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Poly(ADP-Ribose): Release of Template Restriction in HeLa Cells

(NAD/nuclear proteins/DNA polymerase/DNA primer sites)

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ABSTRACT **Evidence is presented to show that ADP-ribosylation of nuclear proteins by poly(ADP-ribose) polymerase enhances template-primer activity of HeLa cell nuclear DNA. We used *Escherichia coli* DNA polymerase I (EC 2.7.7.7; DNA nucleotidyltransferase) as an exogenous probe of nuclear DNA status. Neither NAD nor free poly(ADP-ribose) acts directly on the bacterial enzyme. The stimulation is specific for formation of ADP-ribosylated proteins and is not a generalized polyanion or nucleotide effect on chromatin. The release of template restriction is proportional to the capacity of a given cell line to synthesize poly(ADP-ribose); both the stimulation and poly(ADP-ribose) formation are coordinately proportional to NAD concentration. Binding studies with DNA polymerase indicate exposure or generation of additional 3′-OH primer sites due to ADP-ribosylation in intact nuclei. With intact cells, there appears a correlation among growth, physiological template restriction, and the above effects of ADP-ribosylation.**

Besides DNA and RNA, poly(ADP-ribose) is the third polynucleotide that exists in eukaryote nuclei. A chromatin-bound enzyme, poly(ADP-ribose) polymerase, catalyzes the formation of the homopolymer by the successive elongation of ADP-ribose units, which are derived from the substrate NAD. There is considerable evidence that one function of this reaction is to modify histones and other nuclear proteins by the covalent attachment of poly(ADP-ribose) (1–3). Because of the great interest in ADP-ribosylation of nuclear proteins, several reviews have been recently published outlining the chemistry and biochemistry of this unique polymer (4–6). Our recent interest has centered on the elucidation of possible biological roles of this nuclear protein modification in regulation of DNA replication and NAD levels of cells (7). Recently we noted that in HeLa nuclei, the ADP-ribosylation of nuclear proteins seems related to enhancement of template activation for DNA synthesis when probed by *Escherichia coli* DNA polymerase I (EC 2.7.7.7; DNA nucleotidyltransferase) (8). This apparent stimulation of DNA synthesis due to ADP-ribosylation was detected throughout the cell cycle of HeLa cells, with maximal enhancement noted at G2 phase of the cell cycle. Transcription, as probed in this manner by *E. coli* RNA polymerase, was not affected by poly(ADP-ribose) formation during the cell cycle (8). The present work further explores this stimulation and suggests a possible mechanism for this effect on template activation.

MATERIALS AND METHODS

[*U-adenine*-^{14}C]NAD (136 mCi/mmole) and [*methyl*-^{3}H]-thymidine-5′-triphosphate (23.9 Ci/mmole) were purchased from Amersham Searle Co. *E. coli* DNA polymerase I (5000 units/mg) was purchased from General Biochemicals.

HeLa cells were maintained in spinner cultures (9); nuclei were isolated by the method of Sporn *et al.* (10). Chromatin was prepared by the method of Huang and Huang (11).

The conditions for poly(ADP-ribose) formation have been described (8). Two methods were used to measure the effect of ADP-ribosylation on DNA synthesis: preincubation with NAD, or DNA synthesis in the presence of NAD (Fig. 1).

RESULTS

Poly(ADP-Ribose) and Template Activation. E. coli DNA polymerase I can utilize DNA within the complex nuclear system derived from HeLa cells to catalyze the polymerization of deoxynucleotides (Fig. 1) in a reaction dependent upon the presence of all four deoxynucleoside triphosphates. The labeled product of the reaction bands in CsCl gradients with

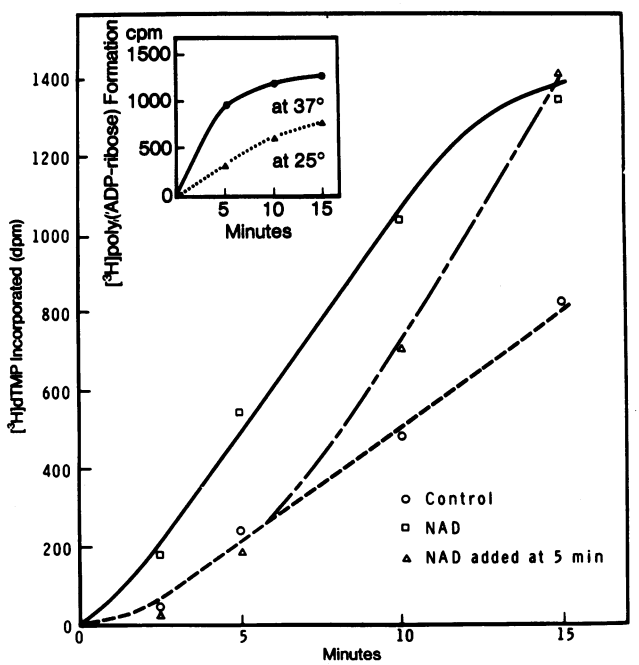

FIG. 1. Kinetics of [^{3}H]dTMP incorporation during poly(ADP-ribose) formation. The normal mixture for DNA synthesis was increased by a factor of five. The reaction was started by addition of 2 × 10^{7} nuclei and incubation was done at 37°. The *inset* shows the amount of poly(ADP-ribose) formed at 37° in the DNA synthesis assay and at 25° in the normal poly(ADP-ribose) polymerase assay.

Abbreviation: ADP-ribose, adenosine diphosphoribose.

TABLE 1. *Effects of NAD and ADP-ribosylation on deoxynucleotide incorporation as catalyzed by DNA polymerase I utilizing various sources of template*

	Source of template	Additions	cpm	%
IA.	Activated calf thymus DNA (50 μg)	None	955	100
		NAD(4 mM)	817	86
		NAD(4 mM) + Nam(40 mM)	878	92
		Nam(40 mM)	905	95
IB.	Activated calf thymus DNA (50 μg)	None	7976	100
		Poly(ADP-ribose) (100 μg)	9051	113
IIA.	Intact nuclei	None	925	100
		NAD(4 mM)	2350	254
IIB.	Sonicated nuclei	None	1030	100
		NAD(4 mM)	2240	218
		NAD(4 mM) + Nam(20 mM)	1380	134
III.	Chromatin (270 μg of protein)	None	757	100
		NAD(4 mM)	2402	318
		NAD(4 mM) + Nam(20 mM)	971	128
IVA.	Intact nuclei	None	608	100
		NAD(4 mM)	1081	178
IVB.	Heated nuclei	None	574	100
		NAD(4 mM)	624	109
V.	Intact nuclei	None	212	100
		NAD(4 mM)	572	270
		DNase	5610	2650
		DNase + NAD(4 mM)	9323	4400

Incorporation of [^{3}H]dTMP into DNA as catalyzed by *E. coli* DNA polymerase I was measured in the standard assay (*Methods*). In Exp. *II*, either 4 × 10^6 intact or disrupted (by sonication) nuclei were used as template. Sheared chromatin (Exp. *III*) was prepared (11); after ADP-ribosylation (8), it was diluted with cold neutral buffer and pelleted by ultracentrifugation before measurement of dTMP incorporation. In Exp. *IV*, nuclei were heated at 75° for 5 min, sufficient to completely inactivate poly(ADP-ribose) polymerase (98% inhibition) before incubation with NAD. DNase I (4 μg/4 × 10^6 nuclei) was used in Exp. *V* for 5 min at 25° in the presence or absence of NAD and subsequently inactivated for 5 min at 75° before DNA synthesis was measured with *E. coli* DNA polymerase I. *Nam*, nicotinamide.

the same density as the bulk HeLa DNA, and is rendered acid-soluble by DNase treatment. Formation of poly(ADP-ribose), as promoted by NAD, stimulates the rate of dTMP incorporation by a factor of about 2-fold. As shown elsewhere in this paper (Table 1) and previously (8), the addition of the end-product of the poly(ADP-ribose) reaction, nicotinamide, inhibits this enhancement of template activity. The data in Fig. 1 also show that the addition of NAD to the reaction subsequent to initial polymerization of deoxynucleotides immediately caused enhancement of HeLa template capacity. The *inset* in Fig. 1 demonstrates that poly(ADP-ribose) is readily synthesized under the conditions optimal for DNA synthesis at 37°, as well as in the normal assay mixture used for the assay of poly(ADP-ribose) polymerase (8) at 25°. In fact, about twice as much poly(ADP-ribose) is formed at 37° in the DNA polymerase mixture.

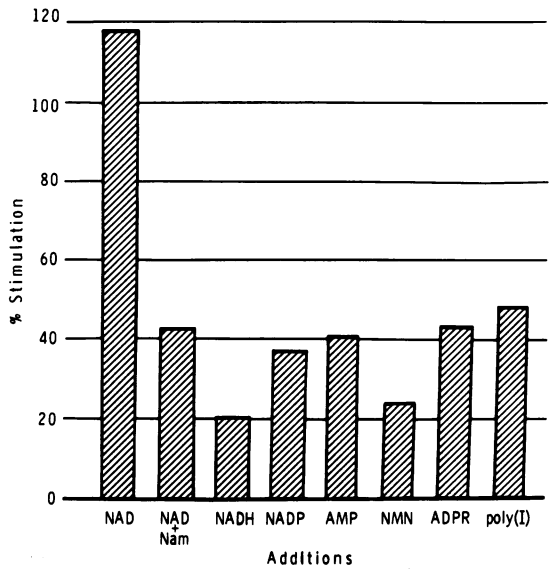

FIG. 2. Effects of NAD, related nucleotides, and poly(I) on the template-primer activity of HeLa cell nuclei. The amount of [^{3}H]dTMP incorporated after 15 min at 37° was determined in one incubation (see *Materials and Methods*). Each compound was present at a final concentration of 4 mM, except for nicotinamide, which was present at 20 mM, and poly(I) (s_{20} = 11), which was present at a final concentration of 40 μg/ml. *Nam*, nicotinamide; *ADPR*, ADP-ribose.

The data in Table 1, Exp. *I*, indicate that, at the concentrations used to effect template activation of nuclei, neither NAD nor nicotinamide have any direct effect on *E. coli* DNA polymerase I itself, when an activated calf thymus DNA template is used in the absence of nuclei. Preliminary experiments with purified poly(ADP-ribose) also demonstrated that the *free* polymer did not affect DNA synthesis when the purified enzyme and calf thymus DNA template were used. The stimulation of dTMP incorporation (Fig. 1) is not due to an enhanced nuclear permeability for deoxynucleotide substrates or the exogenous *E. coli* polymerase, as shown by the results that dTMP incorporation of sonicated nuclei (Table 1, Exp. *II*) and chromatin preparations derived from HeLa cells (Table 1, Exp. *III*) are both stimulated by formation of poly(ADP-ribose). Inhibition of ADP-ribosylation of nuclear proteins by nicotinamide abolished the stimulatory effect in both cases, indicating the relationship between polymer formation and template activation.

Data in Fig. 2 give further support that the enhancement of template activation is specifically due to formation of poly(ADP-ribose). Nucleotides related to NAD that do not function in the poly(ADP-ribose) polymerase reaction caused only negligible increase in deoxynucleotide incorporation promoted by DNA polymerase I, in HeLa nuclei. Polyanions, especially poly(inosinic acid), have been reported to remove template restriction for DNA, presumably by competing with DNA for associated histones (12). However, as indicated in Fig. 2, poly(I) had little effect on the system as probed by exogenous enzyme. These results confirm that poly(ADP-ribose), which occurs naturally in contrast to synthetic polynucleotides, is an extremely sensitive probe of nuclear template status in HeLa cells.

Template Enhancement Related to Ability of System to Form Poly(ADP-Ribose). A correlation is shown by the concentra-

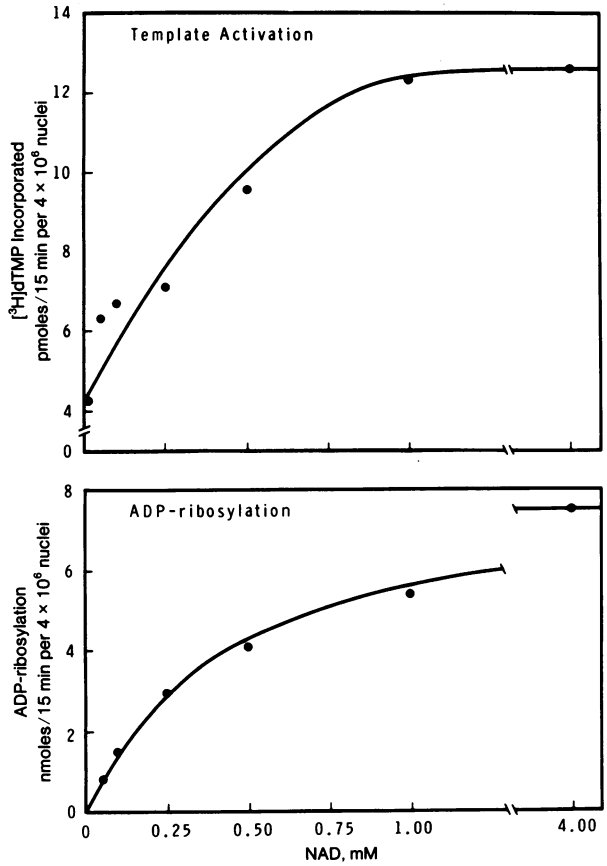

FIG. 3. NAD concentration dependence of poly(ADP-ribose) synthesis and template activation of HeLa nuclei for DNA synthesis mediated by *E. coli* DNA polymerase I.

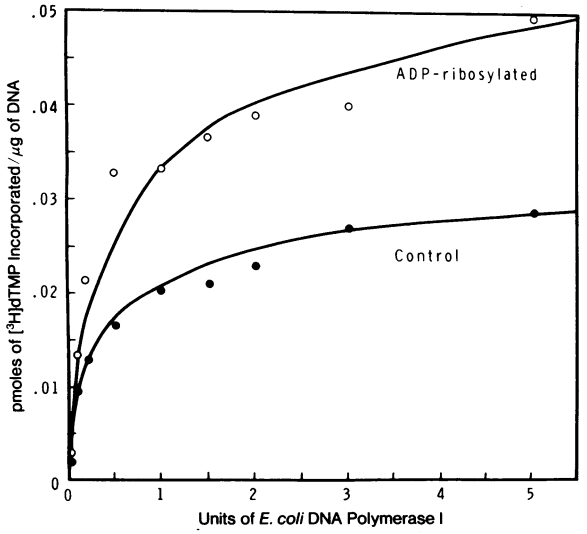

FIG. 4. Incorporation of a single deoxynucleotide as a function of increasing concentration of *E. coli* DNA polymerase I. Aliquots containing 8 × 10^6 nuclei were added to the poly(ADP-ribose) polymerase mixture (1.0 ml final volume) containing either no NAD or 4 mM NAD. After 15 min at room temperature, the solutions were diluted with 5 ml of 0.25 M sucrose containing 2 mM $MgCl_2$ and 40 mM nicotinamide. The nuclei were collected by centrifugation and resuspended in twice-concentrated DNA polymerase mixture containing various amounts of DNA polymerase I and [³H]dTTP but no other deoxynucleoside triphosphates.

tion dependence for NAD of both poly(ADP-ribose) formation and DNA synthesis (Fig. 3). The release of template restriction was proportional to the amount of poly(ADP-ribose) formed; an NAD concentration of 1 mM was sufficient to generate enough poly(ADP-ribose) in 4 × 10^6 nuclei to maximally stimulate DNA synthesis. In addition, when poly(ADP-ribose) polymerase was inactivated by heat, no enhancement of template activity was noted (Table 1, Exp. *IV*).

Preliminary experiments with nuclei derived from human adult fibroblasts, fibroblasts from patients with xeroderma pigmentosum, and HeLa cells indicate that a correlation exists between specific activity of poly(ADP-ribose) polymerase and release of template restriction. The specific activities relative to HeLa cells for the three cell lines above were 0.008, 0.042, and 1.0, respectively, while the effects of NAD on nuclear template activity were 0, 21, and 100%, respectively, over the controls without NAD.

Enhanced Accessibility of DNA by ADP-Ribosylation. Single deoxynucleotide incorporation into DNA as a function of DNA polymerase concentration has been used (13, 14) to measure the number of binding sites in native DNA. Such an experiment is demonstrated with intact HeLa nuclei either in the presence or absence of ADP-ribosylation (Fig. 4). At DNA polymerase I saturation, about twice (25 pmoles/mg of DNA) as much dTMP was incorporated into the DNA of the ADP-ribosylated nuclei compared to the control. This difference represents generation of about 1.6 × 10^{13} new 3′-OH primer sites per mg of nuclear DNA. A similar increase in binding sites of ADP-ribosylated nuclei was found when [³H]dATP or [³H]dCTP was the only radioactively labeled deoxynucleoside triphosphate in the incubation. We have consistently been unable to fully saturate DNA modified by ADP-ribose. One interpretation of these results is that once modified, new 3′-OH sites are constantly being generated (perhaps by endogenous endonucleolytic action).

Increased accessibility of the DNA after ADP-ribosylation is also suggested by another technique. DNA of intact HeLa nuclei can be activated by brief treatment with pancreatic DNase I (Table 1, Exp. *V*) in a manner similar to native DNA (14). A 27-fold increase in template activation is noted by this treatment, while ADP ribosylation caused a 2.7-fold increase in template activation of HeLa nuclei. We observed that prior modification of nuclei by treatment with NAD and subsequent activation with DNase caused a 44-fold activation of template of intact nuclei. The data suggest that this modification alters the nuclear system such as to promote increased ability for DNase activation, probably due to improved accessibility of the DNA for pancreatic DNase I.

If the physiological function of the polymer is to increase the accessibility of the DNA in chromatin, then one might expect a correlation between the template activity of the nuclear DNA and the ability to release template restriction by poly(ADP-ribose). That is, if the template is naturally maximally activated, little additional effect would be expected. This possibility can be explored by using HeLa cells that were maintained under various physiological conditions. As HeLa cells progress from exponential growth through stationary growth, the template activity of nuclear DNA as

probed with *E. coli* DNA polymerase I decreased from 680 cpm of [^{3}H]dTMP incorporated to 250 cpm (Fig. 5). This decrease is presumably due to physiological template restriction and perhaps less accessible 3'-OH primer sites. However, as cells progress toward the stationary phase, the ability to stimulate deoxynucleotide incorporation by ADP-ribosylation increases, i.e., template restriction is reduced. The ability of poly(ADP-ribose) to stimulate DNA synthesis increases from 30% at the beginning of the experiment to 160% at 96 hr of growth. There appears to be an inverse correlation between the ability of ADP-ribosylation to stimulate DNA synthesis, and the status of the nuclear system to be used by *E. coli* enzyme. This is most marked after re-establishment of growth; template capacity is minimal and stimulation is very marked.

DISCUSSION

Our data demonstrate that the formation of poly(ADP-ribose) in HeLa nuclei releases template restriction for DNA synthesis when the nuclei are probed with exogenous, purified *E. coli* DNA polymerase I. The physiological importance of this polymer might be related to our observation (Fig. 5) that this release seems most marked during growth of the intact human cell, when template restriction of chromatin itself was greatest. Furthermore, the extent of template activation seems dependent on the amount of poly(ADP-ribose) formed (Fig. 3) and also on the relative ability of the cell type to synthesize poly(ADP-ribose).

The data suggest that the formation of poly(ADP-ribose) results in increased accessibility of the HeLa DNA to the exogenous DNA polymerase. We find that in control and ADP-ribosylated nuclei dTMP is incorporated into DNA chains of comparable length, but in ADP-ribosylated nuclei the quantity of the chains is increased (data not shown). This is likely due to more chain starts since, after ADP-ribosylation, there are 2-fold more binding sites for the enzyme (Fig. 4). Reports that poly(ADP-ribose) is covalently linked to proteins (1, 2) make it likely that the polymer exerts its action by displacement of chromosomal proteins. Improved accessibility of the DNA to numerous proteins is certainly necessary for purposes of gene transcription and replication and repair of DNA. In contrast to many synthetic polyanions that release template restriction (15, 16), poly(ADP-ribose) has the advantage of being synthesized at the intranuclear site necessary for its action.

We had previously reported on fluctuations of the specific activity of poly(ADP-ribose) polymerase in nuclei isolated throughout the cell cycle (17) and the asynchronous growth cycle (18) of HeLa cells. Our data on the activity of this enzyme system during the cell cycle (8, 17) has been consistent with *either* stimulation or inhibition of DNA synthesis. In addition, we could note no specific pattern of nuclear protein modification by poly(ADP-ribose) during the HeLa cell cycle to account for its physiological role (19).

In contrast to our findings in HeLa cells, considerable evidence has been accumulated by Sugimura and his coworkers (20, 21) and by Burzio and Koide (22, 23) that in isolated rat liver nuclei the formation of poly(ADP-ribose) inhibits the amount of deoxynucleotide incorporation into DNA when nuclei are used both as a source for template and DNA polymerase. The suggested mechanism for this inhibition includes: removal of the endogenous nuclear DNA polymerase from the nuclear DNA template (20, 24); prevention of template

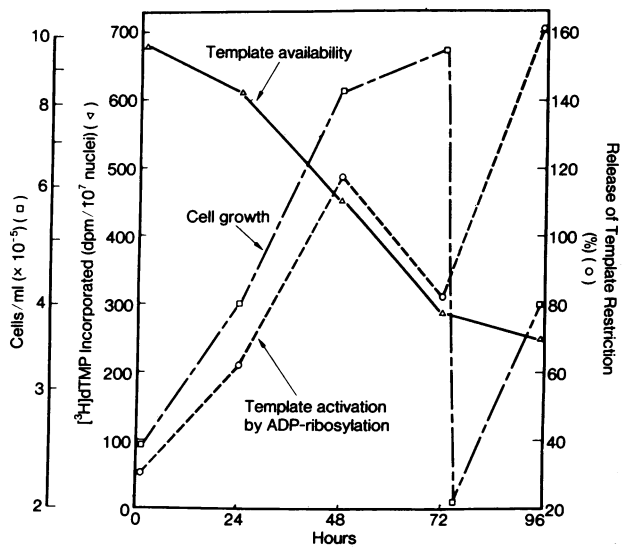

FIG. 5. Relationship of nuclear template activity and release of template restriction by poly(ADP-ribose) formation as a function of the asynchronous growth cycle of HeLa cells. HeLa cells were grown as described (9), and cell samples were collected at the indicated times. At 72 hr, the culture was diluted with fresh medium to restore growth. The template availability (△) of isolated nuclei was determined by measurement of [^{3}H]dTMP incorporation with *E. coli* DNA polymerase I. Release of template restriction (○) is defined as the amount of [^{3}H]dTMP incorporated by *E. coli* DNA polymerase I in the presence of 4 mM NAD divided by the amount of [^{3}H]dTMP incorporated in the absence of NAD multiplied by 100.

activation by endogenous nuclear endonucleases (25); or perhaps a combination of both processes (21). This inhibition of deoxynucleotide incorporation in rat liver nuclei in the presence of poly(ADP-ribose) has been confirmed in several laboratories (3, 20, 26), including our own. Lehmann and Shall (26) have shown that ADP-ribosylation leads to inhibition of endogenous DNA synthesis in rat liver nuclei, but no effect on DNA synthesis was observed in nuclei from lymphoma cells or from lymphocytes stimulated to divide by phytohemagglutinin. Furthermore, in a study where normal and regenerating rat liver nuclei, as well as Novikoff hepatoma nuclei, were all prepared in the same manner, the formation of poly(ADP-ribose) caused inhibition of DNA synthesis in the normal and regenerating systems, but did not affect the nuclei from the hepatoma cells (3).

Further experiments will be required to resolve these differences and to ascertain the mechanisms involved in the different cells, but it is interesting that rat liver nuclei contain a Ca^{++}-Mg^{++}-dependent endonuclease, which has been described by Burzio and Koide (25) and has been suggested to be responsible for activating the rat liver DNA and resulting in enhanced deoxynucleotide incorporation. The role of poly(ADP-ribose) should then be to inhibit this endonuclease activity in rat liver nuclei (25). There is a precedent for poly(ADP-ribose) inhibiting a DNase activity since Yamada *et al.* (27) have reported that this polymer inhibits an exonuclease from rat liver. However, we have been unable to detect a Ca^{++}-Mg^{++}-dependent DNase activity in nuclei from HeLa cells. It is interesting that the Novikoff hepatoma nuclei used by Burzio and Koide (25) apparently lack the presence of a Ca^{++}-Mg^{++}-dependent endonuclease. Although

the precise reasons for these differences in response in nuclei derived from different cells remains to be elucidated, it is clear that subtle perturbations in chromatin structure are being affected and probed by poly(ADP-ribosylation).

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