Biochimica et Biophysica Acta 1858 (2016) 538–545

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Hemolysin of uropathogenic *Escherichia coli*: A cloak or a dagger?☆

Laura C. Ristow, Rodney A. Welch *

Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, WI, USA

ARTICLE INFO

Article history:
Received 2 July 2015
Received in revised form 5 August 2015
Accepted 18 August 2015
Available online 20 August 2015

Keywords:
Hemolysin
RTX toxin
Escherichia coli
UPEC
ExPEC

ABSTRACT

Hemolysin from uropathogenic *Escherichia coli* (UPEC) is a hemolytic and cytotoxic protein active against a broad range of species and cell types. Expression of hemolysin correlates with severity of infection, as up to 78% of UPEC isolates from pyelonephritis cases express hemolysin. Despite decades of research on hemolysin activity, the mechanism of intoxication and the function of hemolysin in UPEC infection remain elusive. Early *in vitro* research established the role of hemolysin as a lytic protein at high doses. It is hypothesized that hemolysin is secreted at sublytic doses *in vivo* and recent research has focused on understanding the more subtle effects of hemolysin both *in vitro* and in elegant infection models *in vivo*, including inoculation by micropuncture of individual kidney nephrons. As the field continues to evolve, comparisons of hemolysin function in isolates from a range of UTI infections will be important for delineating the role of this toxin. This article is part of a Special Issue entitled: Pore-Forming Toxins edited by Mauro Dalla Serra and Franco Gambale.

Contents

1. Introduction

The intestinal commensal bacterium, *Escherichia coli*, can become an opportunistic pathogen upon introduction into the urinary tract, a sterile tissue site. Uropathogenic *E. coli* (UPEC) isolates encode several virulence factors at a higher proportion than intestinal commensal isolates, including the pore-forming toxin, hemolysin. Hemolysin (HlyA) is a potent and ubiquitous cytolysin, the prototypical member of the repeats-in-toxin (RTX) family. Quantification of HlyA expression among commensal *E. coli* isolates reveals that <15% encode *hlyA*. Strikingly, there is a correlation between increased severity of urinary tract infection (UTI) presentation in the clinic and HlyA expression in UPEC isolates[1–3]. Quantification of HlyA expression among UPEC strains reveals a substantial increase in the presence of the *hlyA* operon in the genome as 31–48% of cystitis causing UPEC encode hemolysin[3–5]. Patients presenting with pyelonephritis or bacteremia harbor infectious

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* Corresponding author at: Department of Medical Microbiology and Immunology, University of Wisconsin-Madison School of Medicine and Public Health, 1550 Linden Drive, MSB 6157, Madison, WI 53706, USA.
E-mail address: rawelch@wisc.edu (R.A. Welch).

http://dx.doi.org/10.1016/j.bbamem.2015.08.015
0005-2736/

isolates with up to 78% encoding hemolysin[3–6]. HlyA was identified and named for its property of lysing red blood cells, however it also has cytotoxic activity against a broad range of species and cell types. The genomic organization and regulation of hemolysin expression *in vitro* and *in vivo* have long been the focus of research, but increasingly recent investigations have turned toward understanding the mechanism and consequences of hemolysin action. At high doses, HlyA is thought to form pores in a target cell membrane and lead to lysis of the cell. Studies at lower concentrations of HlyA, thought to be similar to the environment that would be found during an active UTI, reveal more subtle activities of the toxin, for example, the initiation of signaling pathways in host cells to lead to discrete types of cell death or exfoliation of the bladder epithelial cells to allow access to deeper tissue sites. Although HlyA and related RTX toxins have been studied for decades, ambiguity remains in our understanding of the mechanism of intoxication, and even their function in infection. This review will investigate areas of uncertainty as well as highlight some of the recent topics of study as the field continues to evolve.

2. Genomic organization, expression and regulation of the *E. coli* hemolysin

RTX toxins are defined by the presence of a repeating nonapeptide Ca^{2+} binding sequence and are secreted extracellularly via a leader peptide-independent mechanism that depends on recognition of a short C-terminal sequence[7]. The phenylalanine at amino acid (a.a.) residue 989 is crucial for the secretion of hemolysin[8]. In all family members, secretion occurs via type I secretion machinery, using homologs of HlyD, an outer membrane channel forming protein, HlyB, an ABC transporter and TolC, an outer membrane protein for secretion of the effector protein, HlyA [7,9,10]. The traditional RTX operon arrangement of the genes required for HlyA expression, activation and secretion is *hlyCABD*, with *tolC* at a genetically independent locus[9,11–13]. HlyC is required for post-translational activation of HlyA by acyltransferase activity on two lysine residues, converting pro-hemolysin to its active form, which is required for hemolytic and cytotoxic activity, but not for the secretion of the protein[14–20]. In terms of fatty acid chain length, HlyC acyltransferase appears to have a broad range of substrate specificity, resulting in a possibility of 9 unique HlyA covalent structures that equally activate *in vivo*[21]. The resulting HlyA protein is 110-kilodaltons in size, consisting of 1024 a.a. residues[22].

The importance of HlyA in the virulence of different UPEC isolates varies, despite highly similar primary sequences of the gene. Two commonly studied UPEC strains are CFT073, a blood and urine isolate from a patient with pyelonephritis and urosepsis, and UTI89, an isolate from a patient with mild cystitis. These strains are sequenced and are used in many *in vitro* and *in vivo* model systems. Early studies of HlyA regulation demonstrated that swapping promoter regions for *hlyCABD* from strains of varying virulence effectively alters virulence in the rat peritonitis model[23]. Transcriptional regulation of many virulence factors in *E. coli* is partially regulated by RfaH, including hemolysin. RfaH can directly and positively regulate *hlyA* transcription and elongation[24–26]. Indirectly, in a *ΔrfaH* strain, secretion of HlyA is impaired as TolC insertion in the outer membrane in misregulated[27]. Another global regulator of *E. coli*, fumarate and nitrate reduction (FNR), modulates hemolysin expression under anaerobic conditions *in vitro*, similar to conditions that are likely to be encountered *in vivo*[28]. The importance of this regulator was assessed in the CFT073 background. *In vivo*, the *ΔFNR* mutant colonizes the bladder and kidney at 48-fold lower levels than wild-type bacteria, although this phenotype is likely not directly due to the regulation of HlyA, as in the CFT073 background, *ΔhlyA* mutants are not attenuated via intraurethral or intravenous inoculation into mice[28–30].

The BarA–UvrY two-component system responds to changes in the extracellular environment and is known to regulate pathogenicity in other bacteria. In the CFT073 background, mutations in either the sensor kinase, *barA* or the response regulator, *uvrY*, decrease invasion of cultured cells *in vitro* and colonization of the bladder or kidney by one log or more compared to wild-type in the murine UTI infection model[31]. The regulation of several proteins is dependent on the BarA–UvrY system, including hemolysin[31]. In addition to the decrease in hemolysin expression in *ΔbarA* and *ΔuvrY*, the composition of lipopolysaccharide (LPS) is altered, which may affect hemolysin expression. As in the FNR mutants, hemolysin is not solely responsible for the decrease in virulence of either *ΔbarA* or *ΔuvrY* strains in the CFT073 background.

CpxRA is a two-component signal transduction system that senses periplasmic stress and regulates trafficking factors and repair proteins of the periplasm[32]. In the UPEC UTI89 background, CpxR directly interacts with the *hlyCABD* promoter and results in the downregulation of HlyA expression[33]. *ΔcpxR* mutants are attenuated in the mouse model, although wild-type levels of colonization are restored in a *ΔcpxRΔhlyA* mutant [33]. The difference in requirement for virulence factors between UPEC isolates, i.e. UTI89 and CFT073, makes it difficult to pinpoint the importance and regulation of HlyA by the results above, however, it may illuminate differences between the factors critical for the severity of infection. Analyzing hemolysin regulation in several UPEC backgrounds will be required to increase our understanding of the importance of each of the identified regulators.

3. Significance of pore formation?

Unambiguous experimental evidence of a discrete, defined hemolysin pore has proven difficult to acquire. Inferences about the ability of HlyA to form pores are drawn from measurements of host cell membrane instability or permeability. High doses of HlyA lyse cells independent of a receptor, possibly due to the ability to form pores and disrupt the colloidal osmotic pressure[34–36]. Several studies demonstrate that hemolysin can permeabilize lipid bilayers of varying composition[36–38], however, there is not a consensus on whether HlyA acts as a monomer[36,39,40] or multimer[38,41–43] to form pores. The pores formed by HlyA are described as static or appear to increase in pore diameter over time[43,44]. Acylation is not required for either host membrane binding or insertion by HlyA, but this modification is required for eventual lysis of a target cell[16,45]. Sánchez-Magraner et al. investigated the domains of HlyA necessary for inserting into membranes and found that HlyA lacking the N-terminal 601 amino acids is not capable of membrane insertion[46]. As the authors acknowledge, this result is hard to interpret in the context that the N-terminal half of the protein is missing[46]. It may be that the C-terminus is involved in inserting into the membrane in the context of the whole protein, as it is likely that the activities of the protein are not additive, but cooperative, although further studies are necessary to define this function. In support of the role of the N-terminus in pore formation, one of the amphipathic regions in hemolysin, a.a. 272–301, forms an α-helix that is required for pore formation[47]. Our understanding of the structure that HlyA forms at the host cell surface or within the host cell membrane would be greatly improved by electron micrographs or a crystal structure of the protein, although such data are not missing for lack of effort. The fact that any imaging technology has been unable to capture hemolysin as a pore is perhaps evidence that the protein is acting in a manner we have not yet been able to accurately model.

4. Receptor dependence or independence?

4.1. Receptor binding regions

The identification of HlyA functional domains remains an active area of research (Fig. 1). The repeat regions of HlyA bind to calcium before subsequent binding to cell membranes and cytolytic activity[48,49]. Mutagenesis studies reveal important regions of the protein for interacting with red blood cells (RBCs). Amino acids 9–37 have a dampening effect