INFECTION AND IMMUNITY, Mar. 1989, p. 996–998
0019-9567/89/030996-03$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 57, No. 3

Exoenzyme S of *Pseudomonas aeruginosa* ADP-Ribosylates the Intermediate Filament Protein Vimentin

JENIFER COBURN,[1] SIMON T. DILLON,[1] BARBARA H. IGLEWSKI,[2] AND D. MICHAEL GILL[1]*

Department of Molecular Biology and Microbiology, Tufts University Schools of Medicine, Dental Medicine, and Veterinary Medicine, and the Sackler School of Biomedical Sciences, 136 Harrison Avenue, Boston, Massachusetts 02111,[1] and Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642[2]

Received 19 October 1988/Accepted 29 November 1988

Exoenzyme S, which had been thought to be unselective, catalyzes the ADP-ribosylation of only a subset of cellular proteins. The intermediate filament protein vimentin is one of the more abundant substrates. Disassembled vimentin, and proteolytic fragments of vimentin that cannot form filaments, is more readily ADP-ribosylated than is filamentous vimentin.

Pseudomonas aeruginosa secretes two ADP-ribosyltransferases. Exotoxin A modifies elongation factor 2 within cells (5) and thereby blocks protein synthesis. Exoenzyme S (6) is less well understood. Although it has a demonstrated role in pathogenesis (9, 15, 16), its physiological effects have yet to be determined. Identification of its substrates may lead to a clearer understanding of the significance of exoenzyme S in pathogenesis.

We started to characterize the ADP-ribosylation substrates by using NIH 3T3 cells. Frozen pellets (50 μl) were lysed by thawing in 50 mM Tris hydrochloride, pH 8.0–150 mM NaCl–1 mM phenylmethylsulfonyl fluoride (100 μl) and were again frozen and thawed. The lysates were incubated for 5 min at 25°C to allow the consumption of endogenous NAD, after which poly(ADP-ribose) polymerase activity was blocked by the addition of thymidine to 20 mM.

For ADP-ribosylation, a 10-μl portion of this lysate was incubated in 12 μl of a solution containing 5 μM [^{32}P]NAD (New England Nuclear Corp.) and 0.3 μg of exoenzyme S per ml (purified as described elsewhere [8]). After 30 min at 25°C, the reaction was stopped by adding 20 μl of 2% sodium dodecyl sulfate and heating at 100°C for 2 min. The addition of NAD glycohydrolase inhibitors (4) had no effect on the ADP-ribosylation of any substrate. NAD and active exoenzyme S were still present at the end of the incubation. When exoenzyme S was omitted from the reaction, no proteins were ADP-ribosylated.

Although exoenzyme S catalyzes the ADP-ribosylation of many proteins in lysed cells, on two-dimensional gels (2, 11) few of the ADP-ribosylated products correspond to proteins visible by Coomassie blue staining (Fig. 1). Despite the presence of multiple products, it is clear that exoenzyme S does display considerable selectivity. In only one instance was it possible to relate ADP-ribosylated products to stained proteins. Descending from the arrows in Fig. 1a and b are chains of five peptides that become more acidic as they become smaller and that are visible both as stained spots and as radioactive spots. The largest and most basic of these peptides is at the expected molecular weight and pI of vimentin (3). The series is reminiscent of the "staircase" pattern given by fragments of vimentin (10), which is due to proteolytic cleavages by a calcium-dependent protease (7) that removes successive basic peptides from the vimentin amino-terminal "head" region (14). Consequently, we looked for a different labeling pattern when the cells were lysed in the presence of 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid]. The addition of EGTA prevented the production of vimentin fragments and of the corresponding series of ADP-ribosyl products (Fig. 1c and d). Similar results were obtained with lysates of HEp-2 cells, turkey embryo fibroblasts, and pigeon erythrocytes.

Immunoblots reacted with antivimentin serum (ICN Biomedical) followed by horseradish peroxidase-conjugated secondary antibody (Sigma Chemical Co.) revealed a series of immunologically reactive spots (Fig. 2a) which corresponded to the [^{32}P]ADP-ribosylated spots in an autoradiogram of the blot (Fig. 2b). The radioactive proteins were slightly larger and slightly more acidic than the more abundant unmodified proteins, which is consistent with mono-ADP-ribosylation (Fig. 2c). When EGTA was present, vimentin remained intact (Fig. 2d) and was associated with a single radioactive spot (Fig. 2e and f). Control serum did not recognize vimentin or its fragments.

To further investigate the conditions necessary for ADP-ribosylation of vimentin, we compared the labeling reaction in two salt concentrations in which vimentin is either predominantly disassembled (low-salt buffer: 10 mM Tris hydrochloride, pH 8.0–1 mM phenylmethylsulfonyl fluoride–1 mM dithiothreitol–1 mM EGTA) or largely filamentous (physiological buffer: low-salt buffer plus 150 mM NaCl). We first established in an independent system that the low salt reduced overall activity of exoenzyme S by a factor of 9 (Table 1). Pigeon erythrocyte membranes (17) were used as the substrate, and fractionation of the products by gel electrophoresis revealed that labeling of each of the nonvimentin substrates was affected by the salt concentration in the same way.

We then measured the effect of salt concentration on the ADP-ribosylation of purified vimentin (a gift from Peter Steinert), which was renatured from a urea solution by dialysis into low-salt buffer or physiological buffer and reacted under these two ionic conditions (0.3 μg of exoenzyme S per ml–5 μM [^{32}P]NAD–1.7 μg of vimentin per ml, 30 min, 25°C). In both cases it was verified by gel electrophoresis that the labeled vimentin was still intact. After correction for the nonspecific effect of salt on the enzyme,

* Corresponding author.

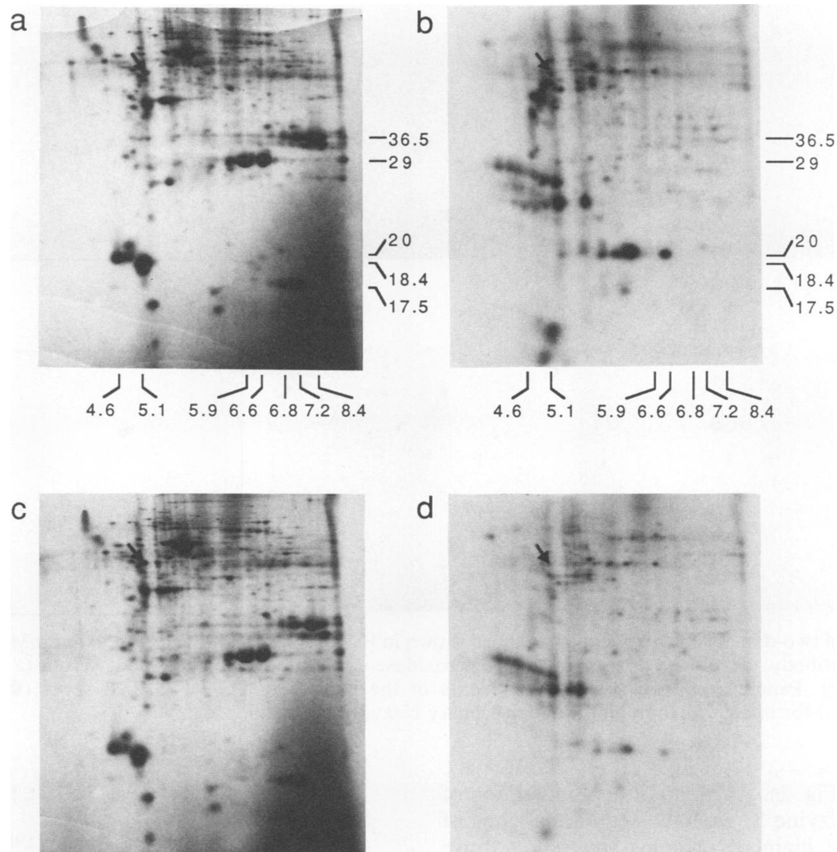

FIG. 1. Two-dimensional gel analysis of the ADP-ribosyl products of exoenzyme S. NIH 3T3 cells were lysed and ADP-ribosylated by using exoenzyme S without EGTA (a and b) or with 5 mM EGTA (c and d). Coomassie blue-stained gels (a and c) and corresponding autoradiograms (b and d) are shown. The marked proteins are internal isoelectric-focusing standard proteins. Molecular weights (in kilodaltons) are marked on the ordinates; pIs are marked on the abscissas. Arrows indicate spots that correspond to the apparent M_r and pI of intact vimentin. Note the chains of spots in the absence of EGTA that become more acid as they decrease in size. These chains are not present in panels c and d, which is consistent with the inhibition by EGTA of the calcium-activated protease that specifically digests vimentin.

the labeling of vimentin was favored by the low-salt condition by at least 14-fold (Table 1; 1.5 divided by 0.11).

This differential may be larger than the apparent 14-fold because the filamentous sample could well have contained some residual nonpolymerized vimentin which would have been ADP-ribosylated preferentially. To investigate this possibility, a portion of NIH 3T3 cell lysate was fractionated by centrifugation after ADP-ribosylation. [^{32}P]ADP-ribosylated vimentin and vimentin fragments were found in the supernatant after centrifugation at 100,000 × *g* (30 min). There was intact vimentin in the pellet, as detected by immunoblotting, but this was not labeled. Thus filamentous vimentin may be an even poorer substrate than calculated or might not be a substrate at all.

If most vimentin is filamentous and thus unavailable as a substrate, the little intact vimentin that is ADP-ribosylated could represent dissociated subunits or perhaps the termini of filaments. In either case, it is conceivable that ADP-ribosylation might have a considerable effect on cell architecture over the long term. Although this remains speculation, there is a strong precedent: *Clostridium botulinum* C2 toxin and *Clostridium perfringens* iota toxin ADP-ribosylate globular actin but not polymerized actin and prevent the repolymerization of globular actin (1, 12, 13). As nonpolymerized actin accumulates, the cellular microfilament network is destroyed.

Although exoenzyme S has appeared to be unselective with respect to its substrates, our evidence shows that several of the more abundant substrates are related (com-

TABLE 1. ADP-ribosylation of vimentin at different salt concentrations[a]

Protein	ADP-ribose incorporation rate[b] at:		Ratio
	Low salt	Physiological salt	
Isolated vimentin	2.0	1.3	1.5
Erythrocyte ghosts (total protein)	0.4	3.7	0.11

[a] Isolated vimentin was ADP-ribosylated in low-salt buffer or physiological salt buffer. Pigeon erythrocyte ghosts (17) were [^{32}P]ADP-ribosylated in the two salt media to determine the effect of salt concentration itself on the ADP-ribosylation rate.

[b] Data are expressed as picomoles of ADP-ribose incorporated per minute per milligram of protein.

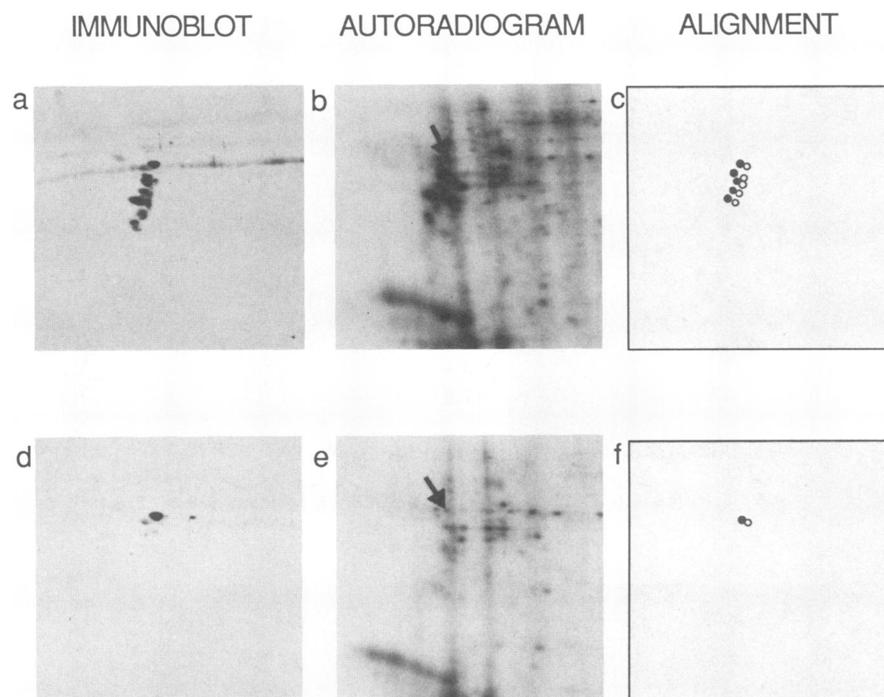

FIG. 2. Immunoblots of two-dimensional gels similar to those shown in Fig. 1. (a to c) No EGTA; (d to f) 5 mM EGTA. Blots were reacted with goat antivimentin antibody, developed with horseradish peroxidase-conjugated rabbit anti-goat serum (a and d), and then exposed with X-ray film (b and e). Panels c and f show the alignments of the centers of the ^{32}P-labeled spots (●) with the centers of the antibody-reactive spots (○) for intact vimentin and the four primary cleavage products.

pare panels d and b in Fig. 1). As an additional indication of the selectivity of exoenzyme S, we have seen no labeling of any other intermediate filament proteins, including those most related to vimentin; desmin and glial fibrillary acidic protein. Thus the substrate diversity of exoenzyme S may not be as great as it has appeared, and it should now be feasible to catalog the principal substrates and to determine which of the modifications are important in pathogenesis.

This work was supported by Public Health Service grants AI 16928 and AI 25669 from the National Institutes of Health.

We thank Peter Steinert for his generous gift of purified vimentin, Jack D. Lile for excellent technical assistance, and Eric Rubin for many helpful suggestions.

LITERATURE CITED

1. **Aktories, K., T. Ankenbauer, B. Schering, and K. H. Jakobs.** 1986. ADP-ribosylation of platelet actin by botulinum C2 toxin. Eur. J. Biochem. **161**:155–162.
2. **Ames, G. F.-L., and K. Nikaido.** 1976. Two dimensional electrophoresis of membrane proteins. Biochemistry **15**:616–623.
3. **Gard, D. J., P. B. Bell, and E. Lazarides.** 1979. Coexistence of desmin and the fibroblastic intermediate filament subunit in muscle and nonmuscle cells: identification and comparative peptide analysis. Proc. Natl. Acad. Sci. USA **76**:3894–3898.
4. **Gill, D. M., and J. Coburn.** 1988. ADP-ribosylation of membrane proteins by bacterial toxins in the presence of NAD glycohydrolase. Biochim. Biophys. Acta **954**:65–72.
5. **Iglewski, B. H., and D. Kabat.** 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. USA **72**:2284–2288.
6. **Iglewski, B. H., J. Sadoff, M. J. Bjorn, and E. S. Maxwell.** 1978. *Pseudomonas aeruginosa* exoenzyme S: an adenosine diphosphate ribosyl transferase distinct from toxin A. Proc. Natl. Acad. Sci. USA **75**:3211–3215.
7. **Nelson, W. J., and P. Traub.** 1981. Properties of a Ca^{2+}-activated protease specific for the intermediate-sized filament protein vimentin in Ehrlich-ascites-tumour cells. Eur. J. Biochem. **116**:51–57.
8. **Nicas, T. I., D. W. Frank, P. Stenzel, J. D. Lile, and B. H. Iglewski.** 1985. Role of exoenzyme S in chronic *Pseudomonas aeruginosa* lung infections. Eur. J. Clin. Microbiol. **4**:175–179.
9. **Nicas, T. I., and B. H. Iglewski.** 1985. Contribution of exoenzyme S to the virulence of *Pseudomonas aeruginosa*. Antibiot. Chemother. **36**:40–48.
10. **Ochs, D. C., E. H. McConkey, and N. L. Guard.** 1981. Vimentin-derived proteins. Exp. Cell Res. **135**:355–362.
11. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
12. **Schering, B., M. Bärmann, G. S. Chhatwal, U. Geipel, and K. Aktories.** 1988. ADP-ribosylation of skeletal muscle and nonmuscle actin by *Clostridium perfringens* iota toxin. Eur. J. Biochem. **171**:225–229.
13. **Simpson, L. L., B. G. Stiles, H. H. Zepeda, and T. D. Wilkins.** 1987. Molecular basis for the pathological actions of *Clostridium perfringens* iota toxin. Infect. Immun. **55**:118–122.
14. **Traub, P., and C. E. Vorgias.** 1983. Involvement of the N-terminal polypeptide of vimentin in the formation of intermediate filaments. J. Cell Sci. **63**:43–67.
15. **Woods, D. E., W. S. Hwang, M. S. Shahrabadi, and J. U. Que.** 1988. Alteration of pulmonary structure by *Pseudomonas aeruginosa* exoenzyme S. J. Med. Microbiol. **26**:133–141.
16. **Woods, D. E., and J. U. Que.** 1987. Purification of *Pseudomonas aeruginosa* exoenzyme S. Infect. Immun. **55**:579–586.
17. **Woolkalis, M., D. M. Gill, and J. Coburn.** 1988. Assay and purification of a cytosolic factor required for the activity of cholera toxin. Methods Enzymol. **165**:246–249.