The International Journal of Biochemistry & Cell Biology 43 (2011) 1792–1801

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Shiga toxin 1 is more dependent on the P proteins of the ribosomal stalk for depurination activity than Shiga toxin 2

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ARTICLE INFO

Article history:
Received 30 March 2011
Received in revised form 27 July 2011
Accepted 26 August 2011
Available online 3 September 2011

Keywords:
Shiga toxin
E. coli O157:H7
Ribosome inactivating protein
Ribosomal stalk
P protein

ABSTRACT

Shiga toxins produced by *Escherichia coli* O157:H7 are responsible for food poisoning and hemolytic uremic syndrome (HUS). The A subunits of Shiga toxins (Stx1A and Stx2A) inhibit translation by depurinating a specific adenine in the large rRNA. To determine if Stx1A and Stx2A require the ribosomal stalk for depurination, their activity and cytotoxicity were examined in the yeast P protein deletion mutants. Stx1A and Stx2A were less toxic and depurinated ribosomes less in a strain lacking P1/P2 on the ribosome and in the cytosol (ΔP2) than in a strain lacking P1/P2 on the ribosome, but containing free P2 in the cytosol (ΔP1). To determine if cytoplasmic P proteins facilitated depurination, Stx1A and Stx2A were expressed in the P0ΔAB mutant, in which the binding sites for P1/P2 were deleted on the ribosome, and P1/P2 accumulated in the cytosol. Stx1A was less toxic and depurinated ribosomes less in P0ΔAB, suggesting that intact binding sites for P1/P2 were critical. In contrast, Stx2A was toxic and depurinated ribosomes in P0ΔAB as in wild type, suggesting that it did not require the P1/P2 binding sites. Depurination of ΔP1, but not P0ΔAB ribosomes increased upon addition of purified P1α/P2β *in vitro*, and the increase was greater for Stx1 than for Stx2. We conclude that cytoplasmic P proteins stimulate depurination by Stx1 by facilitating the access of the toxin to the ribosome. Although ribosomal stalk is important for Stx1 and Stx2 to depurinate the ribosome, Stx2 is less dependent on the stalk proteins for activity than Stx1 and can depurinate ribosomes with an incomplete stalk better than Stx1.

1. Introduction

Shiga toxin (Stx) producing *Escherichia coli* (STEC), such as *E. coli* O157:H7 and other serotypes are the major causes of food poisoning that can lead to either hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS). Stx-mediated HUS is the common cause of renal failure in children in the US (Siegler and Oakes, 2005). A recent HUS outbreak in Germany highlighted the public health impact of this emerging pathogen (Bielaszewska et al., 2011). STEC produce two distinct families of exotoxins, designated Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) that are major virulence factors, essential to the pathogenesis of *E. coli* O157:H7 (Paton and Paton, 1998; Tesh, 2010). There are no specific protective measures or therapeutics effective against infection by STEC. Stx1 and Stx2 are AB_5 toxins consisting of an enzymatically active A subunit associated with a pentamer of receptor binding B subunits. They are also known as type II ribosome inactivating proteins (RIPs) because their A subunits are *N*-glycosidases, which remove a specific adenine (A_{4324} in rat, A_{3027} in yeast, and A_{2660} in *E. coli*) from the universally conserved α-sarcin/ricin loop (SRL) of the large rRNA, inhibiting the elongation step of protein synthesis (Endo et al., 1988). The A subunits of Stx1 and Stx2 can be proteolytically cleaved into an enzymatically active A_1 chain and an A_2 chain, which remains associated with the B pentamer (Garred et al., 1995). In the endoplasmic reticulum (ER) lumen, the A_1 chain is released from the A_2–B_5 complex by reduction of the disulfide bond, and undergoes retrotranslocation from the ER into the cytosol (Sandvig and van Deurs, 2005). Although Stx1 and Stx2 share a common receptor, globotriaosylceramide (Gb3), and indistinguishable activities, they have only 55% and 57% identical amino acid sequences on the A and B subunits, respectively, and are immunologically distinct. Stx2 is more important than Stx1 in the development of HUS (Orth et al., 2007; Boerlin et al., 1999). The German outbreak isolates, which were the deadliest on record, contained only Stx2 and possessed the typical characteristics of enteroaggregative *E. coli* (Bielaszewska et al., 2011). The lethal dose of Stx2 is lower than that of Stx1 in animal models (Tesh et al., 1993; Siegler et al., 2003). However, it has not been possible to demonstrate the increased cytotoxicity of Stx2 in mammalian cell culture models. For example, Stx1 is more toxic to Vero cells than Stx2, while Stx2 is more toxic to mice and non-human primates (Tesh et al., 1993; Siegler et al., 2003). Since $Stx1A_1$ and $Stx2A_1$ are both equally effective in blocking protein

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doi:10.1016/j.biocel.2011.08.018

synthesis *in vitro* (Tesh et al., 1993; Head et al., 1991), the basis for the increased potency of Stx2 is not known. The binding affinity of Stx1 is higher than Stx2 to Gb3-mimicking receptors (Takeda et al., 1999; Nakajima et al., 2001) and the B pentamers of Stx1 and Stx2 show differential stability (Kitova et al., 2009; Conrady et al., 2010).

Accumulating evidence indicates that several RIPs interact with the P proteins of the ribosomal stalk to access the SRL. Trichosanthin (TCS), Stx1 and maize RIP interact with the P proteins *in vitro* (Chan et al., 2007, 2001; McCluskey et al., 2008; Yang et al., 2010). Removal of the last 17 amino acids of P1 or P2 proteins, but not the P0 protein, abolished the interaction between $Stx1A_1$ and human ribosomal stalk proteins, suggesting that the conserved C-terminal domain (CTD) of P1/P2 proteins was critical (McCluskey et al., 2008). TCS binding site on P1/P2 was mapped to the conserved CTD of P proteins by protein crystallography analysis (Too et al., 2009). We have developed a *Saccharomyces cerevisiae* model to examine ribosome interactions and enzymatic activity of RIPs (Hudak et al., 2004; Li et al., 2007; Hur et al., 1995), and demonstrated that ricin A chain (RTA) binds to the P proteins of the ribosomal stalk to depurinate the SRL *in vivo* (Chiou et al., 2008; Li et al., 2009). Using isolated stalk complexes from yeast, we showed that the stalk is the main landing platform for RTA on the ribosome and multiple copies of the stalk proteins accelerate the recruitment of RTA to the ribosome for depurination (Li et al., 2010).

In eukaryotes, the stalk occurs in a pentameric configuration P0–$(P1/P2)_2$ (Guarinos et al., 2003; Grela et al., 2010), where P0 anchors two P1/P2 dimers (Ballesta and Remacha, 1996). In yeast, P1/P2 proteins have diverged into four different polypeptides, P1α, P1β, P2α and P2β. P1α/P2β and P1β/P2α preferentially form heterodimers prior to binding to P0 (Lalioti et al., 2002; Krokowski et al., 2005; Tchorzewski et al., 2000). Presently, the only ribosomal components that are found free in the cytoplasm are the P1/P2 proteins of the ribosomal stalk (Ballesta and Remacha, 1996). Binding to P2 proteins can prevent P1 proteins from degradation in the cytoplasm. In contrast, P2 proteins are stable in the absence of P1 proteins *in vivo* (Nusspaumer et al., 2000). Recent results indicate that the amino terminal end determines the stability of P1 and P2 (Camargo et al., 2011). The N-terminal domains (NTD) of P1/P2 proteins are responsible for dimerization and binding to P0 via the P1 proteins, while the CTD are mobile in the cytosol and interact with the translational GTPases (tGTPases) (Jose et al., 1995; Bargis-Surgey et al., 1999). The last 13 amino acids of the C-termini are identical among all five P proteins in yeast (Ballesta and Remacha, 1996; Gonzalo and Reboud, 2003). The binding sites for P1α/P2β and P1β/P2α proteins on P0 in yeast have been mapped to amino acids 199–230 and 231–258, respectively (Krokowski et al., 2006).

One of the most interesting features of the eukaryotic stalk is its dynamism, where ribosome-bound P1 and P2 are exchanged with the free acidic proteins present in the cytosol and this exchange is increased during protein synthesis (Ballesta and Remacha, 1996; Remacha et al., 1995). This dynamic property of the stalk results in subpopulations of ribosomes containing different amounts of P1/P2 proteins (Guarinos et al., 2003; Saenz-Robles et al., 1990). The biological significance of this exchange is not well understood. It is thought to play a role in regulating the activity of eukaryotic ribosomes (Ballesta and Remacha, 1996; Remacha et al., 1995). Since several RIPs access the SRL by binding to the stalk proteins (Chan et al., 2007, 2001; McCluskey et al., 2008; Yang et al., 2010; Chiou et al., 2008; Li et al., 2009), understanding how this stalk-dependent regulation affects the activity and toxicity of RIPs at the molecular level would be very important for developing protection strategies.

Molecular details of the interaction of $Stx1A_1$ and $Stx2A_1$ with ribosomes *in vivo* and contribution of these interactions to the cytotoxicity of each toxin have not been determined. Furthermore, since the B subunit is required for endocytosis and retrograde trafficking in mammalian cells, it has not been possible to study enzymatic activity of the A subunits in the absence of the B subunits *in vivo*. In a previous study, we identified the amino acids critical for the cytotoxicity of Stx1A and Stx2A and demonstrated that the function of these residues can be differentiated (Di et al., 2011). In this study, using the yeast model, we extend the previous *in vitro* observations with Stx1 (McCluskey et al., 2008) to Stx1 and Stx2 *in vivo*. We demonstrate that cytoplasmic stalk proteins are critical for Stx1A and Stx2A to access the SRL *in vivo* and present the first evidence that Stx1 and Stx2 differ in enzymatic activity towards ribosomes with different stalk composition.

2. Materials and methods

2.1. Yeast strains and plasmids

The *S. cerevisiae* wild type strain was W303 (*MAT*a *ade2-1 trp1-1 ura3-1 leu2-3,112 his-3-11,15 can1-100*). The stalk mutants ΔP1 and ΔP2 were haploid strains described elsewhere (Chiou et al., 2008), while P0ΔAB mutant in the same genetic background was constructed (Cardenas, Revuelta, Remacha and Ballesta, unpublished results). Yeast strains were grown in YPD medium or minimal SD medium containing 2% glucose. Because the different selection markers are available in the yeast strains, RIPs were cloned into yeast vectors that carry either a *URA3* marker (pYES2.1/V5-His TOPO based vector, Invitrogen) or *LEU2* marker (Yep351-based NT198). The *URA3* plasmids containing Stx1A (NT890) and Stx2A (NT901) with a V5 epitope and a 6 × His tag downstream of the *GAL1* promoter were previously described (Di et al., 2011). The *LEU2* plasmids containing Stx1A (NT821) and Stx2A (NT785) were constructed by amplifying the cDNA from Stx1A (NT819) and Stx2A (NT782), respectively, and cloning them into NT198. The *URA3* plasmids were transformed into the wild type, ΔP1 and P0ΔAB strains while the *LEU2* plasmids were transformed into the ΔP2 strain.

2.2. Isolation of monomeric ribosome, membrane and cytosol fractions and immunoblot analysis

Yeast cells were grown in YPD media at 30 °C overnight and collected at log phase. Monomeric ribosomes and cytosol fraction were isolated as described previously (Chiou et al., 2008). The cells were re-suspended in lysis buffer (50 mM Tris–HCl, pH 7.9, 5 mM EDTA, 1% (v/v) Triton X-100, 1 mM PMSF and protease inhibitors), glass beads were added to the mixture, followed by vortexing 6 times for 30 s at maximum speed with 1–2 min on ice between each vortexing cycle. The cell debris was removed by centrifuging at 500 × *g* for 5 min and the supernatant was centrifuged at 100,000 × *g* for 1 h at 4 °C. The pellets containing the membrane fraction and the ribosome fraction were used to detect Stx1 and Stx2 using an immunoblot assay.

For immunoblot analysis the proteins were separated using a 12% SDS–PAGE. Monoclonal antibodies specific for P1β, P2α and P2β were used to detect the P1 and P2 proteins (Vilella et al., 1991). Polyclonal antibody P0 was obtained by injecting rabbits with recombinant P0 protein lacking the last 21 amino acids to avoid the cross-reaction with the acidic P proteins (Santos and Ballesta, 1994). Monoclonal antibodies against L3 (gift of Dr. J.R. Warner), dolichol-phosphate mannose synthase (Dpm1p; Invitrogen) and 3-phosphoglycerate kinase (Pgk1p; Invitrogen) were used as the loading controls for the ribosome, membrane and cytosol fractions, respectively. Polyclonal antibodies against Stx1A and Stx2A were provided by Dr. C. Thorpe (Tufts University Medical Center, Boston, MA).