INFECTION AND IMMUNITY, May 1994, p. 2119–2121
0019-9567/94/$04.00+0

Vol. 62, No. 5

Differential Expression of the Cytotoxic and Hemolytic Activities of the ApxIIA Toxin from *Actinobacillus pleuropneumoniae*

ANH HUE T. TU,[1] CARA HAUSLER,[1] RY YOUNG,[2] AND DOUGLAS K. STRUCK[1]*

Department of Medical Biochemistry and Genetics, Texas A&M Health Science Center, College of Medicine,[1] and Department of Biochemistry and Biophysics,[2] Texas A&M University, College Station, Texas 77843-1114

Received 27 October 1993/Returned for modification 15 December 1993/Accepted 25 February 1994

The ApxIIA protein secreted from *Actinobacillus pleuropneumoniae* is both hemolytic and cytotoxic. However, when the cloned *apxII* operon is expressed in *Escherichia coli*, two forms of the ApxIIA protein can be recovered. Toxin which remains intracellular has hemolytic and cytotoxic activities, while toxin that is secreted is cytotoxic with little or no hemolytic activity. This indicates that the cytotoxicity of ApxIIA is independent of its hemolytic activity.

Actinobacillus pleuropneumoniae is one of a number of gram-negative bacteria that secrete toxins of the RTX (repeat toxin) family (15). To date, three antigenically different RTXs have been found to be produced by different *A. pleuropneumoniae* isolates singly or in combination (6, 10). The genetic determinants for each of these three toxins (ApxI, ApxII, and ApxIII) have been cloned and sequenced (2, 3, 7). Neutralization studies with monoclonal antibodies (10) and direct measurements of the lytic activity of the recombinant toxins expressed in *Escherichia coli* (2, 3, 7) have shown that these three toxins are functionally distinct. ApxIA displays the strongest hemolytic and cytotoxic activity. ApxIIA is also hemolytic and cytotoxic but is claimed to have a lower level of specific activity than ApxIA (10). ApxIIIA, like LktA produced by *Pasteurella haemolytica* and AaLktA from *Actinobacillus actinomycetemcomitans*, is cytotoxic but not hemolytic and thus belongs to the leukotoxin class of the RTX family (2).

The genetic determinants for the prototypical RTX consist of four genes, *CABD*, expressed from a common promoter or promoters (15). The *A* gene encodes the protoxin protein, which requires activation to display its full lytic activity. The activating event appears to be an RTX C-dependent acylation with a long chain fatty acid (8, 9). The exact number of sites for acylation of the protoxin and their location are not known for any of the RTX A proteins. In addition, the temporal relationship between synthesis, acylation, and secretion is also unknown. The RTX *BD* genes encode proteins which have been localized to the bacterial envelope and are required for secretion. For α-hemolysin, an RTX normally expressed by certain strains of *E. coli*, at least three additional genes appear to be required for secretion of active toxin: (i) *acpP*, which encodes acyl carrier protein, the putative acyl donor for activation of the protoxin in vitro (9); (ii) *tolC*, which is required for secretion (13); and (iii) *rfaH*, which has been variously claimed to be necessary for transcription of the *hly* genes (1), secretion of α-hemolysin from *E. coli* (14), or activation of the prohemolysin protein (4). Thus, the secretion of an active RTX is likely to be more complicated than originally envisioned, involving the products of at least seven different genes.

* Corresponding author. Mailing address: Department of Medical Biochemistry and Genetics, Texas A&M University Health Science Center, 440 Reynolds Medical Building, College Station, TX 77843-1114. Phone: (409) 845-9416. Fax: (409) 847-9481.

We have previously reported the cloning of the *apxIICA* genes from *A. pleuropneumoniae* and the production of hemolytically active ApxIIA protein in *E. coli* (3). In this note, we report that the ApxIIA protein recovered from cultures of *E. coli* expressing the *apxIICA* genes appears to exist in two forms which differ markedly in their relative hemolytic and cytotoxic activities.

The ApxIIA protein has been reported to be moderately cytotoxic and weakly hemolytic (10). To make a quantitative comparison of these two activities of ApxIIA, we prepared culture supernatants from an *A. pleuropneumoniae* serotype 7 isolate grown in brain heart infusion broth supplemented with 0.01% NAD. Serotype 7 strains have been shown to secrete only the ApxIIA protein and do not contain the genes for ApxIA or ApxIIIA toxins (6, 10). Using bovine erythrocytes and cells from the bovine leukemic cell line BL3 as target cells, we found the ApxIIA toxin secreted by *A. pleuropneumoniae* to be nearly equipotent on a per-target-cell basis in its ability to lyse these two cell types (Fig. 1). The ratio of hemolytic to cytotoxic activity was invariant during culture growth, with the highest absolute titers occurring as the culture entered the stationary phase (data not shown).

When the *apxIICA* genes are expressed in *E. coli* in the absence of RTX *BD* gene function, lytically active ApxIIA protein accumulates intracellularly and can only be detected after lysis of the bacterial cells by passage through a French pressure cell (3). Intracellular toxin activity peaks at late-log phase and rapidly disappears as the culture enters stationary phase, presumably because of the degradation or denaturation of the accumulated ApxIIA protein (Fig. 2). The ratio of hemolytic to cytolytic activity of the intracellular toxin is approximately 0.5 when assayed against bovine erythrocytes and BL3 leukocytes (Fig. 1). The ratio of intracellular hemolytic to cytolytic activity observed in *E. coli* is unchanged when RTX *BD* genes are expressed in *trans* to *apxIICA* (compare Fig. 1B and 2B). Thus, the activity of the recombinant toxin recovered from the *E. coli* cytoplasm appears similar to that secreted by *A. pleuropneumoniae*. Intracellular hemolytic and cytolytic activities are observed only when *apxIIC* and *apxIIA* are expressed in the same cell. Mixing lysates from cultures in which the two cloned genes were expressed separately does not result in lytic activity against either of the two target cell types.

When *E. coli* cells harboring pYFC37 (*apxIICA*) and either pLG575 (*hlyBD*), pYFC65 (*apxIBD*), or pHT55 (*lktBD*) are plated onto blood agar, the resulting colonies develop small

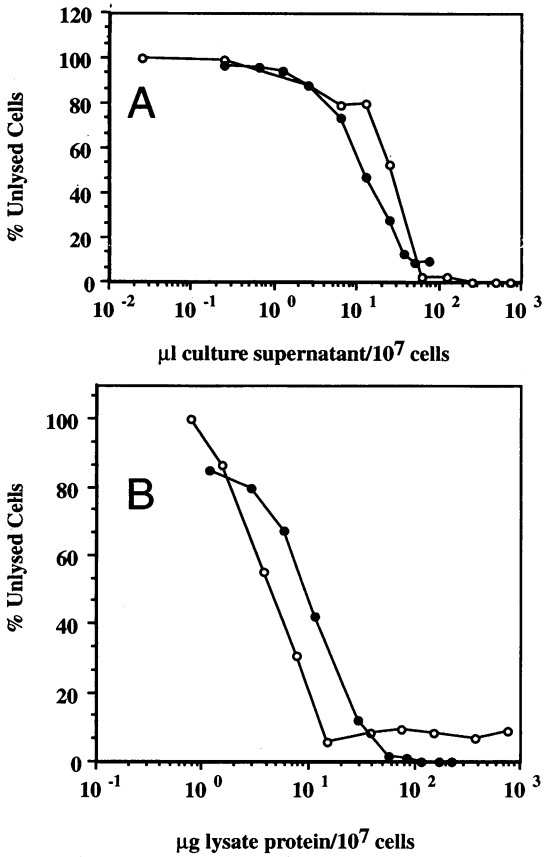

FIG. 1. Lytic activity of the ApxIIA toxin. The toxin was assayed against bovine erythrocytes (solid circles) or bovine leukemia cell line BL3 (open circles), as previously described (11). (A) ApxIIA secreted from a serotype 7 isolate of *A. pleuropneumoniae* exhibiting 50% lethal dose (LD_{50}) values of 100 U/ml for erythrocytes and 50 U/ml for BL3 cells. (B) Intracellular ApxIIA toxin, which accumulates within *E. coli* when the *apxIICA* genes are expressed in the absence of RTX BD function, yielding LD_{50} values of 100 U/µg of lysate protein for erythrocytes and 200 U/µg of lysate protein for BL3 cells.

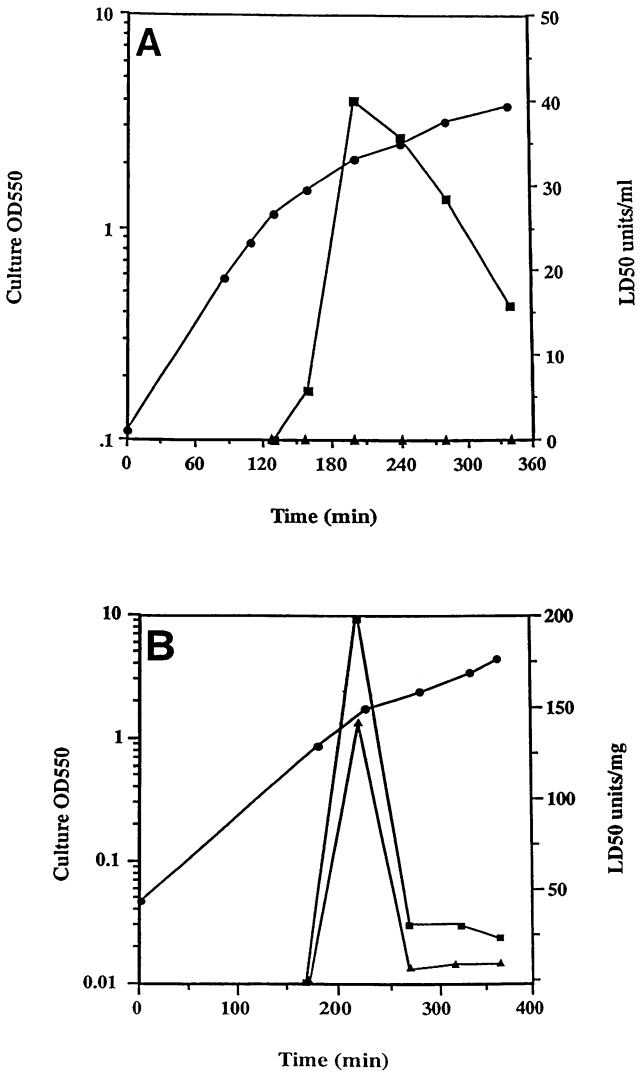

FIG. 2. Lytic activity of the intracellular and secreted forms of the ApxIIA protein from *E. coli*. *E. coli* host cells expressing the *apxIICA* and *lktBD* genes were grown to late log phase (●), and the presence of extracellular and intracellular toxin was assessed periodically by assays with erythrocytes (▲) and the BL3 cell line (■). Fifty percent lethal dose (LD_{50}) values were determined from lysis curves similar to those shown in Fig. 1 for the indicated time points. (A) Secreted toxin. (B) Intracellular toxin. OD550, optical density at 550 nm.

zones of partial hemolysis around their peripheries, suggesting the secretion of hemolytically active ApxIIA. However, when grown in liquid media, no hemolytic activity can be found in cell-free culture supernatants collected in the early, mid, or late phases of logarithmic growth or in the stationary phase (Fig. 2). In contrast, lytic activity against the BL3 cell line is easily detected and peaks at the mid- to late-log phase, approximately the time at which the highest levels of intracellular activity are found (Fig. 2). Even when culture supernatants are concentrated 50-fold by ammonium sulfate precipitation, no hemolytic activity can be detected, despite the fact that cytolytic activity is concentrated >20-fold by this procedure (data not shown). This raises the possibility that the intracellular hemolytic activity we attributed to ApxIIA was actually due to *E. coli*-specific components of the lysate complexing to hemolytically inactive ApxIIA. To control for this, the secreted form of the toxin was mixed with lysates prepared from *E. coli* cells harboring either the vector pAC18 (11) or pDM44, which contains the *apxIIC* gene expressed from the *lac* promoter of pAC18. In neither case could hemolytic activity be conferred upon the secreted ApxIIA protein. Thus, it would appear that the recombinant ApxIIA protein expressed in *E. coli* can be recovered in two forms: (i) an intracellular form whose hemolytic and cytotoxic activities are comparable to those of the ApxIIA toxin secreted by *A. pleuropneumoniae* and (ii) an extracellular form which is cytotoxic with little or no hemolytic activity. It is likely that the intracellular toxin is a mixture of the two forms, so that its hemolytic/cytotoxic ratio is lower than that of ApxIIA secreted from *A. pleuropneumoniae* (Fig. 1).

The inability of *E. coli* to secrete recombinant ApxIIA protein with a hemolytic/cytotoxic activity ratio similar to that which accumulates intracellularly can be explained in one of two ways. First, it is possible that the ApxIIC-dependent activation of pro-ApxIIA is a multistep process and an intermediate form of the toxin with cytolytic, but not hemolytic, properties can be efficiently secreted while the fully activated

TABLE 1. Effect of renaturation from guanidine hydrochloride on activity of secreted toxins

Source of secreted ApxIIA toxin[a]	LD_{50} U[b]/ml of culture supernatant			
	Crude supernatant		After renaturation[c]	
	Hemolytic titer	Cytotoxic titer	Hemolytic titer	Cytotoxic titer
A. pleuropneumoniae	114	83	400	266
E. coli	ND[d]	100	ND	500

[a] Cultures were grown to an optical density at 550 nm of approximately 1.7.
[b] One LD_{50} unit is the amount of culture supernatant required to achieve 50% lysis of 10^7 input cells.
[c] Culture supernatants were precipitated and renatured from guanidine hydrochloride as described previously (12).
[d] ND, no hemolytic activity detected (<1 LD_{50} U/ml).

toxin is inefficiently secreted from the heterologous host. Alternatively, activation of pro-ApxIIA may occur in a single step, but, during the secretory process, the conformation of the toxin is altered so that its hemolytic activity is lost. These distinct models cannot be differentiated until analytical procedures are developed to separate the ApxIIA protein into its chemical or conformational isomers. Recently, it has been found that defects in lipopolysaccharide formation can lead to the secretion of an inactive form of α-hemolysin which becomes hemolytically active after renaturation from 4 M guanidine hydrochloride (12). However, this phenomenon seems unrelated to that reported here, since the ApxIIA toxin secreted by *E. coli* does not regain hemolytic activity after exposure to denaturants even though its cytotoxicity to BL3 cells is enhanced by this procedure (Table 1). In any case, the findings reported here confirm previous reports (5, 11) that the hemolytic and cytotoxic activities of the RTX hemolysins are independent of each other.

This work was supported by USDA Competitive Research grant 92-37204-7954 to D.K.S. and R.Y.

REFERENCES

1. **Bailey, M. J. A., V. Koronakis, T. Schmoll, and C. Hughes.** 1992. *Escherichia coli* HlyT protein, a transcriptional activator of haemolysin synthesis and secretion, is encoded by the *rfaH* (*sfrB*) locus required for expression of sex factor and lipopolysaccharide genes. Mol. Microbiol. **6:**1003–1012.
2. **Chang, Y. F., J. Shi, D. P. Ma, S. J. Shin, and D. H. Lein.** 1993. Molecular analysis of the *Actinobacillus pleuropneumoniae* RTX toxin-III gene cluster. DNA Cell Biol. **12:**351–362.
3. **Chang, Y. F., R. Young, and D. K. Struck.** 1989. Cloning and characterization of a hemolysin gene from *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. DNA **8:**635–647.
4. **Fischer, C., C. K. Holtman, D. K. Struck, and R. Young.** Submitted for publication.
5. **Forestier, C., and R. A. Welch.** 1991. Identification of RTX toxin target cell specificity domains by use of hybrid genes. Infect. Immun. **59:**4212–4220.
6. **Frey, J., M. Beck, U. Stucki, and J. Nicolet.** 1993. Analysis of hemolysin operons in *Actinobacillus pleuropneumoniae*. Gene **123:** 51–58.
7. **Frey, J., R. Meier, D. Gygi, and J. Nicolet.** 1991. Nucleotide sequence of the hemolysin I gene from *Actinobacillus pleuropneumoniae*. Infect. Immun. **59:**3026–3032.
8. **Hardie, K. R., J. P. Issartel, E. Koronakis, C. Hughes, and V. Koronakis.** 1991. In vitro activation of *Escherichia coli* prohaemolysin to the mature membrane-targeted toxin requires HlyC and a low molecular-weight cytosolic polypeptide. Mol. Microbiol. **5:**1669–1679.
9. **Issartel, J. P., V. Koronakis, and C. Hughes.** 1991. Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl-carrier protein-dependent fatty acylation. Nature (London) **351:**759–761.
10. **Kamp, E. M., J. K. Popma, J. Anakotta, and M. A. Smits.** 1991. Identification of hemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* by use of monoclonal antibodies. Infect. Immun. **59:**3079–3085.
11. **McWhinney, D. R., Y.-F. Chang, R. Young, and D. K. Struck.** 1992. Separable domains define target cell specificities of an RTX hemolysin from *Actinobacillus pleuropneumoniae*. J. Bacteriol. **174:**291–297.
12. **Stanley, P. L. D., P. Diaz, M. J. A. Bailey, D. Gygi, A. Juarez, and C. Hughes.** 1993. Loss of activity in the secreted form of *Escherichia coli* haemolysin caused by an *rfaP* lesion in core lipopolysaccharide assembly. Mol. Microbiol. **10:**781–787.
13. **Wandersman, C., and P. Delepelaire.** 1990. TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. Proc. Natl. Acad. Sci. USA **87:**4776–4780.
14. **Wandersman, C., and S. Letoffe.** 1993. Involvement of lipopolysaccharide in the secretion of *Escherichia coli* α-hemolysin and *Erwinia chrysanthemi* proteases. Mol. Microbiol. **7:**141–150.
15. **Welch, R. A.** 1991. Pore-forming cytolysins of gram-negative bacteria. Mol. Microbiol. **5:**521–528.