Regulatory Role of Arginine 204 in the Catalytic Activity of Rat Alloantigens ART2a and ART2b

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ART2a (RT6.1) and ART2b (RT6.2) are NAD glycohydrolases (NADases) that are linked to T lymphocytes by glycosylphosphatidylinositol anchors. Although both mature proteins possess three conserved regions (I, II, III) that form the NAD-binding site and differ by only ten amino acids, only ART2b is auto-ADP-ribosylated and only ART2a is glycosylated. To investigate the structural basis for these differences, wild-type and mutant ART2a and ART2b were expressed in rat mammary adenocarcinoma (NMU) cells and released with phosphatidylinositol-specific phospholipase C. All mutants were immunoreactive NADases. Arginine 204 (Arg204), NH₂-terminal to essential glutamate 209 in Region III, is found in ART2b, but not ART2a. Replacement of Arg204 in ART2b with lysine, tyrosine, or glutamate abolished auto-ADP-ribosylation. Unlike wild-type ART2a, ART2a(R204K) was auto-ADP-ribosylated. The tryptophan mutant ART2b(R204W) was auto-ADP-ribosylated and exhibited enhanced NADase activity. Incubation with NAD and auto-ADP-ribosylation decreased the NADase activities of wild-type ART2b and ART2b (R204W), whereas activity of ART2b(R204K), which is not auto-modified, was unchanged by NAD. Facilitation of auto-ADP-ribosylation by tryptophan 204 suggests that the hydrophobic amino acid mimics an ADP-ribosylated arginine. Thus, Arg204 in ART2b serves as a regulatory switch whose presence is required for additional auto-ADP-ribosylation and regulation of catalytic activity.

Mono-ADP-ribosyltransferases catalyze the transfer of ADP-ribose from NAD to a specific amino acid in an acceptor protein. In place of an amino acid, some of these enzymes utilize water as an acceptor, generating ADP-ribose and nicotinamide from NAD (NAD glycohydrolase activity). The properties of these enzymes have been most studied with bacterial toxins (e.g. cholera toxin, an arginine-specific ADP-ribosyltransferase (ART)) that use ADP-ribosylation to modify proteins and thereby alter activity of critical metabolic or regulatory pathways in mammalian cells (1).

The amino acid sequences of the mammalian ARTs differ significantly from those of the toxins and each other. Analysis of the crystallographic structure of toxin ADP-ribosyltransferases identified three regions involved in formation of the catalytic site, NAD binding, and activation of the ribosyl-nicotinamide bond, which is required for ADP-ribose transfer (2, 3). These regions appear to be present also in the mammalian transferases (4). Region I is defined by an arginine or histidine, Region II, by a sequence rich in hydrophobic amino acids, or by serine X serine, (where X represents threonine, serine, or alanine), and Region III by glutamate (Glu). In the bacterial toxins, ART1, and ART2 (mouse and rat), site-specific mutagenesis of Region III verified the importance of glutamate in catalysis (5–8). Recently, by comparative analysis of crystallographic structures, Han and Tainer (9) extended the significance of the Region III sequences by identifying an ARTT motif (ADP-ribosylating turn-turn motif) that could be implicated in specificity and recognition of the substrate.

Rat ART2a (RT6.1) and ART2b (RT6.2) (for review, see Ref. 10) are encoded by two alleles of a single copy gene (11); the human counterpart has stop signals in the coding region (12). To date, only post-thymic peripheral and intestinal intraepithelial T lymphocytes are known to express ART2 proteins (13). Although their biological functions are unknown, the absence, depletion, or reduction of ART2-expressing T lymphocytes is associated with autoimmune diabetes (14, 15).

Both ART2a and ART2b are linked to the cell surface by GPI anchors, but only ART2a is glycosylated (16, 17). In their mature, processed forms, ART2a and ART2b differ by 10 amino acids. ART2 catalyzes the hydrolysis of NAD to ADP-riboside and nicotinamide but, in contrast to ART1, does not transfer ADP-ribose to arginine or other small guanidine compounds (5). The proteins differ significantly in their abilities to catalyze auto-modification, with ART2b, but not ART2a, capable of auto-ADP-ribosylation (18, 19). In general, the role of GPI-linked NAD metabolizing proteins in T-cell signaling is not clear. Association of ART2 with T-cell src tyrosine kinases was increased by T-cell pretreatment with phorbol 12-myristate 13-acetate (20). NAD and the product of NAD hydrolysis, ADP-riboside, inhibited antigen-induced T-cell proliferation in rats, suggesting that ART2 enzymatic activity may mediate an NAD-dependent immunomodulatory signal (21). The allelic differences between ART2a and ART2b genes result in variations in amino acid sequence and enzymatic properties but the differences in the signaling properties of the isoenzymes is unknown.

To determine the molecular basis for the catalytic diversity of the ART2 proteins and the structural requirements for auto-ADP-ribosylation and its effects on ART2 activity, we compared the amino acid sequences, paying particular attention to the critical catalytic Region III to identify crucial conserved and non-conserved amino acids. Here we report that auto-modification of ART2b is abolished by the replacement of arginine 204, which is located just proximal to the catalytic glutamate and is part of the ARTT motif, and that substitution of arginine

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The abbreviations used are: ART, mono-ADP-ribosyltransferase; ARTT, ADP-ribosylating turn-turn; GPI, glycosylphosphatidylinositol; NMU, rat mammary adenocarcinoma; NADase, NAD glycohydrolase; DPBS, Dulbecco’s phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C.
Role of Arginine 204 in ART2 Auto-ADP-ribosylation

TABLE I

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Primers</th>
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<tbody>
<tr>
<td>ART2a</td>
<td></td>
</tr>
<tr>
<td>58A</td>
<td>N58A</td>
</tr>
<tr>
<td>K59M, S60N, E61A</td>
<td></td>
</tr>
<tr>
<td>M81R</td>
<td></td>
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<tr>
<td>Y204R</td>
<td></td>
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<td>R81K</td>
<td></td>
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<td>R204K</td>
<td></td>
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<tr>
<td>R204E</td>
<td></td>
</tr>
<tr>
<td>ART2b</td>
<td></td>
</tr>
<tr>
<td>R204Y</td>
<td></td>
</tr>
<tr>
<td>R204W</td>
<td></td>
</tr>
</tbody>
</table>

Altered bases are underlined.

Protein expression was induced with 1 mM ADP-ribose, 10 mM [32P]NAD (10 μCi/assay reaction) in DPBS, for 1 h at 30 °C. Where indicated, unlabeled NAD was added and incubation continued for 1 h at 30 °C before termination with the addition of an equal volume of 20% cold trichloroacetic acid. Reaction mixtures were centrifuged (16,000 g for 25 min) and supernatants discarded. Proteins were dissolved in SDS-sample buffer, separated by SDS-PAGE in 12% gels, and transferred to nitrocellulose. Blots were exposed to film (XAR-2, Eastman Kodak Co.) or analyzed by PhosphorImager (Amersham Biosciences). Immunoreactivity was quantified by incubation with rabbit antipeptide antiserum 1126 (Immunogenetech) and antibodies specific for ART2a and ART2b in NMU cells using a dexamethasone-sensitive promoter. The GPI-anchored proteins released from NMU cells were analyzed by SDS-PAGE in 12.5% gels.

RESULTS AND DISCUSSION

The sequences of ART2a and ART2b differ by 14 amino acids, 10 of which are located NH2-terminal to the region excised during addition of the GPI anchor (Fig. 1). In ART2b, but not other ARTs, an arginine is present at position 204 (Arg204) as part of the ARTT motif, located at the amino end of catalytic Region III. A putative consensus N-glycosylation signal is present at positions 58–60 in ART2a but not in ART2b. Both ART2a and ART2b have NADase activity, but only ART2b is significantly auto-ADP-ribosylated. To define the structural basis for these differences in catalytic function, we employed site-specific mutagenesis with synthesis of recombinant ART2a and ART2b proteins in NMU cells using a dexamethasone-sensitive promoter. The GPI-anchored proteins released from cells using PI-PLC to cleave the GPI anchor were incubated with [32P]NAD to assess auto-ADP-ribosylation (Fig. 2A). All
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Fig. 1. Deduced amino acid sequences of rat ART2a (RT6.1) (GenBank™ accession number CAA36301) and rat ART2b (RT6.2) (GenBank™ accession number AAA42085). Identical amino acids are shaded. Arginines 204 (1) and 81 (2) are specific to ART2b. In ART2a, Asn58 (3) and 59KSE61 (4) are in a putative consensus glycoseylation signal not present in ART2b. Regions I, II, and III, believed to participate in formation of the cytotoxic site in the bacterial toxin and mammalian ADP-ribosyltransferases, are indicated by over-lines and the putative catalytic amino acids by an asterisk. Consensus sequence of the ARTT motif involved in substrate specificity and recognition is shown under the corresponding region in rat ART2 proteins (Φ, hydrophobic amino acid; X, any amino acid). Underlines indicate signal sequences, which are excised during the export into the endoplasmic reticulum (amino terminus) and attachment of the GPI anchor (carboxyl terminus).

recombinant proteins had NADase activity (Fig. 3) and all reacted with ART2 antisera, exhibiting the expected size of 29 kDa on immunoblots (Fig. 2B). ART2a, the glycosylated isoform, had an additional band at ~33 kDa, consistent with its single consensus sequence for N-glycosylation.

Multiple species of auto-ADP-ribosylated wild-type ART2b were observed by SDS-PAGE (Fig. 2A). The molecular mass is predicted to increase by 542 Da per addition of each ADP-ribose moiety. The autoradiograms demonstrate the increasing molecular weight of [32P]ADP-ribosylated modified proteins. Replacement of arginine 204 by a conservative lysine (R204K) abolished auto-ADP-ribosylation, whereas substitution of lysine for arginine 81 had no effect, consistent with arginine 204 control by arginine 204.

In ART2a(Y204R), modification of the putative consensus glycosylation site, replacing Asn58 with Ala, or changing 59KSE61 to 59MNA61 prevented glycosylation, resulting in a single immunoreactive band by SDS-PAGE (Fig. 3). Both nonglycosylated species were auto-ADP-ribosylated. The glycosylated ART2a(Y204R), concentrated on concanavalin A-Sepharose, was also auto-ADP-ribosylated (Fig. 4). The glycosylation site did not affect auto-ADP-ribosylation or the regulatory control by arginine 204.

Replacement of Arg204 in ART2b with tyrosine or glutamate abolished auto-ADP-ribosylation as well, although all the mutants retained NADase activity (Fig. 3), demonstrating that the absence of auto-modification was not due to the inability of the proteins to hydrolyze NAD. Surprisingly, ART2b(R204W), ART2b(R204Y), and ART2b(R204E) were also capable of auto-modification (Figs. 3 and 5). The mutant, like the wild type, however, was unable to transfer ADP-ribose to agmatine (data not shown). The auto-modification activity of ART2b(R204W), but not of ART2b(R204Y) or ART2a, suggests that the indolyl side chain of tryptophan in position 204 can promote auto-modification, whereas the phenol group of tyrosine cannot. These data support the hypothesis that the heterocyclic structure of the tryptophan side chain may mimic the purinyl group of ADP-ribo proton, so that the auto-modification of ART2b requires ADP-ribo proton of Arg204.
ADP-ribosylation and were modified by multiple ADP-ribose with 5 mM NAD and separated by SDS-PAGE on 12% gels, transferred carried out in the presence of 1 mM unlabeled ADP-ribose, or (Y204R,59NMA61), and ART2a(Y204R) after incubation and by radiography), followed by addition of 5 mM unlabeled NAD with high specific activity ADP-ribose (for detection of proteins elution from concanavalin A-Sepharose of the glycosylated spe-

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ed. ART2b proteins were incubated with 10 μM [32P]NAD for 1 h at 30 °C, followed by addition of 20% trichloroacetic acid or further incubation with 5 mM NAD for 1 h at 30 °C, before addition of 20% trichloroacetic acid. To concentrate glycosylated proteins before incubation with NAD, supernatants from cells expressing ART2a(Y204R) were incubated with concanavalin A-Sepharose for 1 h. After washing the resin with DPBS, bound proteins were eluted with 0.3 M α-methyl-

o-mannopyranoside in DPBS and incubated for 1 h at 30 °C without or with 5 mM NAD and separated by SDS-PAGE on 12% gels, transferred to nitrocellulose, and quantified with a PhosphorImager (ART2b) or immunoblotting with antibody NAD2 (ART2a) and detection by chemiluminescence. Data are from one experiment representative of four experiments.

To assess the ability of ART2 mutants to be auto-ADP-ribosylated by multiple ADP-ribose moieties, ART2b proteins were incubated first with 10 μM [32P]NAD to label the protein with high specific activity ADP-ribose (for detection of proteins by radiography), followed by addition of 5 mM unlabeled NAD and further incubation (Fig. 4). ART2a(Y204R), ART2a (Y204R),[59NMA61], and ART2a(Y204R) after incubation and elution from concanavalin A-Sepharose of the glycosylated spe-
cies, were incubated with 5 mM NAD (and subsequently detected by immunoreactivity). Wild-type ART2b, ART2b(R81K), and mutants ART2a(Y204R), ART2a[59NMA61] Y204R, and the glycosylated isoforms of ART2a(Y204R) were multiply auto-ADP-ribosylated as evidenced by the bands of lower mobility than the unmodified forms on SDS-PAGE. All of these proteins, including ART2b(R204W) (Fig. 6, inset), were capable of auto-ADP-ribosylation and were modified by multiple ADP-ribose moieties. It is unlikely that auto-modification was due to non-

enzymeatic addition of [32P]ADP-ribose, since reactions were carried out in the presence of 1 mM unlabeled ADP-ribose, or due to NAD binding, since the radiolabeling was not decreased by the presence of 5 mM unlabeled NAD.

A specific ADP-ribose-amino acid linkage can often be iden-
tified by its susceptibility to cleavage by acid, hydroxylamine, and mercuric chloride (Fig. 5). To characterize the multiple ADP-ribose bonds that resulted from incubation of wild-type ART2b, or ART2a(Y204R) with millimolar NAD, their chemical sensitivity was tested. Hydroxylamine completely released the [32P]ADP-ribose from auto-ADP-ribosylated ART2b and ART2a(Y204R) (Fig. 5, IA, IIA, and IIC, lanes 4), consistent with the chemical stability of an ADP-ribose-arginine linkage. [32P]ADP-ribose was not released from auto-ADP-ribosylated ART2b(R204W) by hydroxylamine, mercuric chloride, or acid (Fig. 5, IC), suggesting that the auto-ADP-ribosylated amino acid was not arginine, cysteine, or lysine, respectively. Thus, the tryptophan mutant, although exhibiting auto-ADP-ribosyltransferase activity with multiple modifications, differed from wild-type ART2b in the ADP-ribose acceptor site(s) and, therefore, is only the partial functional equivalent of ADP-ribosyl-arginine.

Comparison of the Vmax kinetic constants determined by Lineweaver-Burk analysis for ART2b(R204K) and ART2b (R204W) showed an increased maximal velocity but similar Km values, when position 204 was occupied by the tryptophan residue with an aromatic side chain (Table II). In agreement, replacement of Tyr204 by Arg in ART2a had little effect on Km values but decreased Vmax consistent with the decreased NADase activity of ART2a(Y204R) observed in Fig. 3. These data support the ARTT motif model in which turn1 contains a conserved aromatic residue 204 thought to be involved in sub-
strate specificity and recognition (9).

The influence of auto-modification on the ability of the ART2 proteins to hydrolyze NAD was investigated. The rate of hy-

drosis by wild-type ART2b appeared to increase during the first hour of incubation with 0.5 mM NAD and then decreased in parallel with the increase in multiply ADP-ribosylated forms of the enzyme seen on SDS-PAGE (Fig. 6). The rate of increase in ART2b NADase during the first hour may reflect the initial transfer of ADP-ribose to arginine 204 and the initiation of additional modifications that inhibit ART2b catalysis. This kinetic pattern is consistent with the non-linear Lineweaver-Burk analysis of ART2b (Fig. 7). The curvature of the analysis suggests that the catalytic activity of the protein is changing during the 1.5 h of the assay due to activation by the initial auto-ADP-ribosylation and inhibition by subsequent additions of ADP-ribose. Thus, it was not possible to calculate the true kinetic constants. In agreement, the activity of ART2b(R204K), which is not auto-ADP-ribosylated, was constant for 4 h. The NADase activity of ART2b(R204W), which was constant for at least 2 h, i.e. with no initial stimulation, decreased like the wild-type ART2b with increasing auto-ADP-ribosylation. The Lineweaver-Burk analysis was linear similar to ART2b

![Fig. 4. Auto-ADP-ribosylation of ART2b, ART2a, and mutant proteins.](Image 85x599 to 279x738)

![Fig. 5. Chemical stability of ADP-ribose-protein bonds in auto-ADP-ribosylated wild-type ART2b, ART2b(R204W), and ART2a(Y204R).](Image 350x544 to 530x737)
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Kinetic constants for NAD glycohydrolase activity of recombinant ART2a, ART2b, and mutants

<table>
<thead>
<tr>
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<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/OD)</th>
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<tbody>
<tr>
<td>ART2a</td>
<td>55 ± 23</td>
<td>15.7 ± 3.0</td>
</tr>
<tr>
<td>ART2a(Y204R)</td>
<td>56 ± 4</td>
<td>0.8 ± 0</td>
</tr>
<tr>
<td>ART2a(N58A,Y204R)</td>
<td>59 ± 18</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>ART2b</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ART2b(R204K)</td>
<td>20 ± 11</td>
<td>3.4 ± 1.4</td>
</tr>
<tr>
<td>ART2b(R204W)</td>
<td>23 ± 11</td>
<td>8.8 ± 3.5</td>
</tr>
</tbody>
</table>

$^a$ OD, optical density in relative units.
$^b$ Lineweaver-Burk analysis was non-linear (see Fig. 7).

Our data indicate that the degree of hydrophobicity of amino acid 204 can regulate the NAD glycohydrolase and auto-ADP-ribosylation activity of the ART2b protein. In the presence of millimolar amounts of NAD, the ADP-ribosylation of arginine can initiate the auto-modifications that also modulate the NADase activity. These data demonstrate the essential function of the amino acid sequence and of the critical role of position 204 in the ARTT motif of Region III. Our results suggest that allotype variations generated during rat ART2 gene evolution have resulted in two differentially regulated NADases that could influence their function in T-cell regulation.

The ability of tryptophan to replace, in part, the function of ADP-ribosylarginine could be of use in protein design. Although ADP-ribosylation has effects on protein function, the modification itself is unstable in biological systems. It can be cleaved by pyrophosphatases, with release of AMP, and the resulting phosphoribosyl protein can be further degraded by phosphatases, yielding ribosyl protein. Obviously, synthesis of an ADP-ribosylated protein requires an additional step(s) fol-
lowing production of a recombinant molecule. In contrast, a protein containing tryptophan can be produced by standard techniques and should have no unusual instability in biological systems. The extent to which ADP-ribose-arginine in protein can be replaced by tryptophan is currently under study.

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REFERENCES
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