RAPID COMMUNICATION Jpn. J. Cancer Res. 88, 921–924, October 1997

Helicobacter pylori Extracts Exhibit Nicotinamide Adenine Dinucleotide-derived Adenylation but Not Mono(adenosine 5′-diphosphate-ribosyl)ation of DNA Ligase

Tadashige Nozaki,[1,6] Mitsuko Masutani,[1] Takeshi Noda,[2] Daizo Saito,[2] Toshiro Sugiyama,[3] Tsuyoshi Takato,[4] Keiji Wakabayashi,[5] Hitoshi Nakagama[1] and Takashi Sugimura[1]

[1]Biochemistry Division, [5]Cancer Prevention Division, National Cancer Center Research Institute, [2]National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, [3]3rd Department of Internal Medicine, School of Medicine, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo 060 and [4]Department of Oral and Maxillo-Facial Surgery, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113

The issue of toxins produced by *Helicobacter pylori* (*H. pylori*) urgently requires clarification given that the bacterium causes gastric epithelial cell damage which may lead to precancerous and cancerous changes. During an investigation of the possibility of mono(adenosine 5′-diphosphate (ADP)-ribosyl)-ation by *H. pylori* products, as observed for other bacterial toxins, we found that radioactivity of [adenylate-^{32}P]nicotinamide adenine dinucleotide (NAD) is incorporated into an *H. pylori* protein of 80 kDa after incubation with crude bacterial extract. In contrast, [carbonyl-^{14}C]NAD did not show any radioactivity incorporation. Unexpectedly, treatment of the modified protein with 0.1 *N* HCl, but not 0.1 *N* NaOH, released the AMP moiety. Such chemical properties are characteristic of bacterial DNA ligase-AMP complexes. We found that an antibody raised against *Escherichia coli* DNA ligase [EC 6.5.1.2] immunoprecipitated the modified 80 kDa protein. Our results indicate that incorporation of radioactivity derived from NAD into the 80 kDa protein was due to adenylation, but not mono-(ADP-ribosyl)ation, of the DNA ligase of *H. pylori*.

Key words: *Helicobacter pylori* — DNA ligase — Mono(ADP-ribosyl)ation — Adenylation — NAD

Helicobacter pylori (*H. pylori*) is now recognized as a factor responsible for development of gastric and duodenal ulcers, chronic gastritis, atrophic gastritis, intestinal metaplasia and gastric cancer.[1–3] There are various strains of *H. pylori*, differing greatly in their virulence. Two genes are known to be involved in the production of toxins; one is *vacA*, coding for a protein which causes vacuolar degeneration in gastric epithelial cells,[4] while the other is *picB/cagE*, which is associated with induction of interleukin 8 in gastric epithelial cells, and is 50% homologous with the gene for the protein of *Bordetella pertussis* (*B. pertussis*), transporting its toxin.[5,6] Many bacterial toxins including *B. pertussis* toxin[7] and diphtheria toxin,[8,9] have been shown to modify host cellular proteins by mono(adenosine 5′-diphosphate (ADP)-ribosyl)ation. In the course of experiments to clarify whether mono(ADP-ribosyl)ation reaction is involved in the pathogenesis of *H. pylori*, as with other bacteria, we found that radioactivity of [adenylate-^{32}P]nicotinamide adenine dinucleotide (NAD), but not [carbonyl-^{14}C]NAD, is incorporated into an 80 kDa protein (p80) in *H. pylori* crude extracts. The acid-labile but alkali-resistant property of this modification is consistent with that of the bacterial DNA ligase-AMP complex.[10] Immunoreactivity of the modified p80 with an antibody against *Escherichia coli* (*E. coli*) DNA ligase indicates that the NAD-derived modification involves adenylation of DNA ligase in *H. pylori*, rather than mono(ADP-ribosyl)ation. Given the importance of epithelial cell damage caused by *H. pylori* infection, leading to chronic gastritis, precancerous and cancerous changes,[1] this finding could be of great significance.

We first prepared extracts of six strains (8570, 8687, MT, 12205, NO and HI) of *H. pylori*, isolated from Japanese patients, and examined the incorporation of radioactivity from NAD. The bacteria were grown on agar plates (Nikken Biomedical Lab., Kyoto) containing 10% bovine blood in a microaerobic atmosphere at 37°C. Liquid culture was done in brucella broth (Difco Lab., Detroit, MI) containing 9% horse serum (Gibco BRL, Gaithersburg, MD). After centrifugation, *H. pylori* cell pellets were suspended in a buffer containing 50 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, 10 μg/ml leupeptin and 1 m*M* phenylmethylsulfonyl fluoride and sonicated in ice-water, then centrifuged at 100,000*g* for 20 min at 2°C. Supernatants were used for the incorporation of NAD radioactivity according to the mono(ADP-ribosyl)ation assay of *B. pertussis* toxin.[11] Briefly, reactions were carried out for 30 min at 37°C in a total

[6] To whom correspondence and reprint requests should be addressed.

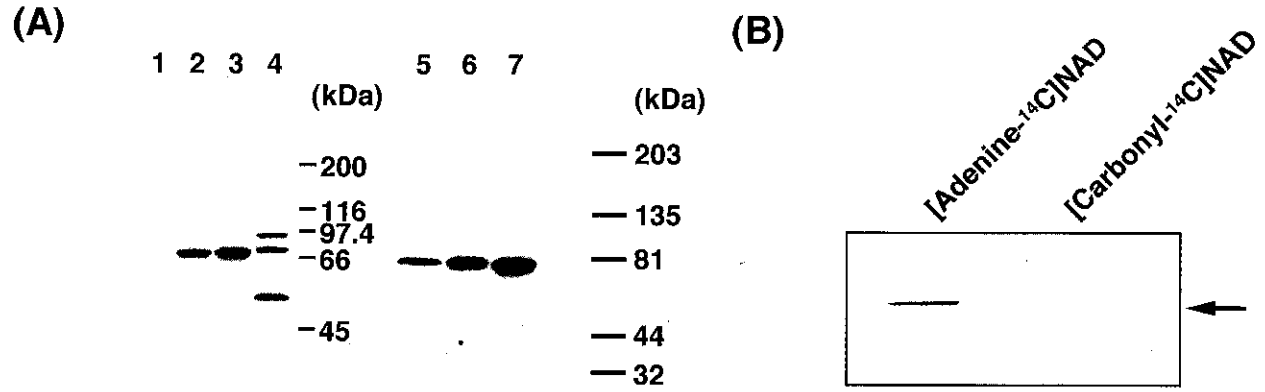

Fig. 1. Incorporation of NAD-derived radioactivity into a protein in *H. pylori* crude extracts. A, *H. pylori* crude extracts (50 μg protein) from six clinical isolates were subjected to reaction with [adenylate-^{32}P]NAD and analyzed by 10% SDS-PAGE. Lane 1: without extract, lanes 2–7: with extracts from six *H. pylori* strains (2: 8570, 3: 8687, 4: MT, 5: 12205, 6: NO, 7: HI). B, [adenine-^{14}C]NAD and [carbonyl-^{14}C]NAD were each used for the reaction with an extract (50 μg protein aliquots) from strain MT.

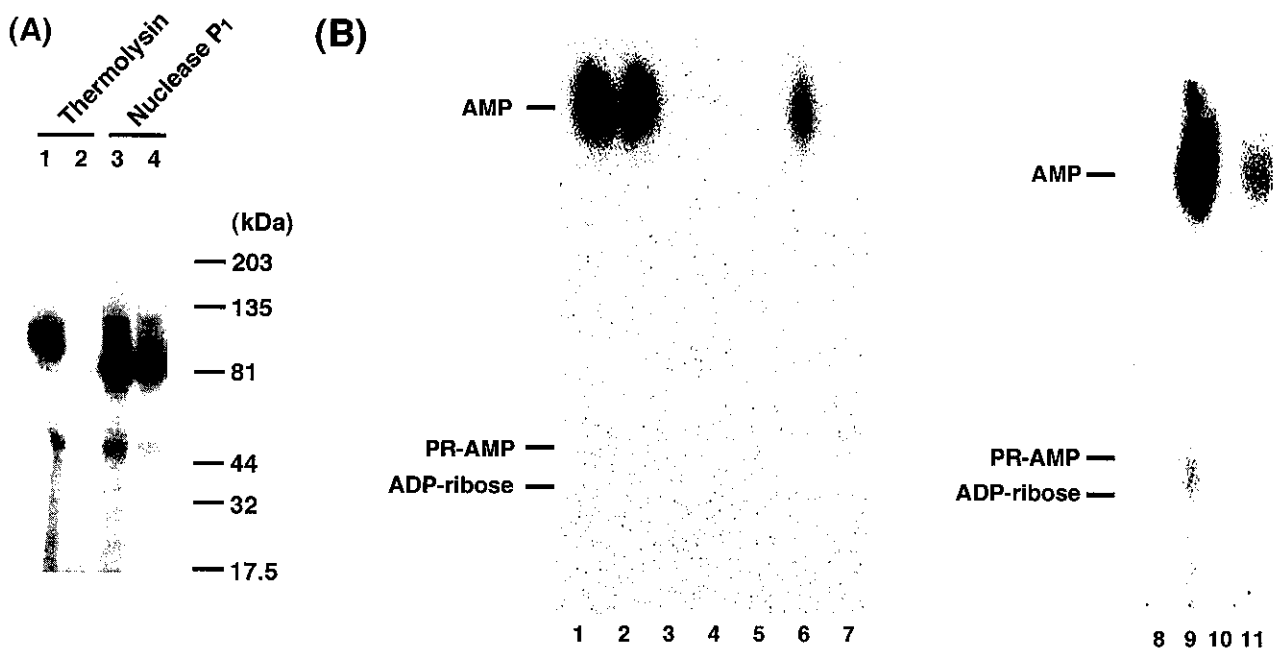

Fig. 2. Enzymological and chemical sensitivity of the [adenylate-^{32}P]NAD-derived moiety of *H. pylori* p80. A, After incubation of *H. pylori* extract (strain MT) with [adenylate-^{32}P]NAD, the reaction mixture was treated either with thermolysin or nuclease P1 and then subjected to SDS-PAGE (1: no addition, 2: 12 μg of thermolysin, 3: no addition, 4: 2.5 μg of nuclease P 1). B, After the reaction with the strain MT extract and fractionation on SDS-PAGE, proteins were transferred to PVDF membranes and the membranes at the position of p80 were cut out. They were subjected to various chemical and enzymological treatments in 20 μl reaction mixtures at 25°C [lane 1] or 37°C [lanes 2–10] for 1 h; lane 1,2: 0.1 *N* HCl, 3: 0.1 *N* NaOH, 4: 0.1 *M* NH_2NH_2, 5: 0.1 *M* NaCl, 6: 0.1% trifluoroacetic acid, 7: methanol, 8: 1 *M* NaCl, 9: 1 *M* NH_2OH, 10: 10 m*M* $HgCl_2$, 11: 0.08 units of snake venom phosphodiesterase I. Following the treatments, portions of the reaction mixtures were spotted onto PEI-cellulose and developed with buffer (0.1 *M* LiCl, 3 *M* acetic acid, 3 *M* urea). TLC was performed with reference compounds, 5′-AMP and ADP-ribose, in each lane. The position of phosphoribosyl AMP (PR-AMP) was determined by digestion of poly(ADP-ribose) with snake venom phosphodiesterase I.

volume of 100 μl containing 50 m*M* Tris-HCl (pH 7.5), 20 m*M* thymidine, 5 m*M* DTT, 1 μ*M* [adenylate-^{32}P]-NAD (800 Ci/mmol, New England Nuclear, Montreal, Canada) and 50 μg protein of *H. pylori* crude extract. [Adenine-^{14}C]NAD or [carbonyl-^{14}C]NAD was used instead of [adenylate-^{32}P]NAD in some experiments as described. After adding 300 μl of 0.2% sodium dodecylsulfate (SDS) to stop the reaction and removal of unincorporated NAD by filtration through Centrisalt C4 (Sartorius, Germany), the supernatants were subjected to 5–20% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Dried gels were then analyzed with an image analyzer, BAS2000 (Fuji Film, Tokyo). As shown in Fig. 1A, strong incorporation of [adenylate-^{32}P]NAD radioactivity was detected with all the extracts of six different strains at approximately 80 kDa. Incorporation of radioactivity was similarly observed at 80 kDa with [adenine-^{14}C]NAD (220 mCi/mmol, Amersham, Bucks., UK), but not with [carbonyl-^{14}C]NAD (30–62 mCi/mmol) (Fig. 1B), suggesting that the NAD-derived modification of the 80 kDa substance involves the adenylate portion of NAD rather than the carbonyl residue of nicotinamide in NAD. As shown in Fig. 2A, the radioactive band proved to be sensitive to protease and thermolysin, but not nuclease P1, indicating that the 80 kDa substance is a protein, not a nucleic acid.

To examine further whether the incorporation of radioactivity from NAD was the result of mono(ADP-ribosyl)ation, the chemical and enzymologic stability of the product was analyzed. After reaction of [adenylate-^{32}P]NAD with crude extract from strain MT, proteins were blotted onto polyvinilidene difluoride (PVDF) membranes (Millipore, Tokyo) and the portions bearing the 80 kDa protein were cut out and subjected to various treatments as shown in Fig. 2B. The compounds released from p80 after these treatments were analyzed by polyethyleneimine (PEI) thin-layer chromatography (TLC). As 0.1 *M* or 1 *M* NaCl and methanol did not elute any radioactivity (lanes 5, 7 and 8), the possibility of non-covalent binding was ruled out. The [adenylate-^{32}P]-NAD-derived radioactive modification was quite sensitive to acidic treatment (0.1 *N* HCl or 0.1% trifluoroacetic acid, lanes 1, 2 and 6), but resistant to alkaline treatment (0.1 *N* NaOH, lane 3). This property precluded the possibility of mono(ADP-ribosyl)ation on arginine residues,[12] although the modification on p80 of *H. pylori* is sensitive to neutral hydroxylamine and snake venom phosphodiesterase I, as is the case for mono-(ADP-ribosyl)ation of arginine. When the membrane was treated with 3.3 μg of purified recombinant ADP-ribosylarginine glycohydrolase,[13] no radioactivity was released (data not shown). The observed acid-sensitive and alkali-resistant properties of the modification are characteristic of DNA ligase-adenylate intermediates.[10]

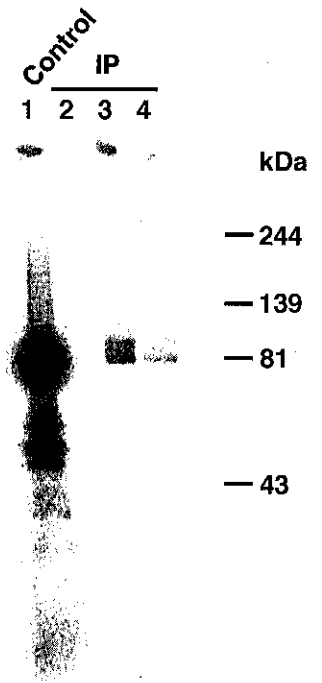

Fig. 3. Immunoprecipitation (IP) of modified p80 from an *H. pylori* extract by antibodies against *E. coli* DNA ligase. After the reaction using strain MT extract (50 μg of protein) and [adenylate-^{32}P]NAD, immunoprecipitation was carried out with either 5 μl of antiserum against *E. coli* DNA ligase or 5 μl of non-immune serum (lane 1: before immunoprecipitation, lane 2: immunoprecipitation with non-immune serum, lanes 3, 4: immunoprecipitation with antiserum against *E. coli* DNA ligase raised in a Balb/c mouse (lane 3) or a C3H mouse (lane 4)).

The size of the modified protein, 80 kDa, is comparable to that of *E. coli* DNA ligase. Bacterial DNA ligase is well known to incorporate AMP moieties, resulting in an adenylated form.[14] When we carried out an NAD-incorporation experiment using purified recombinant *E. coli* DNA ligase, distinct incorporation of radioactivity from [adenylate-^{32}P]NAD was observed at 80 kDa, as in the case of *H. pylori* extracts (data not shown).

To confirm that the modified protein was an *H. pylori* DNA ligase-adenylate complex, we raised polyclonal antibodies in mice against purified recombinant *E. coli* DNA ligase (Takara, Kyoto). After incubation of [adenylate-^{32}P]NAD and strain MT extract, immunoprecipitation analysis was carried out with polyclonal serum against purified *E. coli* DNA ligase or normal mouse serum as a control. Fig. 3 shows the results of SDS-PAGE of the immunoprecipitated proteins. The radiolabeled 80 kDa protein was specifically immunoprecipitated by antiserum against DNA ligase.

Taking all these results into consideration, we conclude that the modified 80 kDa protein in *H. pylori* extracts is a DNA ligase-adenylate intermediate, not a mono(ADP-ribosyl)ation product. Recently Miyake *et al.* reported similar incorporation of [adenylate-^{32}P]-NAD into a similarly sized *H. pylori* protein.[15] The sensitivity of the modification to hydroxylamine and phosphodiesterase I treatment corresponds well with their results. However, the acid-labile and alkali-resistant feature of the modification of p80 shown here is in complete disagreement with their observation of alkali-lability pointing to arginine-specific mono(ADP-ribosyl)ation.

It has been shown that the linkage between lysine residues in DNA ligase and AMP is due to formation of acid-labile phosphoramidate bonds.[14, 16] As shown in Fig. 2A, snake venom phosphodiesterase I could release AMP from the modified p80 of *H. pylori*. When *E. coli* DNA ligase-adenylate was treated with phosphodiesterase I, AMP release was similarly detected. However, it is not clear yet whether the phosphoramidate linkage is sensitive to phosphodiesterase I or whether some other unknown mechanisms may be involved. Further characterization of the modification of p80 is in progress.

We appreciate the assistance of Dr. K. Fukuda (National Cancer Center Research Institute, Tokyo) in the preparation of the antibody and we are indebted to Dr. T. Maehama and Dr. T. Katada (The University of Tokyo, Tokyo) for providing the purified recombinant ADP-ribosylarginine glycohydrolase. We thank Dr. H. Hayatsu (Okayama University, Okayama) for helpful comments on the stability of the phosphoramidate bond. We are also grateful to Dr. M. Nagao (National Cancer Center Research Institute, Tokyo) for constructive suggestions on this study. This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan.

(Received July 17, 1997/Accepted August 6, 1997)

REFERENCES

1) Sugiyama, T. and Yachi, A. Relationship of *Helicobacter pylori* to gastric carcinogenesis. *Ann. Cancer Res. Ther.*, **4**, 77–81 (1995).
2) Parsonnet, J., Hansen, S., Rodriguez, L., Gelb, A. B., Warnke, R. A., Jellum, E., Orentreich, N., Vogelman, J. H. and Friedman, G. D. *Helicobacter pylori* infection and gastric lymphoma. *N. Engl. J. Med.*, **330**, 1267–1271 (1994).
3) Seppala, K. *H. pylori* and gastric ulcer disease. *In* "*Helicobacter pylori*. Basic Mechanisms to Clinical Cure," ed. R. H. Hunt and G. N. J. Tytgat, pp. 429–435 (1994). Kluwer Academic Publishers, Netherlands.
4) Cover, T. L. and Blaser, M. J. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J. Biol. Chem.*, **267**, 10570–10575 (1992).
5) Tummuru, M. K., Sharma, S. A. and Blaser, M. J. *Helicobacter pylori picB*, a homologue of the *Bordetella pertussis* toxin secretion protein, is required for induction of IL-8 in gastric epithelial cells. *Mol. Microbiol.*, **18**, 867–876 (1995).
6) Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R. and Covacci, A. *Cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc. Natl. Acad. Sci. USA*, **93**, 14648–14653 (1996).
7) Sekura, R. D., Fish, F., Manclark, C. R., Meade, B., Zhang, Y. L. *Pertussis* toxin. Affinity purification of a new ADP-ribosyltransferase. *J. Biol. Chem.*, **258**, 14647–14651 (1983).
8) Van Ness, B. G., Howard, J. B. and Bodley, J. W. ADP-ribosylation of elongation factor 2 by diphtheria toxin. NMR spectra and proposed structures of ribosyl-diphthamide and its hydrolysis products. *J. Biol. Chem.*, **255**, 10710–10716 (1980).
9) Van Ness, B. G., Howard, J. B. and Bodley, J. W. ADP-ribosylation of elongation factor 2 by diphtheria toxin. Isolation and properties of the novel ribosyl-amino acid and its hydrolysis products. *J. Biol. Chem.*, **255**, 10717–10720 (1980).
10) Little, J. W., Zimmerman, S. B., Oshinsky, C. K. and Gellert, M. Enzymatic joining of DNA strands, II. An enzyme-adenylate intermediate in the DPN-dependent DNA ligase reaction. *Proc. Natl. Acad. Sci. USA*, **58**, 2004–2011 (1967).
11) Milligan, G., Streaty, R. A., Gierschik, P., Spiegel, A. M. and Klee, W. A. Development of opiate receptors in neonatal rat brain. *J. Biol. Chem.*, **262**, 8626–8630 (1987).
12) Althaus, F. R. and Richter, C. ADP-ribosylation of proteins: enzymology and biological significance. *Mol. Biol. Biochem. Biophys.*, **37**, 1–237 (1987).
13) Maehama, T., Nishina, H. and Katada, T. ADP-ribosylarginine glycohydrolase catalyzing the release of ADP-ribose from the cholera toxin-modified alpha-subunits of GTP-binding proteins. *J. Biochem.*, **116**, 1134–1138 (1994).
14) Modrich, P., Anraku, Y. and Lehman, I. R. Deoxyribonucleic acid ligase. Isolation and physical characterization of the homogeneous enzyme from *Escherichia coli*. *J. Biol. Chem.*, **248**, 7495–7501 (1973).
15) Miyake, M., Hirayama, T., Wada, A., Padilla, P. I., Moss, J., Kato, I. and Noda, M. An arginine-specific mono ADP-ribosyltransferase activity in *Helicobacter pylori* cell lysate. The 12th International Symposium on ADP-ribosylation Reactions: "Bacterial Pathogenesis to Cancer," meeting abstracts, p.30 (1997).
16) Tomkinson, A. E., Totty, N. F., Ginsburg, M. and Lindahl, T. Location of the active site for enzyme-adenylate formation in DNA ligases. *Proc. Natl. Acad. Sci. USA*, **88**, 400–404 (1991).