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Protein D, a Putative Immunoglobulin D-Binding Protein Produced by *Haemophilus influenzae*, Is Glycerophosphodiester Phosphodiesterase

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Protein D of *Haemophilus influenzae* is 67% identical to the periplasmic glycerophosphodiester phosphodiesterase of *Escherichia coli*. Extracts prepared from *E. coli* expressing recombinant protein D had an 8- to 22-fold-higher specific activity of glycerophosphodiester phosphodiesterase compared with extracts of *E. coli* not expressing protein D.

Forsgren and coworkers demonstrated that cells of *Haemophilus influenzae*, *H. haemolyticus*, and *H. aegypticus* bind immunoglobulin D (IgD) (1, 11). They cloned a gene from *H. influenzae* designated *hpd* and demonstrated that the *hpd* gene product, protein D, bound an ^{125}I-labeled IgD myeloma protein (4). Three monoclonal antibodies which recognized protein D were generated. One or more of these murine monoclonal antibodies recognized a protein produced by *H. influenzae*, *H. haemolyticus*, and *H. aegypticus* as well as a protein produced by *H. parainfluenzae*, *H. aphrophilus*, *H. paraphrophilus*, and *Actinobacillus actinomycetemcomitans*. Cells of these latter four species, however, did not bind IgD (1). We demonstrated that cells of serotype b strains of *H. influenzae* bound IgD (12). However, with the exception of myeloma 4490, an IgD myeloma previously reported by Forsgren and coworkers (1, 11) to bind protein D, we were unable to detect IgD binding to cells of nontypeable strains of *H. influenzae* or to *Escherichia coli* cells expressing recombinant protein D (12). We now demonstrate that protein D is glycerophosphodiester phosphodiesterase.

Bacterial strains and plasmids. *H. influenzae* type b MinnA has been previously described (8). It has the outer membrane protein subtype 1H and is representative of the clonal group responsible for the majority of invasive *H. influenzae* type b disease in the United States. *E. coli* BL21(DE3)/pLysS was obtained from F. William Studier (13). The bacteriophage T7 expression vector pT7-7 was obtained from Stan Tabor (14). Plasmid pRSM1021 is the protein D gene (*hpd*) cloned into pT7-7. The construction of pRSM1021 (12) and sequence of the *hpd* gene from strain MinnA have been reported elsewhere (5, 12). The plasmid pRSM1060 contains the *H. influenzae* outer membrane lipoprotein P6 gene under the control of the T7 promoter in pT7-7 (8a, 10).

Glycerophosphodiester phosphodiesterase activity. *E. coli* strains were grown in L broth supplemented with chloramphenicol (25 μg/ml) and/or ampicillin (50 μg/ml) as appropriate. High-level expression of protein D was obtained by the addition of 1 mM isopropylthiogalactopyranoside (IPTG) to mid-log-phase cultures. Cells were then incubated at 37°C for an additional 2 h under high aeration prior to harvesting. *H. influenzae* MinnA was grown to mid-log phase in supplemented brain heart infusion medium as described previously (9). Cells were harvested by centrifugation, and the pellets were frozen. Cells from 35-ml cultures were thawed and resuspended in 5 ml of 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4). All subsequent operations were performed at 4°C. Cells were disrupted by sonication, an aliquot was removed, and the remainder of the extract was spun at 100,000 × *g* for 2 h to pellet the membrane fraction. The pellet was then resuspended in 1 ml of 50 mM HEPES.

Glycerophosphodiester phosphodiesterase activity was determined by a coupled spectrophotometric assay using *sn*-glycerol 3-phosphate dehydrogenase and NAD as described previously (2, 6, 7). Activity in the *E. coli* extracts was dependent on the presence of both L-α-glycerophosphorylcholine and α-glycerophosphate dehydrogenase. One unit is defined as the reduction of 1 μmol of NAD per min. Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, Ill.).

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Hib CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQ   50
Eco            ADSNEKIVIAHRGASGYLPEHTLPAKAMAYAQGADYLEQ   39

Hib DLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQS  100
Eco DLVMTKDDNLVVLHDHYLDRVTDVADRFPDRARKDGRYYAIDFTLDEIKS   89

Hib LEMTENFETKDGKQAQVYPNRFPLWKSHFRIHTFEDEIEFIQGLEKSTGK  150
Eco LKFTEGFDIENGKKVQTYPGRFPMGKSDFRVHTFEEEIEFVQGLNHSTGK  139

Hib KVGIYPEIKAPWFHHQNGKDIAAETLKVLKKYGYDKKTDMVYLQTFDFNE  200
Eco NIGIYPEIKAPWFHHQEGKDIAAKTLEVLKKYGYTGKDDKVYLQCFDADE  189

Hib LKRIKTELLPQMGMDLKLVQLIAYTDWKETQEKDPKGYWVNYNYDWMFKP  250
Eco LKRIKNELEPKMGMELNLVQLIAYTDWNETQQKQPDGSWVNYNYDWMFKP  239

Hib GAMAEVVKYADGVGPGWYMLVNKEESKPDNIVYTPLVKELAQYNVEVHPY  300
Eco GAMKQVAEYADGIGPDYHMLIE.ETSQPGNIKLTGMVQDAQQNKLVVHPY  288

Hib TVRKDALPAFFTDVNQMYDVLLNKSGATGVFTDFPDTGVEFLKGIK      346
Eco TVRSDKLPEYTPDVNQLYDALYNKAGVNGLFTDFPDKAVKFLNKE      333
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FIG. 1. Comparative amino acid sequences of the glycerophosphodiester phosphodiesterases of *H. influenzae* and *E. coli*. Hib, *H. influenzae* MinnA protein D; Eco, periplasmic product of the *E. coli glpQ* gene.

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TABLE 1. Glycerophosphodiester phosphodiesterase activity of protein D

Expt	Strain	Phenotype	Fraction[a]	Activity (U/mg)	Total U (%)
1	BL21(DE3)/pLysS	Control	Sonic extract	0.71	4.0 (100)
			100K sup	0.73	3.5 (88)
			100K pellet	ND[b]	ND (0)
	BL21(DE3)/pLysS/pRSM1021, induced	Protein D$^+$	Sonic extract	6.2	32 (100)
			100K sup	2.4	7.3 (23)
			100K pellet	19	27 (84)
2	*H. influenzae* type b MinnA	Protein D$^+$	Sonic extract	0.09	0.65 (100)
			100K sup	ND	ND (0)
			100K pellet	0.24	0.53 (82)
	BL21(DE3)/pLysS/pT7-7				
	Uninduced	Control	Sonic extract	0.40	2.1
	Induced	Control	Sonic extract	0.51	3.5 (100)
			100K sup	0.89	3.2 (91)
			100K pellet	0.03	0.07 (2)
	BL21(DE3)/pLysS/pRSM1021, induced	Protein D$^+$	Sonic extract	9.0	28 (100)
			100K sup	2.1	3.4 (12)
			100K pellet	19	24 (86)
	BL21(DE3)/pLysS/pRSM1060				
	Uninduced	Control	Sonic extract	0.49	2.3
	Induced	OMP P6^{+c}	Sonic extract	0.18	0.65

[a] 100K sup and 100K pellet refer to the supernatant and pellet fractions of the sonic extract after centrifugation at 100,000 × *g* for 2 h at 4°C.
[b] ND, not detectable.
[c] OMP P6 is a previously described outer membrane lipoprotein produced by *H. influenzae* (10). The P6 gene was cloned and expressed in the T7 expression vector pT7-7.

Comparison of the sequences of protein D and the *glpQ* gene product. A search of the current GenBank/EMBL data base by using the TFASTA algorithm (3) revealed that protein D had high homology to the *glpQ* gene product of *E. coli* (15). Protein D and *E. coli* glycerophosphodiester phosphodiesterase are 67% identical and 78% similar. The comparative amino acid sequences are shown in Fig. 1. Larson and coworkers identified the product of the *glpQ* gene as a periplasmic phosphodiesterase which catalyzes the hydrolysis of the deacylation products of glycerophospholipids to glycerol phosphate and an alcohol (6, 7). The *E. coli* enzyme did not show high specificity for the alcohol portion of the substrate and was readily assayed by the generation of glycerol phosphate from glycerophosphorylcholine.

Protein D has glycerophosphodiesterase activity. To determine whether protein D had glycerophosphodiester phosphodiesterase activity, we used the assay as optimized for the *E. coli* enzyme. Activity was readily demonstrable in the sonic extracts of *E. coli* and *H. influenzae* (Table 1). The *Haemophilus* activity was found predominantly in the 100,000 × *g* pellet, whereas the *E. coli* activity was found in the 100,000 × *g* supernatant. This localization is consistent with the reported periplasmic and cytoplasmic localization of the two identified glycerophosphodiester phosphodiesterases of *E. coli* and the membrane localization of the lipoprotein, protein D, of *H. influenzae*.

After induction of T7 RNA polymerase, the protein D gene in *E. coli* BL21(DE3)/pLysS/pRSM1021 is efficiently transcribed from the T7 promoter, and protein D accumulates (12). In extracts prepared from these cells, the specific activity of glycerophosphodiester phosphodiesterase was 8- to 22-fold greater than that observed in extracts of *E. coli* not producing protein D. As anticipated, the recombinant activity was primarily localized to the 100,000 × *g* pellet (Table 1). To demonstrate that the increased activity observed after induction of the T7 expression system was not indirectly due to the T7 expression system itself or, alternatively, due to the overexpression of a membrane lipoprotein, we analyzed extracts from strains BL21(DE3)/pLysS/pT7-7 and BL21(DE3)/pLysS/pRSM1060. This latter strain produces the *H. influenzae* outer membrane lipoprotein P6 under the control of the T7 promoter. Extracts prepared from these strains had specific activities of the glycerophosphodiester phosphodiesterase which were comparable to or lower than those observed with strain BL21(DE3)/pLysS.

In summary, we have demonstrated that protein D of *H. influenzae* has high homology to the product of the *E. coli glpQ* gene, a periplasmic glycerophosphodiester phosphodiesterase. The increased activity in *E. coli* extracts producing recombinant protein D and the subcellular localization of the recombinant activity are consistent with the hypothesis that protein D is *H. influenzae* glycerophosphodiester phosphodiesterase.

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REFERENCES

1. **Akkoyunlu, M., M. Ruan, and A. Forsgren.** 1991. Distribution of protein D, an immunoglobulin D-binding protein, in *Haemophilus* strains. Infect. Immun. **59:**1231–1238.
2. **Bublitz, C., and O. Wieland.** 1962. Glycerokinase. Methods Enzymol. **5:**354–361.
3. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
4. **Janson, H., L. O. Hedén, A. Grubb, M. Ruan, and A. Forsgren.** 1991. Protein D, an immunoglobulin D-binding protein of *Haemophilus influenzae*: cloning, nucleotide sequence, and expression in *Escherichia coli*. Infect. Immun. **59:**119–125.
5. **Janson, H., M. Ruan, and A. Forsgren.** 1992. High degree of conservation of protein D genes from nontypeable and type b strains of *Haemophilus influenzae*, abstr. D-244. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol.
6. **Larson, T. J., M. Ehrmann, and W. Boos.** 1983. Periplasmic glycerophosphodiester phosphodiesterase of *Escherichia coli*, a new enzyme of the *glp* regulon. J. Biol. Chem. **258:**5428–5432.

7. **Larson, T. J., and A. T. van Loo-Bhattacharya.** 1988. Purification and characterization of *glpQ*-encoded glycerophosphodiester phosphodiesterase from *Escherichia coli* K-12. Arch. Biochem. Biophys. **260:**577–584.
8. **Munson, R. S., Jr., B. Brodeur, P. Chong, S. Grass, D. Martin, and C. Proulx.** 1992. Outer membrane protein P1 and P2 of *Haemophilus influenzae* type b: structure and identification of surface-exposed epitopes. J. Infect. Dis. **165S:**S86–S89.
8a. **Munson, R. S., Jr., and S. Grass.** Unpublished data.
9. **Munson, R. S., Jr., J. L. Shenep, S. J. Barenkamp, and D. M. Granoff.** 1983. Purification and comparison of outer membrane protein P2 from *Haemophilus influenzae* type b isolates. J. Clin. Invest. **72:**677–684.
10. **Nelson, M. B., R. S. Munson, Jr., M. A. Apicella, D. J. Sikkema, J. P. Molleston, and T. F. Murphy.** 1991. Molecular conservation of the P6 outer membrane protein among strains of *Haemophilus influenzae*: analysis of antigenic determinants, gene sequences, and restriction fragment length polymorphisms. Infect. Immun. **59:**2658–2663.
11. **Ruan, M., M. Akkoyunlu, A. Grubb, and A. Forsgren.** 1990. Protein D of *H. influenzae*; a novel bacterial surface protein with affinity for human IgD. J. Immunol. **145:**3379–3384.
12. **Sasaki, K., and R. S. Munson, Jr.** 1993. Protein D of *Haemophilus influenzae* is not a universal immunoglobulin D-binding protein. Infect. Immun. **61:**3026–3031.
13. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
14. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
15. **Tommassen, J., K. Eiglmeier, S. T. Cole, P. Overduin, T. J. Larson, and W. Boos.** 1991. Characterization of two genes, *glpQ* and *ugpQ*, encoding glycerophosphodiester phosphodiesterases of *Escherichia coli*. Mol. Gen. Genet. **226:**321–327.