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ADP-ribosylation of guanosine by SCO5461 protein secreted from *Streptomyces coelicolor*

Tsuyoshi Nakano [a,*], Yuko Matsushima-Hibiya [a,1], Masafumi Yamamoto [a,2], Azusa Takahashi-Nakaguchi [a,3], Hirokazu Fukuda [a], Masaya Ono [b], Takeji Takamura-Enya [c], Haruyasu Kinashi [d], Yukari Totsuka [a]

[a] *Division of Cancer Development System, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[b] *Division of Chemotherapy and Clinical Research, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[c] *Department of Applied Chemistry, Kanagawa Institute of Technology, 1030 Shimo-Ogino, Atsugi 243-0292, Japan*
[d] *Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan*

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ABSTRACT

The *Streptomyces coelicolor* A3(2) genome encodes a possible secretion protein, SCO5461, that shares a 30% homology with the activity domains of two toxic ADP-ribosyltransferases, pierisins and mosquitocidal toxin. We found ADP-ribosylating activity for the SCO5461 protein product through its co-incubation with guanosine and NAD^+, which resulted in the formation of N^2-(ADP-ribos-1-yl)-guanosine (^{ar2}Guo), with a K_m value of 110 μM. SCO5461 was further found to ADP-ribosylate deoxyguanosine, GMP, dGMP, GTP, dGTP, and cyclic GMP with k_{cat} values of 150–370 s^{-1}. Oligo(dG), oligo(G), and yeast tRNA were also ADP-ribosylated by this protein, although with much lower k_{cat} values of 0.2 s^{-1} or less. SCO5461 showed maximum ADP-ribosylation activity towards guanosine at 30 °C, and maintained 20% of these maximum activity levels even at 0 °C. This is the first report of the ADP-ribosylation of guanosine and guanine mononucleotides among the family members of various ADP-ribosylating enzymes. We additionally observed secretion of the putative gene product, SCO5461, in liquid cultures of *S. coelicolor*. We thus designated the SCO5461 protein product as *S. coelicolor* ADP-ribosylating protein, ScARP. Our current results could offer new insights into not only the ADP-ribosylation of small molecules but also signal transduction events via enzymatic nucleoside modification by toxin-related enzymes.

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Abbreviations: MTX, mosquitocidal toxin from *Bacillus sphaericus* SSII-1; dGuo, 2′-deoxyguanosine; Guo, guanosine; ^{ar2}Guo, N^2-(ADP-ribos-1-yl)-guanosine; ^{r2}Guo, N^2-(ribos-1-yl)-guanosine; NAD^+, β-nicotinamide adenine dinucleotide; cGMP, guanosine 3′,5′-cyclic monophosphate.

* Corresponding author. Present address: Central Research Laboratories, Sysmex Corporation, 4-4-4 Takatsukadai, Nishi-ku, Kobe 651-2271, Japan. Tel.: +81-78-992-5988; fax: +81-78-992-3284.
E-mail addresses: tnakano@pp.iij4u.or.jp, Nakano.Tsuyoshi@sysmex.co.jp (T. Nakano).
[1] Present address. Division of Refractory Cancer Research, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
[2] Present addresses. Division of Cancer Prevention Research, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Central Animal Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Central Institute for Experimental Animals, 3-25-12 Tonomachi, Kawasaki-ku, Kawasaki 210-0821, Japan.
[3] Present address. Medical Mycology Research Center, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan.

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1. Introduction

ADP-ribosylation is the post-translational modification of proteins and involves the transfer of an ADP-ribose moiety from β-nicotinamide adenine dinucleotide (NAD^+) to specific residues in target proteins. Mono-ADP-ribosyltransferase activity is well-known to be present in several bacterial toxins that effectively target G proteins, elongation factors, and actins [see (Aktories and Just, 2000) for review]. Emerging studies have also revealed the existence of non-toxic mono-ADP-ribosyltransferases. Nitrogenases in *Azospirillum brasilense*, *Azospirillum lipoferum*, and *Rhodospirillum rubrum* are regulated by dinitrogenase reductase ADP-ribosyltransferase (DraT) and dinitrogenase reductase-activating glycohydrolase (DraG) during nitrogen fixation (Huergo et al., 2009; Masepohl and Hallenbeck, 2010), and vertebrate ecto ADP-ribosyltransferases (ARTs) target human neutrophil peptide-1 and cell surface $P2X_7$ receptors (Scheuplein et al., 2009; Stevens et al., 2009). Poly(ADP-ribose) polymerase 10 (PARP-10/ARTD10) also shows mono-ADP-ribosylation activity towards histones (Messner and Hottiger, 2011).

Some of the ADP-ribosyltransferases also target non-protein molecules. The pierisins, originally identified from *Pieris rapae* and *Pieris brassicae* as proteineous toxin against mice and cell lines (Marsh and Rothschild, 1974; Feltwell, 1982; Watanabe et al., 1999), target the N^2 amino groups of 2′-deoxyguanosine in double stranded DNA, causing mutations and an apoptotic response in cultured cells (Carpusca et al., 2006; Matsumoto et al., 2008; Orth et al., 2011; Yamamoto et al., 2009). The non-toxic CARP-1 from shellfish *Meretrix lamarckii* also target the same bases of DNA *in vitro* (Nakano et al., 2006). In contrast, tRNA 2′-phosphotransferases initially ADP-ribosylate a 2′-phosphate at the splice junction of pre-tRNA, then remove it by forming ADP-ribose 1″-2″ cyclic phosphate, resulting in the formation of a correct tRNA anticodon loop (Kato-Murayama et al., 2005; Sawaya et al., 2005; Steiger et al., 2005). Some small molecules can also be targets for ADP-ribosylation. For example, both Arr and Arr2 from opportunistic pathogens inactivate rifampicin through ADP-ribosylation (Baysarowich et al., 2008). In addition, some ADP-ribosyltransferases show low NAD^+ glycohydrolase activity that targets water molecules.

We observed from a BLAST search that the SCO5461 protein product, annotated as a secretion protein in the genome of *Streptomyces coelicolor* A3(2) (Bentley et al., 2002), shares homology with the activity domains of the pierisins and the mosquitocidal toxin from *Bacillus sphaericus* SSII-1 (MTX). MTX is an NAD^+:arginine ADP-ribosyltransferase that kills mosquito larvae (Schirmer et al., 2002a,b; Thanabalu et al., 1993), whereas pierisins are NAD^+:DNA(guanine-N^2) ADP-ribosyltransferases that induce apoptosis or gene mutation in mammalian cells in culture and *in vivo* (Shiga et al., 2006; Takamura-Enya et al., 2001; Totsuka et al., 2003; Watanabe et al., 2004). *Streptomyces* are gram-positive, soil-bacteria, and are unique organisms in terms of their metabolite profiles, most notably in relation to antibiotics, and in their properties as soil cleaners (Chater et al., 2010; Hodgson, 2000). In our present study, we demonstrated the ADP-ribosylating activity of SCO5461 and found that it has strong activity against the N^2 amino groups of guanine residues in nucleosides and mononucleotides. This is therefore the first report of an ADP-ribosyltransferase that mainly targets nucleosides, mononucleotides, and their 5′-phosphorylated forms. We also discuss the physiological roles of the ADP-ribosylation of nucleosides and mononucleotides.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and a plasmid

S. coelicolor A3(2) M145 ($SCP1^-SCP2^-$) was grown on Tryptic Soy Broth (Difco, Detroit, MI), with shaking in a Sakaguchi-flask at 28 °C. *Escherichia coli* K-12 JM109 (Toyobo, Osaka, Japan) was grown on LB for subcloning; *E. coli* K-12 ER2508 (New England Biolabs, Ipswich, MA) was grown on Terrific Broth for protein expression. A plasmid vector, pMALp2x (New England Biolabs), was used for subcloning and protein expression.

2.2. cDNA subcloning and expression of ScARP

We performed genome DNA extraction, PCR cloning and subcloning of cDNA using standard protocols (Kieser et al., 2000; Sambrook and Russell, 2001). SCO5461 and SCO5461(43–204) genes were ligated into pMALp2x. We introduced point mutations into these genes via overlap-PCR (Nakano et al., 2006). Proteins encoded in pMALp2x vectors were expressed as maltose-binding protein (MBP)-fused products in *E. coli* (Riggs, 1990). Following affinity purification, the MBP tag was cleaved from these recombinant products with factor Xa protease, followed by Mono-S column chromatography. Details of all of these procedures are included with the Supporting information.

2.3. ADP-ribosylation of nucleic acids

The standard reaction conditions employed for nucleosides and mononucleotides were as follows: nucleosides (final 1 mM) were incubated with SCO5461(43–204) protein (final 0.2 nM) and NAD^+ (final 0.01–3 mM) in 200 μl of 50 mM Hepes–NaOH pH 7.0 and 50 mM NaCl, for 10 min at 30 °C. The reaction mixture was immediately injected into an HPLC column. When oligo- or polynucleotides were used as substrates, reacted nucleotides (final 0.1 mg/ml) were injected into HPLC columns after digestion with micrococcal nuclease, phosphodiesterase II, and alkaline phosphatase (Nakano et al., 2006). The products were quantified from the A_{257} values in a standard curve generated using an equimolar mixture of ADP-ribose and Guo. Details of the digestion and HPLC conditions are included with the Supporting information.

2.4. Chemical synthesis of N^2-(D-ribofuranos-1-yl)-guanosine

The chemical synthesis of N^2-(D-ribofuranos-1-yl)-guanosine was performed in accordance with the synthesis route determined previously for N^2-(D-ribofuranos-1-yl)-2′-deoxyguanosine (Takamura-Enya et al., 2001). The

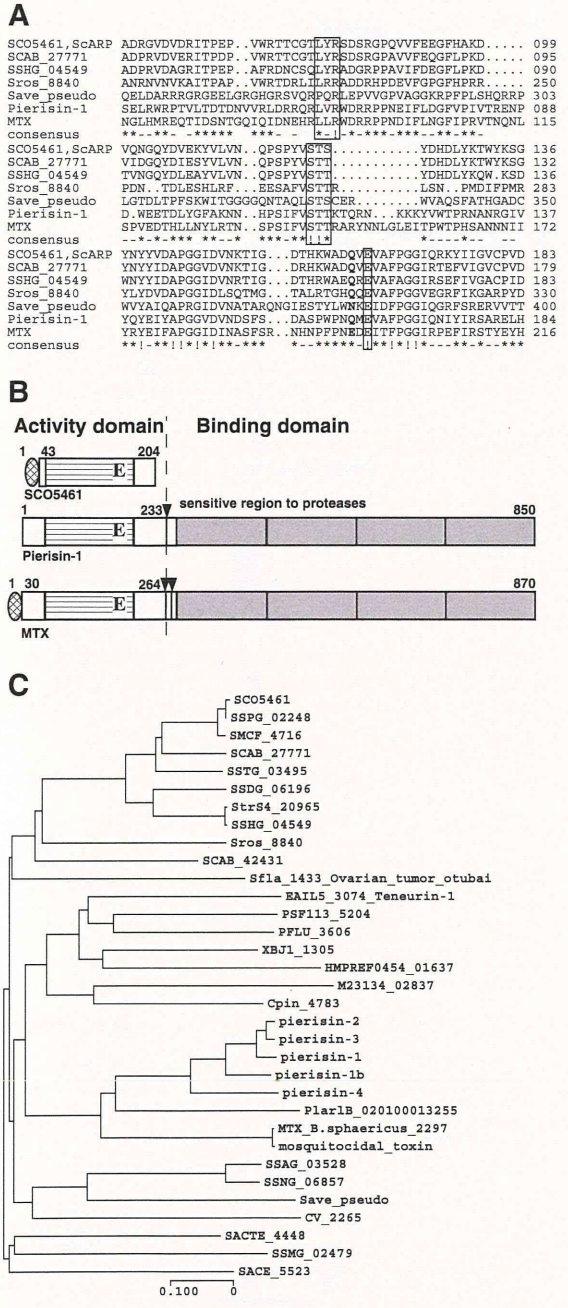

Fig. 1. Similarities between SCO5461 and its homologues. (A) Multiple alignments of the deduced amino acid sequences of SCO5461 and its homologues. Conserved motifs (LXR, STT, and reaction center glutamic acids) were boxed. Amino acid residues located two residues upstream of the reaction center are indicated in bold type. The source organisms and DDBJ/EMBL/GenBank accession numbers are indicated in Table S1. Note that SSPG_02248 from *S. lividans* TK24 (EFD66608) corresponds exactly to SCO5461 in this region and is not included in the alignment. (B) Schematic representations of the primary structure of SCO5461 and its homologues. Boxes with horizontal lines, activity domains; dotted boxes, ricin-fold like receptor-binding domains; cross-hatched ovals, secretion signals; "E", reaction center glutamic acids; arrowheads, regions sensitive to proteases. For details on binding domains, see Kanazawa et al. (2001) and Carpusca et al. (2006). (C) Dendrogram for amino acid sequences having homology to ScARP. Sequences were chosen from the DDBJ/EMBL/GenBank database using the BLASTp program (Altschul et al., 1990), and similarities were calculated within the highly-homologous regions by ClustalW (Thompson et al., 1994). A phylogenetic tree was drawn using DendroMaker for Macintosh ver. 4.1 (Tadashi Imanishi, http://www.cib.nig.ac.jp/dda/timanish/dendromaker/home.html). The scale represents the number of substitutions per site. Names of genes, source organisms, and INSDC accession numbers are listed in Table S1 of the Supporting information.

details of these procedures and the ^{1}H NMR and MS data are described in the Supporting information.

3. Results

3.1. Subcloning of the SCO5461 gene, a putative ADP-ribosyltransferase from S. coelicolor

SCO5461, a putative gene from *S. coelicolor*, encodes a protein (204 amino acids, MW 22385.60) that comprises a secretion signal motif (amino acids 1–42) and a possible ADP-ribosyltransferase domain (amino acids 43–204, MW 18426.99). The latter activity domain has a 30% homology with the activity domains of the mosquitocidal toxin from *B. sphaericus* SSII-I (MTX) and pierisin-1 from *P. rapae* (Fig. 1A and B). MTX and pierisin-1 have lectin-like receptor-binding domains at their C-termini which facilitate their entry into eukaryotic cells and repress the ADP-ribosyltransferase activity of these proteins until cleaved by proteases (Carpusca et al., 2006). However, SCO5461 has no putative receptor-binding domain.

Recent genome projects have revealed homologues of SCO5461 in some actinomycete genomes (Fig. 1A and C, and Table S1 on Supporting information), the products of which contain three characteristic motifs of cholera toxin-like ADP-ribosyltransferase motifs; an LXR motif, an STT motif, and a catalytic center glutamic acid (Domenighini and Rappuoli, 1996; Otto et al., 2000). All SCO5461 homologues in actinomycete lack putative binding domains, and those in *Streptomyces lividans*, *Streptomyces scaviei*, and *Streptomyces albus* harbor secretion signals at their N-terminal ends. However, a SCO5461 homologue in *Streptosporangium roseum* lacks a putative secretion signal. Interestingly, avermectin-producing *Streptomyces avermitilis* has a pseudogene lacking a clear ORF, and streptomycin-producing *Streptomyces griseus* has no SCO5461 homologue. All known arginine ADP-ribosyltransferases, including MTX, have a glutamic acid located two residues upstream of their catalytic center glutamic acid (Laing et al., 2011). On the other hand, pierisin-1 and most SCO5461 homologues have glutamine, instead of glutamic acid, two residues upstream of their catalytic center (Fig. 1A, bold type).

3.2. ADP-ribosylation of nucleic acids by ScARP, the SCO5461 gene product

We purified the activity domain of the SCO5461 gene product as an MBP-tagged form following its expression in *E. coli* and then removed the MBP tag with factor Xa. The resulting peptide (SCO5461(43–204), MW 18903.51), which

had a preceding ISEF residues derived from the multi-cloning site sequence on its N-terminus, was confirmed to be the single predominant band on an SDS-PAGE gel (Figure S1 on Supporting information) and was therefore used in the subsequent experiments.

When SCO5461(43–204), expressed in *E. coli*, was incubated for 4 h with calf thymus DNA in the presence of NAD^+, two new peaks appeared at the same retention times seen for N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine by HPLC analysis (Fig. 2A-I). Both peaks were clearly enhanced by co-injection of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine, synthesized by the incubation of deoxyguanosine and pierisin-1 in the presence of NAD^+ (Figure S2 on Supporting information). Since these peaks were much smaller than those made by pierisin-1 and calf thymus DNA, we searched for more desirable substrates. Many RNA species, including tRNA and oligo$(G)_{14}$, and oligo$(dG)_{14}$ can be more easily modified compared with calf thymus DNA, suggesting that SCO5461(43–204) recognizes single stranded oligonucleotides as substrates (Fig. 2A; details described later).

Interestingly, when SCO5461(43–204) was incubated for 10 min with guanosine (Guo) and NAD^+, two new HPLC peaks appeared (Fig. 2B-I). UV spectra of these two new peaks were found to be similar to that of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (Fig. 2B-I). Similar results were obtained with deoxyguanosine (dGuo; Fig. 3 and detailed analysis described later). However, when SCO5461(43–204) was incubated with (deoxy)adenosine, (deoxy)cytidine, thymidine, or uridine, in the presence of NAD^+, no new peaks appeared (Figure S3A on Supporting information).

The replacement of glutamic acid at the putative reaction center of the SCO5461 product with aspartic acid (Figure S1 on Supporting information) resulted in a >90%-decrease in the peaks (Fig. 2B-III), and no new peaks appeared with the use of ADP-ribose instead of NAD^+ (Fig. 2B-IV), suggesting that the SCO5461 product had ADP-ribosylated guanine residues. We hereafter refer to the SCO5461 protein product as *S. coelicolor* ADP-ribosylating protein (ScARP).

We further analyzed the structure of compounds in the two newly appeared peaks in the reaction of Guo, NAD^+, and ScARP (Fig. 2B-I, indicated with an arrow and an arrowhead). As shown in Figure S3B on Supporting information, the area of both peaks on the HPLC charts increased linearly up to 1 h. The ratio of the backward peak (minor peak, indicated by an arrowhead) to the forward peak (major peak, indicated by an arrow) from the reaction of Guo, NAD^+, and ScARP then increased during a 4-h reaction. When the isolated forward peak was re-analyzed by HPLC after incubation at 30 °C, the ratio changed to about 1:1 within 4 h, suggesting that both peaks can easily anomerize to each other (Figure S3C on Supporting information). Moreover, LC-ESI-MS analysis indicated that both the compounds in these peaks have a molecular ion peak at m/z 825, corresponding to ADP-ribosylated guanosine, and an ion peak at m/z 693, arising from the loss of a ribose moiety commonly observed in nucleoside separation, and an ion peak at m/z 926 corresponding to a triethylamine addition, derived from the HPLC eluent, to the parent mass at m/z 825 (Fig. 4A). We concluded that both peaks were ADP-ribosylguanosine and

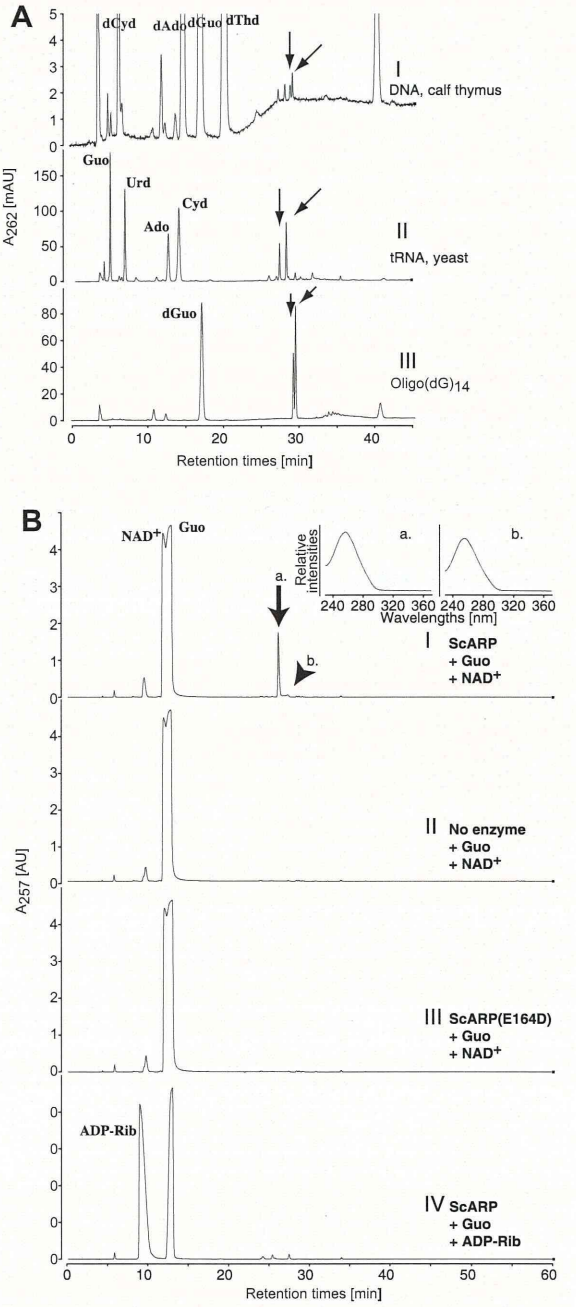

Fig. 2. Structural analysis of ADP-ribosylated nucleic acids formed by SCO5461(43–204) (ScARP). (A) HPLC elution patterns of hydrolysates of (I) calf thymus DNA, (II) tRNA, and (III) oligo$(dG)_{14}$, after incubation with ScARP (final 625 nM) and NAD^+ (final 2 mM) for 4 h. The arrows indicate newly appearing peaks. Note that unreacted NAD^+ was removed by ethanol precipitation prior to nuclease digestion. (B) HPLC elution patterns of guanosine incubated with (I) ScARP (0.2 nM) and NAD^+ (1 mM), (II) NAD^+ (1 mM), (III) ScARP(E164D) (0.2 nM) and NAD^+ (1 mM), and (IV) ScARP (0.2 nM) and ADP-ribose (1 mM). The arrow and arrowhead indicate newly appearing peaks. UV absorption spectra of compound **a** (λmax 257 nm) and **b** (λmax 256 nm), generated using a photodiode array detector, are superimposed in the elution pattern I.

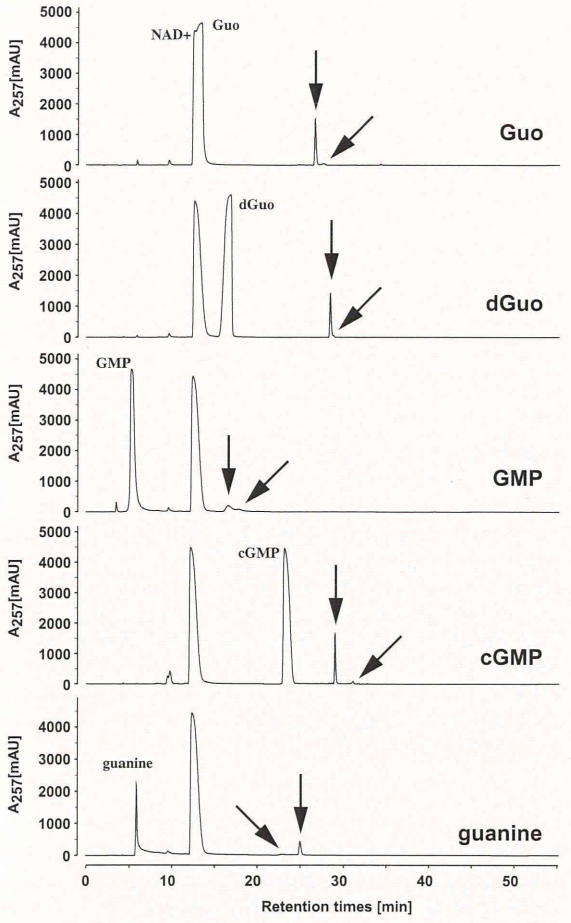

Fig. 3. ADP-ribosylation of monomers of nucleic acids by ScARP. HPLC elution patterns of Guo (top), dGuo (upper middle), GMP (middle), cGMP (lower middle), and guanine (bottom) incubated for 10 min (30 min for guanine) with ScARP (0.2 nM) and NAD^+ (1 mM) are shown.

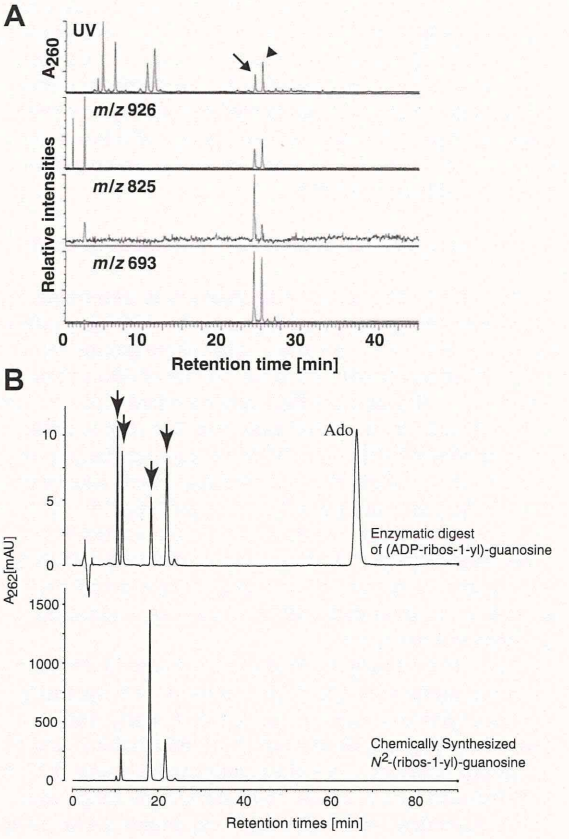

Fig. 4. Structural confirmation of N^2-(ADP-ribos-1-yl)-guanosine. (A) LC-ESI-MS elution patterns of reaction products formed from tRNA (0.1 mg/ml), NAD^+ (2 mM), and ScARP (625 nM). The HPLC profile (UV) and the ion chromatograms (m/z 926, 825, and 693) are shown. Both compounds in these peaks had a molecular ion peak at m/z 825 corresponding to [ADP-ribosyl-Guo + H^+]$^+$, an ion peak at m/z 693 arising from the loss of a ribose moiety, and an ion peak at m/z 926 corresponding to a triethylamine addition, derived from the HPLC eluent, to the parent mass at m/z 825. The forward peak is indicated by an arrow, and the backward peak is indicated by an arrowhead. (B) HPLC elution patterns of hydrolysate of (ADP-ribos-1-yl)-guanosine (top) and chemically synthesized N^2-(ribos-1-yl)-guanosine (bottom). Ado, adenosine.

the initial-reaction product was the forward peak, whereas the backward peak was its anomerized form at the C1′-position of ADP-ribose.

We further confirmed the structure of ADP-ribosylated Guo using HPLC, LC-MS, and ^{1}H NMR. We first digested ADP-ribosyl-Guo to ribosyl-Guo using phosphodiesterase I and calf intestinal alkaline phosphatase, and compared the resulting products by HPLC with independently synthesized N^2-(ribos-1-yl)-Guo (Scheme S1 on Supporting information). As shown in Fig. 4B, the retention times of the four peaks from enzymatic digest coincident with those of the synthesized (α- and β-ribofuranos-1-yl)-Guo and their further anomerized form, (α- and β-ribopyranos-1-yl)-Guo, as found for N^2-(ribos-1-yl)-2′-deoxyguanosine (Takamura-Enya et al., 2001). ^{1}H NMR analysis of ADP-ribosyl-Guo revealed that most of the signals could be assigned to the same as those of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (Takamura-Enya et al., 2001), except presence of a newly absorbance peak of around 4.5 ppm derived from a proton at the 2′ position of the ribose moiety with missing protons around 2.00 ppm derived from two protons of 2′ and 2″ position of the deoxyribose moiety that was presented in the ADP-ribosylated 2′-deoxyguanosine (Figure S4 on Supporting information). This result strongly indicated that the product was N^2-(ADP-ribos-1-yl)-guanosine (Fig. 5). Similar results were obtained using either HPLC or LC-MS analysis when dGuo was used instead

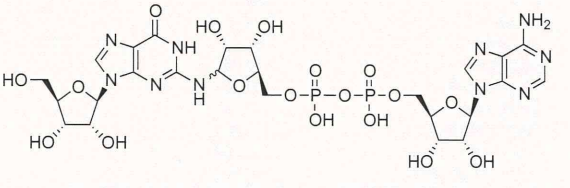

Fig. 5. Structure of N^2-(ADP-ribos-1-yl)-guanosine.

of Guo. Moreover, retention times for two peaks corresponding to ADP-ribosylated dGuo were the same as those of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (i.e. the enzymatic digest of ADP-ribosylated DNA produced by pierisin-1). We thus concluded that ScARP ADP-ribosylates the N^2 amino groups of guanine residues in Guo and dGuo in the same manner as pierisin-1 does for the N^2 amino groups of guanine residues in dsDNA.

3.3. Kinetics of NAD^+:guanosine ADP-ribosylation by ScARP

We next performed kinetic analyses to determine the structural preferences of nucleic acids for ADP-ribosylation by ScARP. Neither the 3′- and 5′-phosphate groups showed much effect on the ADP-ribosylation rate of Guo and dGuo, and cyclic GMP was also effectively modified. Guanine was also modified but at a lower rate than Guo (Fig. 3, Table 1). All of the tested DNAs and RNAs containing guanine residues were modified more slowly than their monomers; 50% of the bases in both oligo(G)$_{14}$ and oligo(dG)$_{14}$ were modified at a relatively faster rate than yeast tRNA, *E. coli* rRNA, mRNAs from HeLa cells, and synthesized dsRNA, but only a small amount of guanine residues in calf thymus dsDNA were modified (Table 1, Fig. 2A, Figure S2 on Supporting information).

We next tried to optimize the reaction conditions for the kinetic analysis of ScARP, NAD^+, and Guo. The quantity of ADP-ribosylated products increased linearly for up to 60 min (see Figure S3B on Supporting information), and the enzymatic kinetics were thus determined using 0.2 nM ScARP in a 10-min reaction. The optimum pH range was 6–7, but activities were relatively low under weak acidic conditions compared with weak basic conditions. The optimum temperature was 30 °C, and 20% of the maximum activity was retained even at 0 °C. The optimum NaCl concentration was 50 mM but the effect of the NaCl concentration was not appreciable within the 0–300 mM range (Figure S5 on Supporting information). Similar activities were observed in the presence of KCl or Na_2SO_4 (50 mM each) in the place of NaCl, and neither the addition of Ca^{2+}, Mg^{2+}, EDTA, EGTA, NH_4Cl, nor dithiothreitol (5 mM each) interfered with this activity. We used Lineweaver–Burk plot analysis to determine the K_m value for NAD^+ at 110 μM, and the k_{cat} value for Guo at 325 s^{-1} (Fig. 6; Table 1). K_m values for Guo and dGuo are 31 μM and 52 μM, respectively, supporting that both Guo and dGuo are equally targeted. Both the 3′- or 5′-phosphate groups showed little effect on the k_{cat} values. These results suggest that ScARP has relatively high activity compared with the activity domains of MTX and pierisin-1, which have k_{cat} values of 2.5 ± 1 min^{-1} for soybean trypsin inhibitor (Schirmer et al., 2002a) and 55 s^{-1} for dsDNA (Watanabe et al., 2004) respectively.

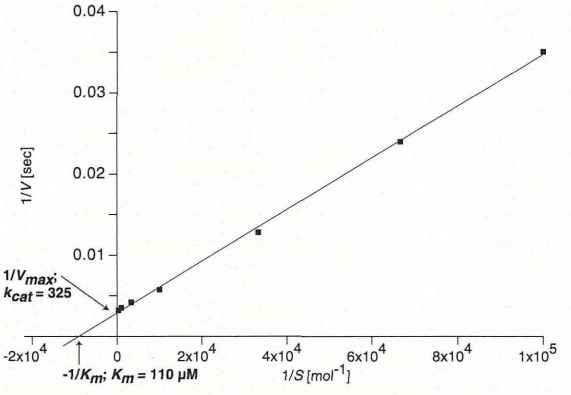

Fig. 6. Lineweaver–Burk plot analysis of ADP-ribosylation of 1 mM Guo by 0.2 nM ScARP in the presence of 0.01–3 mM NAD^+, in a 50 mM Hepes-NaOH/50 mM NaCl buffer for 10 min at 30 °C. Product amounts were determined by the A_{257} values.

Table 1
Acceptors for ADP-ribosylation by ScARP.

Acceptors	k_{cat} [s^{-1}]	K_m [μM]
Guo	325	31
dGuo	278	52
GMP	204	
dGMP	512	
cGMP	148	
GTP	460	
dGTP	379	
guanine	63	174
oligo(G)14	0.26	
oligo(dG)14	0.20	
tRNA, yeast	0.14	
DNA, calf thymus	0.001	
Ado, Cyd, Urd	<0.0001	
dAdo, dCyd, dThd	<0.0001	

k_{cat} and K_m values were obtained using Lineweaver–Burk plot analysis.

3.4. Secretion of ScARP from S. coelicolor

This is the first report that characterizes a mono-ADP-ribosylating enzyme for guanine nucleosides and mononucleotides i.e. ScARP. Although Widdick et al. may have previously detected secreted ScARP, their reporter-gene assay could not determine twin-arginine translocation pathway-dependent secretion from a plate culture of *S. coelicolor* (Widdick et al., 2006). Since there is no UUA^{Leu} codon in the SCO5461 gene, the tRNA of which is specifically expressed upon differentiation (Lawlor et al., 1987), ScARP must not be differentiation-specific. We thus validated its ADP-ribosyltransferase activity from liquid culture medium of growing-phase *S. coelicolor* using in-gel enzymatic analysis and LC-ESI-MS (Figure S6A on Supporting information). When the active fraction was separated with an SDS-PAGE gel co-polymerized with yeast tRNA, and ADP-ribosylated by soaking the gel with [^{32}P]NAD^+ and radioluminographed, a band corresponding to an ADP-ribosyltransferase was detected at 18-kDa, with the same mobility as that of the activity domain of ScARP (Fig. 7). We also purified cultured medium partially, and the active fractions with or without ScARP, as the internal standard, were measured in duplicate by nano LC-MS/MS system (2DICAL, Ono et al., 2009, 2006). As shown in Figure S6B and C on Supporting information, the peak of 499.57 *m/z* (RT 35.455 min) matched the doubly-charged ITPEPVWR sequence with an iScore of 31 and an expect score of 44.0, and the peak of 482.526 *m/z* (RT 36.310 min) matched the triply-charged GPQVVFEEGFHAK sequence with an iScore of 37 and an expect score of 6.5 of SCO5461/gi21223819 in

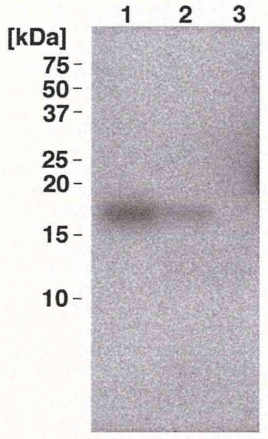

Fig. 7. In-gel analysis of NAD^+:tRNA ADP-ribosyltransferase activity from the culture medium of *S. coelicolor*. Lane 1, total protein from the culture medium of growing phase of *S. coelicolor*, concentrated with an Amicon Ultra MWCO 10 kDa devise (10 μg); lane 2, secretion-form, 18-kDa (10 ng) ScARP; lane 3, secretion-form, 18-kDa ScARP(E164D) (10 ng).

the MASCOT database. Both peaks were clearly enhanced in the samples supplemented with ScARP, indicating that *S. coelicolor* secreted ScARP protein in liquid culture medium.

4. Discussion

We revealed in our current study that ScARP is an enzyme lacking receptor-binding domains, secreted from *S. coelicolor*, and that ADP-ribosylates the N^2 amino groups of guanine nucleosides as well as mononucleotides. This is therefore the first report to identify an ADP-ribosyltransferase that mainly targets mononucleotides and nucleosides, since pierisin-1 shows weak ADP-ribosylation activity on dGuo (Figure S2 on Supporting information; Takamura-Enya et al., 2001), but the ADP-ribosylation of Guo by MTX was undetectable at least in the condition used in present study (Figure S3D on Supporting information). Hence, ScARP could be classified as a pentosyltransferase [EC 2.4.2.x] with a systematic designation of NAD^+:guanine-N^2-ADP-D-ribosyltransferase, or as an N-glycosidic cholera toxin-like-ADP-ribosyltransferase catalyzing mono-ADP-ribosylation [EC 2.4.2.30.1.X.1.2] with a systematic name NAD^+:mono-ADP-D-ribosyl-guanine-N^2-ADP-D-ribosyltransferase (GADPRT), according to the new extended EC numbering system proposed by Hottiger et al. (2010).

Pallen et al. (2001) have also predicted by PSI-BLAST analysis that a 219-aa secretion protein (ID 7105990), identified in the unfinished genome project of *S. coelicolor*, could be an ADP-ribosyltransferase. However, their predicted reaction center in the β-5 strand of this protein product corresponds to E189, and not E164, in the SCO5461 product. This somewhat contrasting finding may be due to the preliminary nature of the sequences in unfinished genome projects. Previous reports have described the existence of NAD^+:protein ADP-ribosyltransferases and ADP-ribosylated proteins not only in *S. coelicolor* (Penyige et al., 2009; Shima et al., 1996; Sugawara et al., 2002) but also in *Streptomyces grieus*, a species that has no SCO5461 homologue (Ochi et al., 1992; Penyige et al., 1996). Since ScARP also targets basic proteins, such as soybean trypsin inhibitor with a k_{cat} value of less than 0.001, ScARP might not ADP-ribosylate protein substrates that were previously identified in *S. coelicolor*.

The formation of only a single isomer and the ready anomerization of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine has been proposed as the mechanism underlying the ADP-ribosylation of deoxyguanosine residues in DNA by pierisins (Takamura-Enya et al., 2004). Our present study revealed that ScARP produces only a single isomer of N^2-(ADP-ribos-1-yl)-guanosine, and it anomerized within 4 h. Although we couldn't determine which isomer had been the initial product, an inversion at the C1′-position of ADP-ribose moiety from β to α occurs in some ADP-ribosyltransferases [see (Laing et al., 2011) for review]. Future structural analyses of ScARP co-crystallized with Guo and NAD^+ will likely elucidate the reaction mechanism.

ScARP is a secreted protein without a receptor-binding domain, but the accumulated data indicate that the C-terminal receptor-binding domain is indispensable for toxic activities of MTX and pierisins (Carpusca et al., 2006). Moreover, ScARP seems not to be an acute toxic enzyme, since it could be expressed in *E. coli*; even expression vectors for pierisins have not been successfully generated, possibly due to the toxic effects of their basal expression during subcloning (Yamamoto et al., 2009). Nevertheless, the wide distribution of the ScARP homologues lacking a C-terminal receptor-binding domain in streptomycetes suggests that they must have an important function. If ScARP is an enzyme which functions extracellularly, it could be possible that ADP-ribosylguanosine (^{ar2}Guo) functions in response to environmental changes. Many bacteria use guanosine derivatives in response to environmental changes, such as guanosine penta- or tetraphosphate ((p)ppGpp)-mediated stringent responses (Srivastava and Waters, 2012), or cyclic di(3′-5′)GMP (c-di-GMP)-mediated regulation of motility and biofilm formation (Krasteva et al., 2012). In this respect, it is interesting to speculate that ScARP disregulates these signals by direct ADP-ribosylation of these guanosine derivatives, or by synthesizing ^{ar2}Guo as mimicking or competitively inhibiting molecules. Since the damaged nucleotide pool induces genomic instability and gene mutation [for reviews, see Henderson et al. (2010)], it might be possible to speculate the ^{ar2}Guo-mediated increasement of DNA replication errors in bacteria living near *S. coelicolor*.

Although streptomycetes are known to secrete a large amount of extracellular enzymes to obtain soluble nutrients (Chater et al., 2010), there is a "topological paradox", similar to that found in vertebrate ARTs and CD38 (De Flora et al., 1997; Koch-Nolte et al., 2011), since both NAD^+ and guanosine mainly exist inside cells. Possible sources for NAD^+ and guanosine are dead or damaged bacteria and eukaryotes, the major nutrition source for streptomycetes. The other possible sources for guanosine are secreted extracellular nucleic acids [see (Kikuchi and Rykova, 2010) for review]. Further studies, such as screens for naturally formed ADP-ribosylated guanine nucleotides, elucidation of the responses of bacteria to ADP-ribosylated guanosine,

and phenotypic analysis of a ScARP-deletion strain will be necessary in the future to better understand the biological significance of ScARP.

The other remaining question is the origin of ADP-ribosyltransferase such as ScARP. The ScARP homologue in *S. avermitilis* (Save_pseudo in Fig. 1A and C) is overlapped by the SAV_1763 lipoprotein gene and therefore has an imperfect ORF. Save_pseudo is located on the border between the region for non-essential secondary metabolite genes and the region for essential genes (Ikeda et al., 2003). Our present hypothesis is that this gene could have once been distributed among some *Streptomyces* strains, but then disappeared by recombination with the other gene in *S. avermitilis*. There are many actinomycete genome projects currently underway, reflecting the importance of these bacteria in ecology, pharmacy, and industry. Both phylogenetic and biological studies of these microorganisms may help to elucidate the origin and physiological roles of nucleoside- and mononucleotide-specific ADP-ribosyltransferases.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary information

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxicon.2012.11.019.

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