Microbial Pathogenesis 51 (2011) 89–95

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

Yersinia pestis TIR-domain protein forms dimers that interact with the human adaptor protein MyD88

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ARTICLE INFO

Article history:
Received 27 September 2010
Received in revised form
13 May 2011
Accepted 19 May 2011
Available online 27 May 2011

Keywords:
Innate immune evasion
TIR domain protein
Yersinia pestis
Pathogenic
MyD88

ABSTRACT

Recent research has highlighted the presence of Toll/Interleukin 1 receptor (TIR)-domain proteins (Tdps) in a range of bacteria, suggested to form interactions with the human adaptor protein MyD88 and inhibit intracellular signaling from Toll-like receptors (TLRs). A Tdp has been identified in *Yersinia pestis* (YpTdp), a highly pathogenic bacterium responsible for plague. Expression of a number of YpTIR constructs of differing lengths (YpTIR1, S130-A285; YpTIR2, I137-I273; YpTIR3, I137-246; YpTIR4, D107-S281) as fusions with an N-terminal GB1 tag (the B1 immunoglobulin domain of Streptococcal protein G) yielded high levels of soluble protein. Subsequent purification yielded 4–6 mg/L pure, folded protein. Thrombin cleavage allowed separation of the GB1 tag from YpTIR4 resulting in folded protein after cleavage. Nuclear magnetic resonance spectroscopy, size exclusion chromatography, SDS-PAGE analysis and static light scattering all indicate that the YpTIR forms dimers. Generation of a double Cys-less mutant resulted in an unstable protein containing mainly monomers indicating the importance of disulphide bonds in dimer formation. In addition, the YpTIR constructs have been shown to interact with the human adaptor protein MyD88 using 2D NMR and GST pull down. YpTIR is an excellent candidate for further study of the mechanism of action of pathogenic bacterial Tdps.

1. Introduction

Toll-like receptors (TLRs) are key components of the innate immune system, which detect a range of pathogen associated molecular patterns (PAMPs) including lipopolysaccharide (LPS), bacterial cell wall components and nucleic acids [1], and initiate the first line of host defence against infection. TLRs have a conserved domain architecture comprised of a large extracellularly located leucine rich repeat (LRR) domain [2] and an intracellular Toll/Interleukin 1 receptor (TIR) domain [3] linked by a single pass transmembrane region. TLRs are suggested to exist as dimers, which can be either heterotypic, homotypic or both depending on the receptor [4,5]. Upon interaction with a PAMP the TLR dimer is thought to undergo a molecular rearrangement of the intracellular TIR domains to generate an active interaction domain [4–6] allowing recruitment of intracellular adaptor proteins; MyD88, MyD88 adaptor like (MAL, also called TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo-motif containing protein (SARM) [7]. All of the adaptors also contain TIR domains and in some cases other protein–protein interaction domains important in signal transduction [8].

Broadly there are two signal transduction pathways, the MyD88 dependent pathway utilized by all TLRs except TLR3 and the MyD88 independent pathway utilized by TLR3 and also TLR4 [7,9]. The recruitment of adaptor proteins initiates the complex signaling cascades which upregulate gene expression of nuclear factor kappa B (NF-κB) and interferon response factor (IRF) controlled genes. This ultimately initiates an innate immune response through the release of proinflammatory cytokines [7].

Key components of the signaling cascade are the heterotypic interactions between the TIR domains upon receptor activation and adaptor recruitment. The importance of the TIR domains in the innate immune response has made them the subject of intense study. The structures of the monomeric forms of the TIR domains of TLR1 and TLR2 [10] revealed a conserved architecture comprised

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0882-4010/$ – see front matter
doi:10.1016/j.micpath.2011.05.004

of a central five-stranded parallel β-sheet surrounded by five α-helices. These structures highlighted the presence of a flexible region, the BB loop connecting strand βB and helix αB, which projects away from the main TIR domain. Mutational analysis revealed that several residues within this loop have important roles in signal transduction [10], most notably a proline residue. Mutation of the equivalent residue, Pro712, to histidine in TLR4-TIR results in mice unable to initiate the innate immune response upon stimulation with LPS [11]. The BB loop also plays a role in mediating the interactions between the monomers of the dimer of TLR10-TIR [12]. The recent NMR structure of the adaptor MyD88-TIR domain confirmed the conserved architecture of the TIR domain family and the flexible nature of the BB loop [13].

Bioinformatics analysis has identified TIR containing proteins in a range of both pathogenic and non-pathogenic bacteria. The first studied example of such a protein was the TIR-like protein A (TlpA) identified in *Salmonella enterica* serovar Enteridis [14]. *In vitro* analysis showed that this protein was able to reduce the ability of TLR4 and MyD88 to stimulate NF-κB activity. Furthermore bacteria with disrupted *TlpA* genes exhibited reduced pathogenicity in mice, suggesting that TlpA is an important virulence factor. These data indicated that the bacterial TIR-like proteins were likely to have a role in evasion of the innate immune system. Studies on equivalent proteins, from *Brucella abortus* (Btp1), the uropathogenic *Escherichia coli* strain CFT073 (TcpC) and *Brucella melintensis* (TcpB) have supported a role in immune system evasion [15–17].

A recent in-depth bioinformatic analysis [18] identified 922 TIR-domain proteins in a range of fungi, archaea, viruses and pathogenic and non-pathogenic bacteria. The high incidence of these proteins in non-pathogenic species suggests that these proteins may have a range of functions, including evasion of the innate immune system, depending on the organism in question. However it has been shown that the TIR-domain protein from the non-pathogenic thermophilic bacterium, *Paracoccus dentrificans*, PdTLP interacts with the human adaptor protein MyD88 *in vitro* [19,20]. In addition, the crystal structure of PdTIR displays a similar fold to the known human TIR domains [20]. Given the benign nature of this organism it would seem unlikely that this protein has a role in innate immune system evasion, therefore making it of limited use as a model system for studying the interactions between bacterial TIR proteins and human TIR proteins. Further work is required to reveal the precise function of PdTIR.

Our bioinformatic analysis [18] identified a TIR domain protein in the pathogenic bacterium, *Yersina pestis* (YpTdp). *Y. pestis* is a facultative intracellular bacterium that causes the zoonotic diseases bubonic and pneumonic plague [21]. The main reservoirs of the disease are rodents, birds, farm animals and their associated fleas and the disease is transmitted to humans via flea or animal bites. Most famously associated with the Black Death pandemics of the Middle Ages, *Y. pestis* continues to cause a significant number of deaths every year mainly in Africa and Asia [22,23]. YpTdp is a 41 kDa protein containing five cysteines. Other work in our group has shown that when over-expressed *in vitro* YpTdp is able to disrupt immune signalling pathways but that its removal has no obvious effect on virulence and instead affects the characteristics of *Y. pestis* growth (Spear et al., manuscript submitted). Additionally, microarray studies have shown that the gene encoding YpTdp is expressed *in vitro* in various conditions [24,25]. In order to understand more about the role of YpTdp in immune system evasion, we have attempted to produce the TIR domain of YpTdp (YpTIR) with a view to obtaining high quality samples for functional and structural studies. Here, we have expressed and purified YpTIR as a fusion with an N-terminal GB1 tag. The resulting protein is folded and exists in solution as a dimer that interacts with the TIR domain of human adaptor protein MyD88.

2. Results

2.1. Expression and purification of GB1-tagged YpTIR constructs

The region of the YpTdp containing the TIR domain (YpTIR1; residues S130 to A285) was estimated based on sequence alignments with TIR domains of known structure (Fig. 1). A construct based on this region and two shorter YpTIR constructs, corresponding to residues I137-I273 (YpTIR2) and I137-N246 (YpTIR3), were generated. YpTIR3 lacks the region of the protein corresponding to the Box 3 motif (Fig. 1). All three gene fragments were cloned into the GEV2 vector as a fusion with an N-terminal GB1 tag (the B1 immunoglobulin domain of Streptococcal protein G) [26] and a C-terminal His tag. High-level expression was achieved for GB1-YpTIR1 and GB1-YpTIR2 but not for GB1-YpTIR3. Affinity chromatography followed by size exclusion chromatography (SEC) of all three constructs produced highly pure protein although the final yield of GB1-YpTIR3 was extremely low (Fig. 2ab). The final yields of pure protein obtained were 5.7, 4.6 and 0.6 mg/L for GB1-YpTIR1, GB1-YpTIR2 and GB1-YpTIR3 respectively. The size exclusion profiles of GB1-YpTIR1 and GB1-YpTIR2 indicated that both proteins were monodispersed (Fig. 2a). However in both cases the protein had a lower retention volume (~14 ml) than expected for a ~25 kDa protein suggesting that the protein was present in a higher oligomeric form, possibly a dimer. The GB1-YpTIR3 eluted as a broad peak from the SEC column suggesting that this protein was aggregating possibly as the result of partial unfolding. The high yield, purity and quality (Fig. 2ab) of GB1-YpTIR1 and GB1-YpTIR2 allowed further analysis of the proteins.

2.2. Removal of the GB1 tag

Whilst the expression and isolation of the GB1-tagged constructs is useful, it is important to cleave the GB1 tag for certain downstream applications including some structural studies. Attempts to cleave the GB1 tag from GB1-YpTIR1 and GB1-YpTIR2 were unsuccessful even after extended periods of incubation with high concentrations of thrombin protease, probably as the result of close association of the GB1 and YpTIR domains. In order to allow efficient thrombin cleavage a further longer YpTIR (GB1-YpTIR4, residues D107-S281) construct was designed. This was also cloned into GEV2 and expressed and purified as described in Section 2.1 (Fig. 2cd). Cleavage was followed by Co^{2+}-IMAC to separate the His-tagged YpTIR4 from the GB1 tag resulting in pure, cleaved protein with a yield of 2.5 mg/L.

2.3. YpTIR is a dimer in solution

In order to assess the folded state of the YpTIR constructs prior to further studies, the GB1-YpTIR protein constructs were analysed using 1D NMR spectroscopy. All GB1-YpTIR constructs displayed folded domains in addition to GB1 (Fig. 3). YpTIR4 after thrombin cleavage also displayed folded domains. Resonance linewidths from the 1D and 2D $^{1}H-^{15}N$ HSQC NMR spectra of ^{15}N-GB1-YpTIR1 (Fig. 3) strongly suggest that the protein is dimeric, as suggested by SEC. This was further supported by analysis using a SEC column together with static light scattering (SLS) which indicated that the approximate molecular weights of the different YpTIR proteins were ~40–54 kDa. The measured molecular weight of 40.9 kDa for YpTIR4 (predicted molecular weight of the monomer = 20.8 kDa) following removal of the GB1 tag (Fig. 4) strongly indicated that dimer formation is mediated through the YpTIR domain. The YpTIR contains two Cys residues; Cys90 and Cys132. In order to investigate the role of these residues in the formation of a stable dimeric protein we generated both single and a double Cys-less mutant.