the target protein by the ADP-ribosyltransferase (ART) enzymes. Lysine residues can also be post-translationally modified by acetylation, which is catalysed by acetyltransferases. All of these post-translational modifications are reversible. Thus phosphorylation is reversed by phosphateses, ADP-ribosylation by specific hydrolases (ARH and macro-proteins), and protein lysine acetylation by deacetylases (HDACs and sirtuins). In addition to the transfer and binding of small molecules to proteins, post-translational modifications also include the binding of macromolecules to proteins, such as polymers of ADP-ribose, certain lipids (prenylation, glycosylphosphatidylinositol (GPI)-anchoring), small proteins (ubiquitinylation, sumoylation), and carbohydrates (glycosylation). Taken together, these modifications are basically involved in all cellular functions, including the cell cycle, apoptosis, autophagy, aging, angiogenesis, metabolism, signal transduction, cell proliferation and cancer.

This review is focused on mono-ADP-ribosylation, a post-translational modification of proteins that was first described more than five decades ago (2). Here we report on the pathogenic role of viruses and of bacterial toxins with ART activity, and then we discuss recent advances in the biological roles of mono-ADP-ribosylation of cellular proteins, as catalysed by the eukaryotic ART enzymes. Thus, this review is focused on the active members of the ARTCs (clostridia toxin like) ectoenzymes that modify various cell surface proteins, and on the active ARTDs (diphtheria toxin like), as intracellular enzymes that act on a variety of nuclear and cytosolic proteins. Sirtuins that show ART activity are also briefly introduced.

This is a challenging and exciting time in the field of mono-ADP-ribosylation, as new enzymes that can catalyse this post-translational modification, and new ADP-ribosylated proteins with central roles in signal transduction and intracellular trafficking, are being discovered. Thus, we can expect many other exciting discoveries in the study of mono-ADP-ribosylation that will lead to new knowledge in the near future. Therefore, this is the right time to more heavily invest in a better understanding of how these ART enzymes work inside and outside the cell, and in their regulation of cell physiology and pathology.

3. MONO-ADP-RIBOSYLATION AS A PATHOGENIC MECHANISM

Mono-ADP-ribosylation is a phylogenetically ancient and crucial mechanism for the regulation of protein function. This post-translational modification was originally identified as the mechanism of action of the bacterial toxins (3); however, it is also used by viruses, like bacteriophage T4, to regulate transcription, and thus to impose their genetic programme over that of an infected host cell (4). It is believed that the bacterial ADP-ribosylating toxins themselves derive from the genome of lysogenic phages, on the basis that some bacterial toxins are encoded by lysogenic phages incorporated into the DNA of their host bacteria. Proof of concept is provided by cholera toxin, which is encoded by a lysogenic bacteriophage. The genes encoding the cholera enterotoxin A and B components (see below) exist in *Vibrio cholera* as a prophage, the integrated form of the phage genome in the host bacterial chromosome (5).

Bacteriophage T4 is a ubiquitous phage, even if its main host is *Escherichia coli*, and it comprises the head, which contains the dsDNA, and the contractile tail. It encodes three ARTs: the 76 kDa Alt, and the two 24 kDa Mod enzymes (ModA, ModB). These enzymes participate in the regulation of the T4 replication cycle through ADP-ribosylation of a distinct group of host proteins (reviewed by (6)). The main target for Alt and ModA is *E. coli* RNA polymerase (7). Alt is a component of the phage head and enters the host cell in the process of infection, together with the phage DNA, to immediately ADP- ribosylate one of the two α-subunits of the host RNA polymerase on arginine 265; the newly synthesised ModA then completes the modification of the other α-subunit at the same arginine (8). This ADP-ribosylation of the host RNA polymerase enhances viral transcription from T4 early promoters

(9). In all, 27 and 8 proteins have been identified as the putative targets of Alt and ModB, respectively, by mass spectrometry (10). The Alt enzyme not only ADP-ribosylates the a-subunit of RNA polymerase, but also its β-, β'- and σ70 subunits (11), along with proteins of the translational apparatus, including elongation factor EF-Tu, the chaperone known as the trigger factor, prolyl-tRNA synthetase, and GroEL (10). Both EF-Tu and the trigger factor are modified also by ModB, which in addition ADP-ribosylates the ribosomal protein S1 (11). It has been shown that ModB modifies the trigger factor on its arginine 45, which is part of a domain that is known to interact with the ribosomal protein L23 (i.e., Phe44-Arg45-Lys46). Thus, this modification might affect the interactions of the chaperone with ribosomes, to inhibit protein translation. The ADP-ribosylation of EF-Tu and of ribosomal proteins, in addition to ribosome-associated proteins, can further affect protein synthesis in the infected cell.

Similar to the viruses, diphtheria, cholera, pertussis, clostridia and many other bacterial toxins are mono-ARTs that can modify specific host proteins after their translocation into the mammalian host cell, which results in pathological situations. Diphtheria toxin is secreted by *Corynebacterium diphtheriae*, and it was the first toxin found to act as an ART. In the cytosol of the host cell, diphtheria toxin catalyses ADP-ribosylation of the diphthamide residue 175 of elongation factor 2 (EF-2) (12), which is an essential component of the translational machinery, and thus this ADP-ribosylation inhibits protein synthesis. Following diphtheria toxin, other bacteria (e.g., *Vibrio cholerae*, *Bordetella pertussis*, *Clostridium difficile*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) were discovered to secrete toxins that catalyse ADP-ribosylation of key host-cell proteins, including again EF-2, and also the α subunit of the heterotrimeric guanine-nucleotide-binding (G) proteins, the small GTPases Rho and Rac, and monomeric actin (see (13-24), and REFERENCES therein), thus causing diseases such as diphtheria, diarrhoea and whooping cough. The main features of these bacterial toxins have been expertly and intriguingly reviewed recently, and thus we refer the reader to these reviews for discussions about both structural aspects (25) and their mechanisms of action (23, 24, 26).

Briefly, diphtheria toxin and exotoxin A, which is secreted by *Pseudomonas aeruginosa*, ribosylate the same diphthamide residue of EF-2. These toxins consist of a single polypeptide with AB structure–function properties, which is composed of three distinct domains. In the case of diphtheria toxin, these are: an N-terminal ART domain (the A component), a central translocation domain, and a C-terminal receptor-binding domain (which together form the B component) (27). The B component of diphtheria toxin binds to the cell surface heparin-binding epidermal growth factor (HB-EGF)-like precursor (28), thus forming a complex that is internalised by receptor-mediated endocytosis. The A component is then translocated to the cytosol of the host cell, causing the human disease known as diphtheria. The same domains, but in the opposite order, characterise exotoxin A. This toxin binds to LDL receptor-related protein 1 (29), enters the cell via receptor-mediated endocytosis, and then reaches the endoplasmic reticulum (ER) by retrograde transport, from where the A component is translocated into the host cytoplasm (reviewed by (23, 24)). ART activity has been found also in ExoS and ExoT from *Pseudomonas aeruginosa*, two toxins that can be delivered directly into a host cell using the bacterial type III secretion apparatus, and that modify Ras on arginine 41 and CT10-regulator of kinase (Crk), respectively (18).

A second group of toxins with ART acivity include *Clostridium botulinum* C2 toxin, *Clostridium perfringens* Iota toxin, *Clostridium spiroforme* toxin, *Clostridium difficile* toxin, and *Bacillus cereus* vegetative insecticidal protein (VIP2). All of these toxins consist of two separate A and B components that act in concert to intoxicate eukaryotic cells. Thus, the B component forms a complex with the A component (the ART domain) and mediates toxin binding to a cell-surface receptor, which then activates receptor-mediated endocytosis, allowing the translocation of the A component from the acidic endosomes into the cytosol. All of these toxins ADP-ribosylate actin on arginine 177, then modified actin binds to the fast polymerising barbed ends of actin filaments while leaving the pointed end of the actin filaments free, where actin usually depolymerises, thus inhibiting actin polymerisation and destroying the actin cytoskeleton (reviewed in (23, 24)).

The cholera and the heat-labile enterotoxins LT-1 and LT-2 from *E. coli* ADP-ribosylate arginine 201 of the α subunit of the Gs protein, which inhibits its GTPase activity, while pertussis toxin ADP-ribosylates a cysteine of the α subunit of the Gi, Go and Gt proteins. These toxins are members of the AB5 family that is characterised by a monomeric A subunit and a pentameric B component. As before, the function of the B component is to mediate the

binding of these toxins to specific components on the target cell, thus initiating toxin internalisation and translocation into the cytosol (30). The detailed mechanisms underling intoxication by these toxins have been reviewed previously (26).

Studies of the structure, intracellular trafficking, and identification of novel targets of the ART bacterial toxins have provided continuous advances in our understanding of their mechanisms of action. Bacterial toxins have been used as vaccines, and more recently, for the development of novel therapies to treat human diseases, by taking advantage of their delivery into human cells. For instance, Exotoxin A fusion proteins have been used in conjugate immunotoxin therapies to target and kill cancer cells (31-34).

4. MONO-ADP-RIBOSYLATION AS A PHYSIOLOGICAL REGULATORY MECHANISM IN PROKARYOTES

Of note, bacteria use mono-ADP-ribosylation not only as a pathogenic mechanism, but also as a physiological mechanism, to regulate certain of their own cellular functions, and in this last case, the mono-ADP-ribosylation has been shown to be reversible.

The first studies started 40 years ago, and were promoted by Ludden and coworkers in their elucidation of the regulatory mechanism of nitrogen fixation through mono-ADP-ribosylation in the photosynthetic bacterium *Rodospirillum rubrum* (35, 36). The activity of the dinitrogenase reductase is controlled by reversible mono-ADP-ribosylation that is catalysed by an ART activity, known as DRAT (dinitrogenase reductase ADP-ribosyltransferase), that modifies arginine 101 of dinitrogenase reductase (37, 38). The dinitrogenase reductase is then fully reactivated by an ADP-ribosylarginine-hydrolase known as DRAG (dinitrogenase reductase activating glycohydrolase). The molecular and functional details of this cycle were recently reviewed by (39). Thus, *R. rubrum* represents the first example of how a vital biochemical process like nitrogen fixation is tightly regulated through an ADP-ribosylation cycle.

5. MONO-ADP-RIBOSYLATION IS A PHYSIOLOGICAL REGULATORY MECHANISM IN EUKARYOTES

Molecular cloning and three-dimensional structural analyses have revealed enzymes that are related to bacterial ARTs in all kingdoms of life. In eukaryotes, these enzymes transfer one (mono) or several (poly) units of ADP-ribose from βNAD^+ to a specific amino acid of the target protein, with the formation of an *N*- or *S*-glycosidic (mono) or an *O*-glycosidic (poly) bond and the release of nicotinamide (40-42). These ARTs ADP-ribosylate their target proteins on specific amino acids, including diphthamide, arginine, cysteine, asparagine, threonine, glutamine, lysine and glutamate, as well as nucleotides and antibiotics. In line with the concept that more than 200 genes in the human genome are of bacterial origin, Pallen hyphothesised that the human mono-ARTs were acquired by horizontal gene transfer from bacteria to vertebrates (14).

Post-translational ADP-ribosylation of the various cellular protein targets is associated with the regulation of many intracellular pathways that are critical for gene transcription, cell signalling, mitosis, metabolism and apoptosis. There are also ever-increasing numbers of reports that link mono-ADP-ribosylation to human diseases, including inflammation, neurodegeneration and cancer metastasis. The ARTC ectoenzymes and the ARTDs intracellular enzymes, which are named according to the prototype bacterial toxin that their structural aspects resemble (43), are the main regulators of these functions. In addition, SIRT4 and SIRT6, two members of the sirtuin family, have been reported to have ART activity.

5.1. The mono-ADP-ribosylation cycle

Both mono- and poly-ADP-ribosylation are reversible reactions. Specific hydrolase enzymes can cleave these covalent bonds to release the target protein in its unmodified form, which is then potentially ready for the next round of modifications. Poly-ADP-ribose (PAR)-glycohydrolase (PARG) can specifically hydrolyse the ribose–ribose bonds in PAR chains (44), while the ADP-ribosylarginine hydrolase ARH1 cleaves the mono-ADP-ribose–protein bond (45).

Recently, it was shown that ARH1 activity can counteract the ADP-ribosylation of $G\alpha s$ that is catalysed by cholera toxin, and that cellular knocked-down for ARH1 shows increased sensitivity to the deleterious effects of this toxin (46); however, when disease occurs, the extremely efficient ART activity of cholera toxin cannot be reversed by ARH1. In addition to ARH1, the ARH family includes two other members: ARH2, which does not hydrolyse any known substrate (47), and ARH3,

which catalyses the hydrolysis of poly-ADP-ribose and O-acetyl-ADP-ribose, the metabolite generated during sirtuin-mediated deacetylation (48-50). Thus, the hydrolysis of PAR chains is catalysed by PARG and by structurally unrelated ARH3, both of which, however, cannot cleave the terminal ADP-ribose of the chain that is directly linked to the protein (48, 51). The enzymatic activity responsible for this reaction has been recently revealed to be a function of certain macro-domain–containing proteins, such as MACROD1, MACROD2 and TARG1 (52), which can reverse mono-ADP-ribosylation on glutamate and lysine.

The first macro-domain was discovered more than 20 years ago, but only in more recent years has the discovery that the macro-domain can bind ADP-ribose (53) opened up new and different research areas, thus definitively establishing the connection between macro-domains and ADP-ribosylation reactions. Members of the macro-domain family have been conserved throughout evolution, with homologues identified in viruses, archea, bacteria, *Drosophila melanogaster*, *Xenopus laevis*, plants and mammals, which include 11 human members (54-56). Different macro-domains can interact with monomeric, polymeric, or both forms of ADP-ribose, or with other NAD derivatives (57, 58). We have shown that the macro-domain of the Af1521 protein from *Archaeoglobus fulgidus* can bind not only ADP-ribose, but also ADP-ribosylated proteins (59), which is in line with the concept that macro-domains represent protein–protein interaction domains. Indeed, the macro-domain of the histone variant macroH2A1.1. and the chromatin remodeller ALC1 are binding modules for PAR (52, 60, 61).

Moreover, while ARTD8, MACROD1, MACROD2 and TARG1 (52, 58, 62-64) have been associated with mono-ADP-ribosylation and demonstrated to reverse this reaction, other macro-domains include O-acetyl-ADP-ribose binding modules that can efficiently catalyse the hydrolysis of O-acetyl-ADP-ribose (65-67). Thus, all of these macro-domain–containing proteins are mono-ADP-ribosylhydrolases that can reverse mono-ADP-ribosylation.

5.2. The mono-ADP-ribosylation orphan of ART

Several proteins with key roles in various cellular functions have been reported to be targets of mono-ADP-ribosylation reactions. In most cases, the modified amino acids have been identified, while the specific enzymes that are responsible for these modifications are still not known. For instance, we have reported on the enzymatic mono-ADP-ribosylation of arginine 129 of the G-protein β subunit, cellular ADP-ribosylation/ de-ADP-ribosylation cycle controls the functional activation/ inactivation cycle of the G$\beta\gamma$ heterodimer, thus regulating intracellular signalling events that are initiated by the activation of G-protein–coupled receptors (42, 68-70). Another yet-to-be identified cellular ART modifies EF-2 and inhibits *in-vitro* protein synthesis in mammals (71). Also, actin and other components of the actin regulatory machinery have been reported to be mono-ADP-ribosylated. In human HL-60 cells, inhibitors of the arginine-specific mono-ART have been reported to block the depolymerisation of actin (72). Similarly, ADP-ribosylation of tubulin inhibits its assembly, which suggests that this modification can control cytoskeletal organisation in the brain (73). Moreover, ADP-ribosylation factor 6 (ARF6) is a protein that is involved in the reorganisation of the cortical actin cytoskeleton (74-76), and it is also modified by mono-ADP-ribosylation on arginine 110 (42, 70). For a recent review of the many targets of mono-ADP-ribosylation and their roles in cell biology, see (42).

6. THE MONO-ADP-RIBOSYLTRANSFERASES

These endogenous ARTs comprise three distinct families of enzymes, and each family includes both active ARTs and inactive proteins, which raises the possibility that the inactive members have evolved to perform distinct functions that are often related to the functions of the active members. In the following sections, we will discuss the mono-ADP-ribosylation reactions that are catalysed by the active ectoenzymes of the ARTC family, and by the active intracellular mono-ARTs of the ARTD family. The ARTC family is composed of a small group of proteins (four in human) that are either active mono-ARTs (human (h)ARTC1, hARTC5) or inactive proteins (hARTC3, hARTC4). The larger family of the ARTDs is composed of 17 proteins, of which some are poly-ARTs (PARP1 to PARP5; also known as ARTD1 to ARTD6), the majority are mono-ARTs (ARTD7, ARTD8, ARTD10 to ARTD12, ARTD14 to ARTD17), and two are inactive proteins (ARTD9, ARTD13). Finally, mono-ART activity has been reported also for two members of the sirtuin family. The sirtuin family is composed of seven proteins, which again include active and inactive ARTs, where the non-ART sirtuins are deacetylases (see Table 1 for the active ARTs).

Table 1. Active mono-ARTs

Family	Nomenclature (alternative names)	Catalytic motif	Cellular localization	Target	Activators	Physiological roles	Diseases	Ref.
ARTC	ARTC1 (ART1)	R-S-E	Cell membrane, Endoplasmic reticulum	Integrin α7, HNP-1, GRP78**	FMLP, platelet-activating factor, IL8, Stress	Myotube differentiation, Pro-inflammatory, Protein folding and ER stress response	Cancer (colon carcinoma), Inflammation	92, 93
ARTC	ARTC2* (ART2/RT6)	R-S-E	Cell membrane	P_2X_7	LPS, IFN	Pro-inflammatory	Inflammation	91
ARTC	ARTC5 (ART5)	R-S-E	Secreted	Arginine	-	Unknown	Unknown	
ARTD	ARTD7 (PARP15/BAL3)	H-Y-L	Stress Granules	Auto-modification	IL4	Stress Granules Assembly	Cancer (DLBCL)	121, 122
ARTD	ARTD8 (PARP14/BAL2/ CoaSt6)	H-Y-L	Nucleus (main), Cell periphery, Stress Granules	HDAC2/3, p100	IL4	IL4- and Stat6-dependent transcription	Cancer (DLBCL; B-lymphoma), Inflammation (AAD)	124, 125, 128-131
ARTD	ARTD10 (PARP10)	H-Y-I	Cytoplasm (main), Nucleus	GSK3β, NEMO, Ran	IFN	Inhibits Cell proliferation, Pro-apoptotic	Neurological disease (ALS), Inflammation	58, 114, 115, 117, 119
ARTD	ARTD11 (PARP11)	H-Y-I	Nucleus (main), Cytoplasm (additional)	Unknown	-	Unknown	Unknown	
ARTD	ARTD12 (PARP12/ ZC3HDC1)	H-Y-I	Golgi apparatus, Stress Granules	Unknown	IFN	Stress Granules Assembly	Alphaviruses clearance	150, 151
ARTD	ARTD14 (PARP7/ TiPARP/RM1)	H-Y-I	Nucleus	PEPCK	IFN TCDD	Gluconeogenesis	Unknown	145-149
ARTD	ARTD15 (PARP16)	H-Y-Y	Endoplasmic reticulum	IRE1α, PERK, Kapβ1	Stress	Nucleo-cytoplasmic transport, UPR	Inflammation	132, 141
ARTD	ARTD16 (PARP8)	H-Y-I	Nucleus (main), Cytoplasm (additional)	Unknown	-	Unknown	Unknown	
ARTD	ARTD17 (PARP6)	H-Y-I	Cell membrane (main)	Unknown	-	Inhibits cell cycle progression	Cancer (colorectal)	144
SIRTUINS	SIRTUIN4 (SIRT4)	-	Mitochondria	GDH	Calorie restriction	Insulin secretion, Glutamate metabolism	Metabolic disease	155
SIRTUINS	SIRTUIN6 (SIRT6)	-	Nucleus	ARTD1/ PARP1	-	DNA repair	Cancer	157-159

6.1. The mono-ARTCs

The mammalian ARTC family is composed of the ecto-ARTs, which are enzymes that have the catalytic domain oriented to the outside of the cytosolic compartment. There are four hARTC proteins, in contrast to the well-studied murine

family, which is composed of six subtypes (ARTC1, 2.1., 2.2., 3, 4, 5). Thus, in human, ARTC expression includes the two arginine-specific ARTs, hARTC1 and hARTC5, and the two catalytically inactive proteins hARTC3 and hARTC4 (77); for the gene encoding ARTC2, which is duplicated in mouse (78), this is instead a pseudogene in human (79). These ARTCs are GPI-anchored proteins, with the only exception of ARTC5, which is a secreted enzyme (80-83). These proteins are characterised by an α-helix–rich N-terminal region, which represents a signal sequence for extracellular proteins, and by a C-terminal region folded into β-sheets, which is characteristic of GPI-anchored membrane proteins. Human and mouse ART5 lack this hydrophobic C-terminal signal sequence, and thus are secreted enzymes (84-86). The ARTC family is structurally related to the clostridial toxins and is characterised by a catalytic domain that is encoded by a single exon and is composed of 70-100 amino acids that can be divided into three regions: an N-terminal region, which contains a conserved arginine residue; a central region, which is characterised by the serine-x-serine motif (where x can be threonine, serine or alanine) and is involved in the binding of NAD^+; and a C-terminal highly acidic region, which contains the catalytically crucial glutamate residue (87, 88). The sequence arginine-serine-glutamate-glutamine-glutamate (R-S-EQE; which spans regions 1–2–3, respectively) is typical of the arginine-specific ARTs, and it is found in all of the active ARTC enzymes: ARTC1, 2 and 5. ARTC3 and ARTC4 do not have measurable activities (89, 90), and thus their roles in cell biology are independent of ART activity, a situation that is also seen in the ARTD family, which includes the inactive ARTD9 and ARTD13 members, and in the sirtuin family, which is composed of seven members, only two of which are active as mono-ARTs.

Thus, the ARTC family can be regarded as a family of arginine-specific ARTs that can modify soluble or plasma-membrane-associated proteins. Human ARTC1 and murine ARTC2 are the most studied members, and they can modify human neutrophil protein 1 (HNP1) and the P2X7 ATP-gated cation channel, respectively. These thus possibly have roles in inflammatory and immune responses. Moreover, the mono-ADP-ribosylation of integrin a7, which is catalysed by hARTC1, has been defined as having a role in myotube differentiation (91-93).

The first relationship between ARTC and inflammation was shown for the two murine ARTC2 isoforms that are expressed in T cells and macrophages (only the 2.1. isoform) (94). Moreover, mediators of inflammatory responses up-regulate ARTC2.1. in macrophages and down-regulate ARTC2.2. in T-cells, such as lipopolysaccharide (LPS) and interferon (IFN)-β and IFN-γ. When activated by NAD^+, ARTC2.2. catalyses mono-ADP-ribosylation of arginine 127 of P2X7; this ARTC2.2./P2X7 pathway leads to cell death, which is a crucial component in immune/ inflammatory responses. The concentration of extracellular NAD^+ in serum is low (0.1.-0.3. μM) and thus it is below the Km of the ARTs, which suggests that under basal conditions, the ARTCs are inactive and that their activation can only occur when the levels of extracellular NAD^+ increase. Intracellular NAD^+ (ca. 500 μM) can be released into the extracellular space under physiopathological conditions caused by tissue injuries, like acute inflammation. Under these conditions, the cells that express higher levels of P2X7 can become more prone to cell death. This is the case for regulatory T cells, which express higher levels of P2X7 than effector T cells, and which have an inhibitory function towards effector T cells. Under conditions that lead to an extracellular NAD^+ increase, the regulatory T cells can be selectively reduced in number by activation of this ARTC2.2./P2X7 pathway, thus promoting an immune/ inflammatory response. Furthermore, a pro-inflammatory environment can result in the accumulation of reducing agents at inflammatory sites, and can thus also lead to activation of ARTC2.1., which specifically requires the reduction of a disulfide bridge in this isoform.

It appears reasonable to consider ARTC1 as the human counterpart of ARTC2, as they share some substrates, like integrin. ARTC1 is widely expressed, although at low levels. Of note, ARTC1 can participate in immune/ inflammatory responses through its ability to mono-ADP-ribosylate the defensin HNP-1 (92, 95), an arginine-rich peptide that is considered to be the major component of innate antimicrobial immunity (96, 97). ADP-ribosylated HNP-1 is generated during inflammatory responses, and once modified, it loses its antimicrobial and cytotoxic activity, although it can still function as a chemoattractant for recruiting leukocytes (92, 95). Moreover, the presence of ARTC1 on the cell surface of leukocytes has been shown to be rapidly induced as a consequence of the exposure of polymorphonuclear leukocytes to the chemotactic peptide formyl-Met-Leu-Phe, or to platelet-activating factor or the chemokine interleukin (IL)-8 (98). This thus suggests that

hARTC1 is involved in chemotactic responses of polymorphonuclear leukocytes, events that are central to immune and inflammatory processes.

Intriguingly, we have very recently demonstrated that ARTC1 localises not only to the cell periphery, but also to the ER. In line with the concept that the catalytic domain is oriented outside the cytosolic compartment, this ARTC1 catalyses mono-ADP-ribosylation of the ER luminal chaperone GRP78/BiP (Fabrizio *et al*., manuscript in preparation), a protein that has crucial roles in protein folding and ER stress responses, and that has also been implicated in inflammation and cancer. Elegant experiments performed in the Ziegler laboratory by means of targeted expression of the catalytic domain of PARP1/ARTD1 have allowed the visualisation of intracellular NAD^+ pools in different compartments, including the ER (99). A role for ARTC1 in colorectal carcinoma has also been very recently highlighted, with higher expression of ARTC1 (in addition to PARP1/ARTD1) shown in 63 human colon carcinoma tissue samples. In CT26 cells, which represent a murine model of colon adenocarcinoma, silencing of hARTC1 or inhibition of its catalytic activity decreases the level of PARP1/ARTD1 and apparently sensitises these CT26 cells to cisplatin-induced cell death (100, 101).

6.2. The mono-ARTDs

PARP1 (also known as ARTD1) is the founding and most extensively studied member of the PARP family (102, 103). PARP1 is activated by DNA strand breaks, and its role in cellular responses to genotoxic and oxidative stress is widely recognised and has been widely studied, with various PARP inhibitors under evaluation in several clinical trials as potential anti-inflammatory and anti-cancer therapies (104-109). In human, of the 17 proteins that constitute the ARTD family (43, 110, 111), nine have been reported to act as mono-ARTs (112). While ARTD1-6 are typical PARPs, the other members have lost the conserved glutamate residue of the H-Y-E triad, which is crucial for polymer elongation, and so they are either inactive or are active as mono-ARTs. In the following sections we will discuss only those ARTD members that can act as mono-ARTs.

6.2.1. ARTD10

ARTD10 is a 150-kDa enzyme that comprises several domains of potential functional relevance, and it was the first ARTD to be characterised as a mono-ART (112). In addition to the C-terminal ART catalytic domain (amino acids 818-1025), the ARTD10 sequence is characterised by further domains: an RNA-recognition motif (RRM; amino acids 11-85) and a glycine-rich domain (amino acids 281-399) that are involved in RNA binding; a glutamic acid (Glu)-rich region (amino acids 588-697) that contains two ubiquitin interaction motifs (UIM; amino acids 650-667, 673-690); and a leucine-rich nuclear export sequence (NES; amino acids 598-607). ARTD10 is predominantly cytosolic under basal conditions, but it can shuttle between the cytoplasmic and the nuclear compartments (113). The nuclear export of ARTD10 is mediated through its NES, while a region that acts as a nuclear localisation signal defines its nuclear import, which has been mapped in the middle of ARTD10 (112).

ARTD10 was initially isolated as a partner of the oncoprotein c-Myc, and it was shown that its overexpression interferes with cell proliferation (114). Moreover, the catalytic activity of ARTD10 is strictly required to inhibit cell proliferation, as a catalytically inactive mutant of ARTD10 is not effective, and this inhibitory effect of ARTD10 appears to be a consequence of its induction of apoptosis (115). Although, it remains unknown which protein(s) are mono-ADP-ribosylated by ARTD10 to mediate this growth inhibitory phenotype, a possible candidate is GSK3β, which is known to regulate cell proliferation (reviewed in (116)), and the kinase activity of which is inhibited once it is modified by ARTD10 (117).

ARTD10 has a role in the nuclear B family is?B) transcription factor signalling. The NF-?factor-kappaB (NF- involved in cell proliferation, innate and adaptive immune response, and further crucial processes, like inflammation and tumorigenesis (reviewed in (118)). An intriguing model proposed by Verheugd and colleagues (119) shows that by means of its UIM motifs, ARTD10 binds K63-poly-ubiquitin chains that can be generated by tumour necrosis factor (TNF) receptor-associated factor (TRAF) proteins. Thus, in response to two structurally different but functionally overlapping cytokines, IL-1β and TNFα, the TRAFs are activated, and by binding poly-ubiquitin and by ADP-ribosylating NEMO, ARTD10 inhibits NEMO K63-poly-ubiquitination and its downstream signalling pathway, B target genes. Thus, ARTD10?which results in reduced activation of NF- B signalling.?definitively represents a novel regulator of NF-

6.2.2. ARTD8

Three members of the ARTD family are characterised by the presence of N-terminal

macro-domains, with two in ARTD9 (BAL1/PARP9) and ARTD7 (BAL3/PARP15), and three in ARTD8 (BAL2/PARP14); these are collectively known as the macro-domain containing mono-ARTDs. The gene encoding ARTD9 was originally identified in a genome-wide search for genes related to the risk for diffuse large B cell lymphoma (DLBCL), the most common non-Hodgkin lymphoma (120). ARTD9 does not show any ART activity, which is in contrast to ARTD8 and ARTD7 that have been reported to have mono-ART activity (121). For ARTD7, all that is known is that it localises to stress-granules along with ARTD8 (122). ARTD9 and ARTD8 are over-expressed in DLBCL (121), with the expression of ARTD9 being particularly elevated in lymphoma characterised by high immune infiltrates (123).

When stably overexpressed in DLBCL cells, ARTD9 stimulates cell migration, and this led to the hypothesis that ARTD9 promotes malignant B-cell migration and dissemination in high-risk DLBCL (120). Then, in a pilot series of primary DLBCLs, ARTD9 expression was shown to be significantly higher in more aggressive and chemoresistant tumours than in low-risk tumours (123). In this context, ARTD9 and ARTD8 function as transcriptional activators. Similar to the other ART enzymes, the expression of ARTD9 can be induced by cytokine, and specifically by the inflammatory IFN-γ, which is known to be secreted by host-activated tumour-infiltrating T lymphocytes, and to induce expression of ARTD9 and its interactor BBAP (BAL1 binding partner; an E3 ubiquitin ligase) in DLBCL cell lines. In turn, this induced ARTD9 promotes the transcription of interferon-controlled genes (123). Thus, by inhibiting the host immune response against the lymphoma, ARTD9 can function as a transcriptional activator of tumour genes in an inflammatory environment.

ARTD8 was identified as a collaborator of Stat6 (signal transducer and activator of transcription) and thus it is also known as CoaSt6, and as an activator of IL-4– and Stat6–dependent transcription (124). ARTD8 catalyses ADP-ribosylation of p100 (124), a protein that interacts with RNA polymerase II and functions as a bridging factor between Stat6 and the transcription machinery (125). The functional consequences of this modification remain to be characterised in detail, unlike ARTD8-mediated ADP-ribosylation of the HDAC2 and HDAC3 histones deacetylases. Indeed, in the presence of IL-4, the ART activity of ARTD8 is activated, and HDAC2 and HDAC3 are ADP-ribosylated and released from their promoters, thus allowing the binding of Stat6 and the consequent transcription (125). In line with this, a catalytically inactive mutant of ARTD8 did not enhance Stat6-mediated transcription, and ART inhibitors blocked IL-4–dependent transcription (124). Moreover, in response to IL-4, ARTD8 is also involved in proliferation and survival of B lymphocytes, with a role in the regulation of the glycolytic activity of these cells (126, 127). This is in line with the requirement for a major supply of cellular energy to sustain continuous cell growth and proliferation of cancer cells. ARTD8 also interacts with and stabilises the phosphoglucose isomerase/ autocrine motility factor (by inhibiting its ubiquitination), a cytosolic and secreted enzyme that is essential for glycolysis and gluconeogenesis and that is involved in tumour progression and metastasis (128).

More recently, a central role has been demonstrated for ARTD8 in the promotion of the survival of myeloma cells (129). Jun N-terminal kinase (JNK)2 is required for the survival of myeloma cells and constitutively suppresses JNK1-mediated apoptosis by inducing the expression of ARTD8, which binds to and inhibits JNK1 (129). Intriguingly, ARTD8 has been found to be localised not only in the nucleus, but also at the cell periphery, together with ARTD9, where it associates with actin fibers, as a component of focal adhesions (130). Human actin has been previously reported to be modified not only by this bacterial toxin, but also by an endogenous enzyme that has never been identified, and thus it would be interesting to determine whether the ART activity of ARTD8 can modify actin, and thus affect actin polymerisation, and hence cell proliferation. Moreover, in line with the regulatory role of ARTD8 on IL-4– and STAT6–dependent transcription, and with the roles that IL-4 and STAT6 have in asthma, the ART activity of ARTD8 has been seen to be involved in the pathogenesis of asthma, using a murine model of allergic airway disease (131).

6.2.3. ARTD15

ARTD15 is the only known ARTD family member with a putative C-terminal transmembrane domain, and we have shown that ARTD15 associates with membranes of the nuclear envelope and the ER, using immunofluorescence and electron microscopy (132). ARTD15 is a single-pass transmembrane protein with the N-terminal region (amino acids 1-280) positioned towards the cytoplasm, and the very short C-terminal tail (amino acids 300-322) in the ER lumen (132). In line with this orientation towards the cytosolic compartment

of the ARTD15 catalytic domain, we identified cytosolic importinβ1/ karyopherin β1 (Kapβ1) as a target of ARTD15 (132). Considering that Kapβ1 is a carrier protein with a pivotal role in the transport of various cargo proteins through the nuclear pore complex (133-138), we can hypothesise that its mono-ADP-ribosylation regulates the nucleocytoplasmic shuttling of cellular proteins. Moreover, Kapβ1 regulates multiple aspects of mitosis (139, 140), in which the ARTDs have various roles. However, the functional consequences of ARTD15-mediated mono-ADP-ribosylation of Kapβ1 remain to be defined. Instead, there is a role for ARTD15-mediated mono-ADP-ribosylation of two proteins that localise to the ER membranes (as does ARTD15 itself): the double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK), and the inositol-requiring enzyme 1α (IRE1α) (141), two of the three sensors of ER stress (142, 143). Upon conditions that cause ER stress, a cell response that involves three signalling pathways is initiated, with PERK inhibiting protein synthesis through eIF2α phosphorylation, and IRE1α leading to increased translation and transcription of proteins, including GRP78, which promotes protein folding. As ARTD15-mediated ADP-ribosylation activates PERK and IRE1α, Jwa and colleagues hypothesised that ARTD15 has a role in the unfolded protein response (UPR), to maintain a state of activation of these two sensors of ER stress that can be instrumental in the restoration of ER homeostasis (141). While the role of ARTD15 in the UPR needs further investigation, in addition to the nucleus, the ER is emerging as a new platform for ADP-ribosylation reactions. Indeed, the most recent data clearly indicate that two members of two different families of mono-ARTs, namely ARTC1 and ARTD15, localise and function at the ER.

7. OTHER ACTIVE ARTDs

In addition to the macro-ARTDs, ARTD10 and ARTD15, there are five ARTD proteins for which mono-ART activities have been either demonstrated, at least in terms of auto-modification, or only hypothesised. Similar to the situation with the ARTD7 macro-ARTD, nothing is known about ARTD11 and ARTD16, and limited studies are available for the other three ARTD members: ARTD17, ARTD14 and ARTD12 (Table 1).

ARTD17 is a mono-ART enzyme with possible involvement in cancer. Tuncel and colleagues showed that the catalytic activity of ARTD17 is required for negative regulation of cell-cycle progression in HeLa cells. Moreover, the immunohistochemical analysis of human colorectal cancer specimens have shown that ARTD17 expression is inversely correlated with Ki-67, which is a well-known proliferation marker, and is associated with a good prognosis. Thus, we would suggest that ARTD17 expression levels might become a prognostic biomarker for improved survival of patients with colorectal cancer (144).

ARTD14 is also known as TiPARP (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible PARP), and it is a 75-kDa nuclear mono-ART. Its expression is induced by TCDD, which is a carcinogen and a potent activator of the ligand-activated transcription factor aryl hydrocarbon receptor (AHR) (145, 146). The effects of TCDD, as well as of environmental dioxin, include decreased gluconeogenesis, which is at least in part due to repression of AHR-mediated transcription of phosphoenolpyruvate carboxykinase (PEPCK). ARTD14 overexpression reproduces the TCDD effects on glucose, which suggests that ARTD14 mediates these TCDD effects (146). TCDD-dependent transcriptional induction of ARTD14 leads to ADP-ribosylation of cytosolic and mitochondrial PEPCKs. However, as AHR suppression also enhances ADP-ribosylation, it is clear that the complex modulatory effects on ADP-ribosylation by ARH are far from being defined at present (147). Moreover, ARTD14 has been shown to act as a transcriptional repressor of AHR, which thus reveals a negative feedback loop in AHR signalling (148). ARTD14 also acts with a different mechanism from that of AHR repressor (AHRR), as only the silencing of ARTD14, and not that of AHRR, increases TCDD-induced AHR protein levels, while silencing of both ARTD14 and AHRR enhances AHR transactivation (149).

Inhibitory effects on the replication of several different types of RNA viruses has been reported for ARTD12 (150, 151). IFN-stimulated long-isoform ARTD12 (ARTD12L), and also ARTD14 and ARTD10, inhibit cellular translation and virus replication. The ARTD12L-mediated translation inhibition and antiviral activity depend on its binding to polyribosomes, via its RNA-binding domain, and requires its catalytic activity (151). Of note, a prominent inhibitory effect on the synthesis of virus-specific, rather than host-specific, proteins has been reported (151), thus defining these ARTDs as critical enzymes for virus clearance.

8. SIRTUINS

Sirtuins are NAD^+-dependent protein deacetylases that use NAD^+ to deacetylate lysine and to simultaneously transfer the acetyl group onto ADP-ribose, thus generating O-acetyl (OA)-ADP-ribose and releasing nicotinamide (152, 153). The sirtuins are a highly conserved class of enzymes that are found in a variety of phylogenetically distributed species, including archea, eubacteria, yeast, mammals, and even viruses. The mammalian sirtuin family is composed of seven proteins, SIRT1-7, with different intracellular localisations: SIRT1 and SIRT2 have been detected in the nucleus and cytosol, SIRT3, SIRT4 and SIRT5 are found in mitochondria, while SIRT6 and SIRT7 are predominantly nuclear. All the members of the sirtuin family share a conserved core domain that comprises approximately 200-275 amino acids, and which consists of a NAD^+ binding site and a catalytic domain. Two sirtuins can catalyse the mono-ADP-ribosylation reaction, demonstrating that the same protein family include members that can perform more than one biochemical reaction (154, 155).

In mitochondria, SIRT4 has been identified as being responsible for mono-ADP-ribosylation of glutamate dehydrogenase (GDH), and is thus a cysteine-specific, mono-ART (156). GDH converts glutamate to α-ketoglutarate. ADP-ribosylated GDH is inhibited with the consequent reduction of α-ketoglutarate production (156). This SIRT4-mediated modification of GDH is involved in the regulation of insulin secretion in pancreatic β cells. In SIRT4-deficient pancreatic β-cells, GDH activity increases, which leads to stimulation of insulin secretion in response to glutamine. Therefore, SIRT4 has an inhibitory effect on amino-acid–stimulated insulin secretion.

SIRT6 has been reported to catalyse mono-ADP-ribosylation of PARP1/ARTD1 in human cells, which promotes DNA double-strand break repair by homologous recombination in response to oxidative stress (157, 158). SIRT6 has also been associated with aging and cancer. SIRT6 expression diminishes with cellular senescence, and its overexpression in non-senescent cells strongly stimulates homologous recombination repair, in a PARP1/ARTD1-dependent manner (158). Thus, SIRT6 might contribute to counteract age-related genomic instability and the higher incidence of cancer with age. SIRT6 overexpression induces apoptosis in different cancer cell lines (159). Other sirtuins, including SIRT1, SIRT2, SIRT3 and SIRT7, have been linked to carcinogenesis, with roles as tumour suppressors or cancer promoters, although this is due to their deacetylase activity (161). Interestingly, the activity of SIRT6 tumour suppression has been associated with its mono-ART activity (159, 160), while the deacetylase activity of SIRT1, SIRT2, SIRT3 and SIRT7 has been linked to carcinogenesis, as part of the role of the sirtuins as tumor suppressors or as cancer promoters (161, 162).

9. THE MONO-ADP-RIBOSYLTRANSFERASES AS THERAPEUTIC TARGETS

Mono-ADP-ribosylation has been associated with increasing numbers of biological roles, with potentially yet more to be discovered. Many of these recall as many human diseases, which include neurodegenerative and inflammatory diseases, and the onset and progression of cancers. A role in the development of some human diseases has been showed for some active ART members of the ARTD family, including ARTD8, ARTD15 and ARTD10 in particular (see Table 1). In the tumour context, ARTD8 and its ART activity have crucial roles in pro-survival signalling in DCBCL, through induction of the expression of tumour-specific factors. In contrast, ARTD10 can act as a tumour suppressor through induction of apoptosis in osteosarcoma cancer cells and through inhibition of proliferation of cancer cells. Of interest, both of these enzymes (ARTD8, ARTD10) can modulate cell metabolism. In B lymphocytes, ARTD8 facilitates tumour survival through modulation of cell metabolism: it has a central role in the regulation of glycolytic activity (126, 127) and it also stabilises phosphoglucose isomerase/autocrine motility factor, which is involved in tumour progression and metastasis, through inhibition of its ubiquitination (128). The role of ARTD8 is not exclusive to DCBCL, as it has been shown to be highly expressed also in myeloma cells, and to be associated with disease progression and poor survival (129). Thus, considering the widely studied roles of ARTD8 in cancer and also its emerging role in allergic airway disease (131), the targeting of ARTD8 activity appears to be of particular therapeutic relevance for lymphoma, myeloma and asthma.

Similarly, the target of ARTD10, GSK3β, appears to have a role in neurodegenerative disorders, as its overexpression causes neuronal cell death (recently reviewed by (163)). GSK3β has been implicated in the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS),

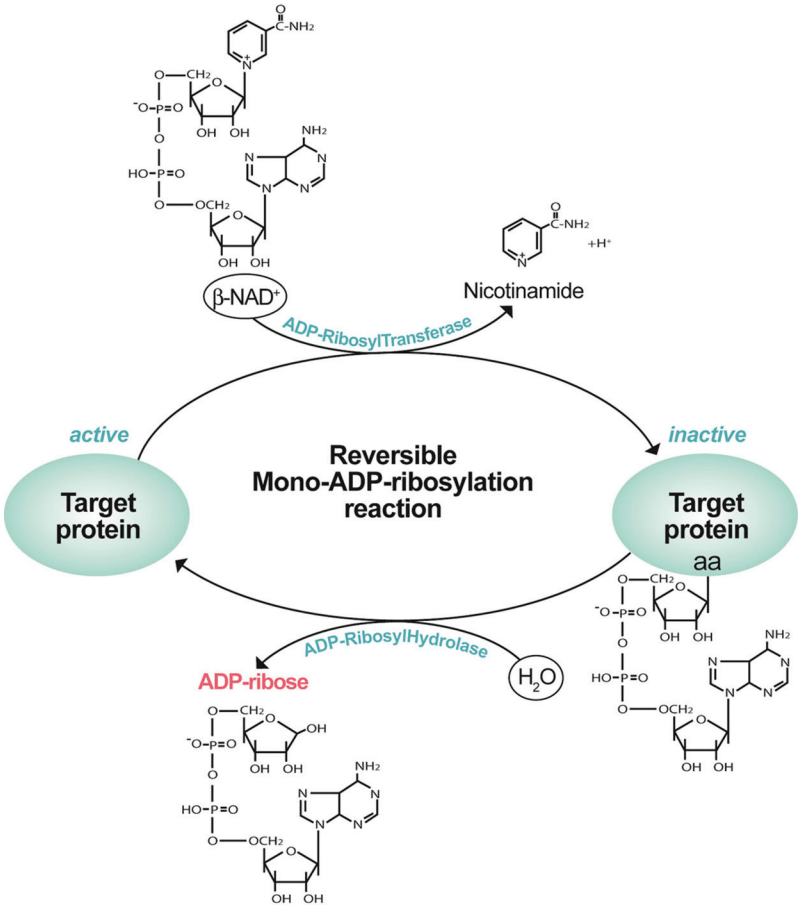

Figure 1. Schematic representation of the reversible mono-ADP-ribosylation reaction. Mono-ARTs transfer the ADP-ribose moiety from βNAD$^+$ to a specific amino acid of the target protein, releasing nicotinamide. This modification in general leads to inactivation of the target protein. Specific hydrolase enzymes can cleave the covalent bond between the ADP-ribose and the side chain of the target protein, thus restoring normal protein function (see text for details).

which is characterised by degeneration of motor neurons, which results in progressive motor paralysis. Mutations in the gene coding for superoxide dismutase (SOD1) are associated with approximately 20% of familial ALS. Studies have shown that GSK3β inhibition can prevent motor neuron cell death in an *in-vitro* ALS model that is characterised by expression of the G93A mutant of human SOD1 (164). Thus, through mono-ADP-ribosylation of GSK3β, ARTD10 can act as an inhibitor of cell proliferation and also as a regulator of neuronal cell death. Moreover, in addition to its role in cell signalling pathways that regulate proliferation and apoptosis, ARTD10 expression can be induced by LPS and IFNα, which indicates its involvement in immunological processes.

ARTD15 represents an additional attractive therapeutic target. Through its ability to modify Kapβ1, PERK and IRE1α, ARTD15 has roles in the regulation of nucleo-cytoplasmic trafficking and in the UPR. Both of these cellular processes are involved in inflammation, neurodegeneration and cancer. Indeed, impaired cytoplasmic–nuclear transport can induce mislocalisation of tumour suppressors and oncogenes, and aberrant intracytoplasmic accumulation of proteins in some neurological diseases (165, 166). Despite the central role of GRP78 in the UPR and cancer, knowledge of the mechanisms that regulate the expression and function of this chaperone are limited (167-169). Mono-ADP-ribosylation of GRP78 by ARTC1 regulates the availability of functional GRP78, and mono-ADP-ribosylation of PERK and IRE1α by

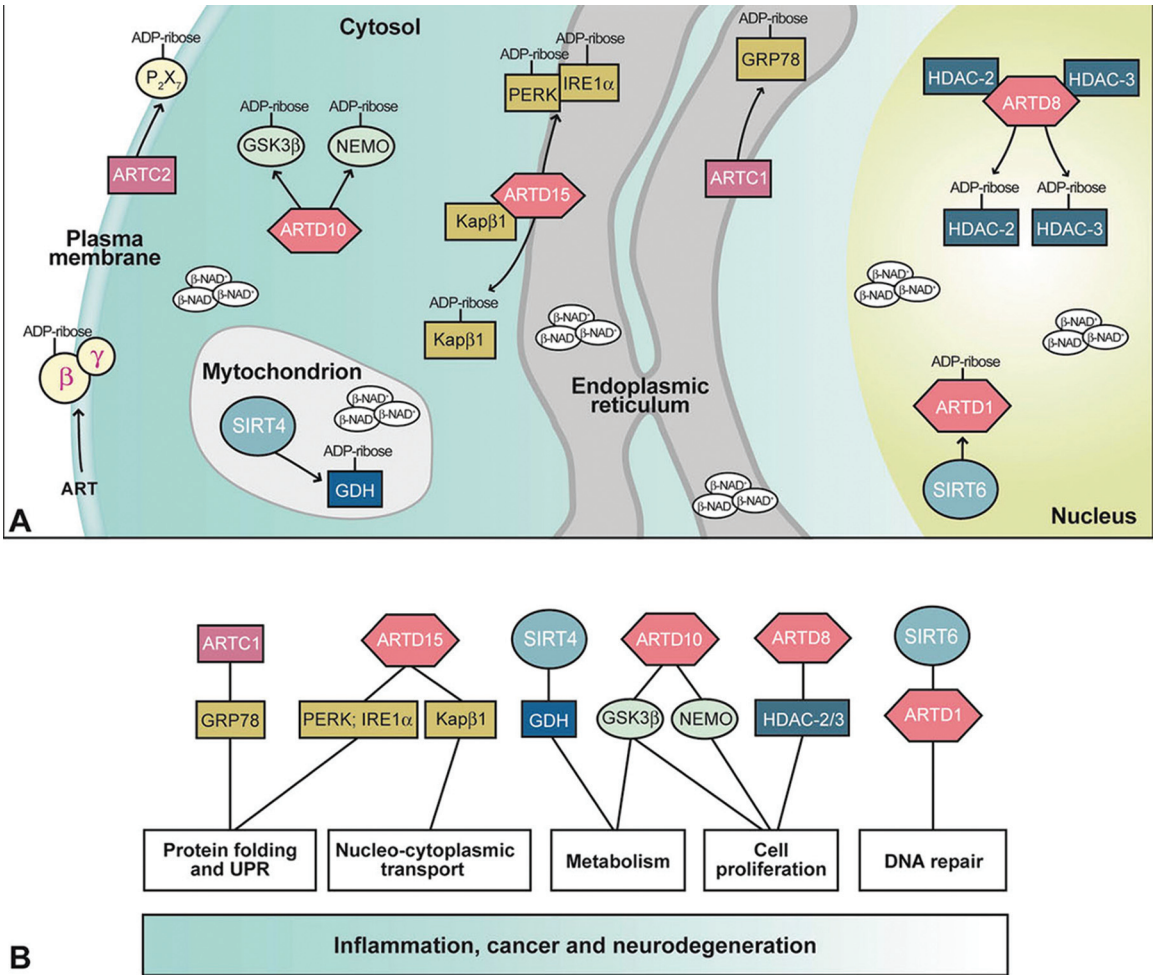

Figure 2. Schematic representation of the mono-ADP-ribosylation reactions. (A) The different subcellular compartments where mono-ADP-ribosylation reactions can occur. At the cell surface, mono-ADP-ribosylation is catalysed by ARTC2 and a not-yet-identified mono-ART; in the cytosol, by ARTD10; in the mitochondria, by SIRT4; in the endoplasmic reticulum, by ARTC1 and ARTD15; and in the nucleus, by ARTD8 and SIRT6. (B) Scheme of the physiological roles of the mono-ADP-ribosylation reactions and of their possible involvement in disease (see text for details).

ARTD15 activates the UPR, thus participating to the regulation of ER homeostasis.

Therefore, many ART enzymes of the different families represent new therapeutic targets for cancers and inflammatory diseases.

10. CONCLUSIONS

It has been demonstrated that mono-ADP-ribosylation by the ARTC, ARTD and sirtuin enzymes is involved in many pathophysiological conditions, like inflammation, cancers and neurodegeneration, and so drug discovery approaches aimed to target these mono-ARTs should be of high priority. The development and characterisation of inhibitors that are specific for these individual ARTCs, ARTDs and sirtuins can lead to the discovery of novel compounds that should provide new insights into the mechanisms of crucial cellular functions, which include nucleo-cytoplasmic trafficking, UPR signalling and the regulation of cell survival and cell death. Further improved understanding of how these enzymes are regulated and how the reaction they catalyse impacts upon these cellular functions has obvious clinical relevance for the treatment of cancers and of inflammatory diseases. A large amount of data has been generated that demonstrates the efficacy of

PARP1/ARTD1 inhibitors as anti-inflammatory and anti-cancer drugs. We expect that new and more efficacious drugs can be generated through the targeting of these new ARTs. For instance, ARTD15 can be taken as a new drug target considering that different lines of research have revealed that ER stress is involved in the pathogenesis of several diseases, including obesity, type 2 diabetes, and cancers.

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Send correspondence to: Maria Di Girolamo, Laboratory of G-Protein-Mediated Signalling, Fondazione Mario Negri Sud, Via Nazionale 8/A, 66030 Santa Maria Imbaro (CH), Italy, Tel: 39-0872-570348, Fax: 39-0872-570412, E-mail: mdigirolamo@negrisud.it