An efficient method to produce recombinant bacterial effector Tir coupled cytoskeleton protein (TCCP) complexed with host protein insulin receptor tyrosine kinase substrate (IRTKS)

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Accepted 10 May, 2013

An important pathogenic process in enterohemorrhagic Escherichia coli (EHEC) associated diseases is the formation of attaching and effacing (A/E) lesions, which are a typical pathological change in host cells. The classical pathway for A/E lesion formation requires the participation of proteins from both bacteria and host cells, namely intimin, translocated intimin receptor (Tir), insulin receptor tyrosine kinase substrate (IRTKS), Tir coupled cytoskeleton protein (TCCP), ARP3/2, and N-WASP. The interaction between IRTKS and TCCP is mediated by the binding of the SH3 domain of IRTKS (SH3_IRTKS) to the proline rich repeat (PRR) domain of TCCP, which is important for the induction of A/E lesions. The inability to efficiently produce the purified target complex has hindered the structural determination of these protein complexes. Here, we report an effective method for the generation of a complex consisting of SH3_IRTKS with three PRR_TCCP domains. Two recombinant fragments, TCCP3R and TCCP5R, as well as SH3_IRTKS, were successfully expressed in soluble form and purified. In addition, methods were established to prepare two different protein complexes, SH3_IRTKS-TCCP3R and SH3_IRTKS-TCCP5R. These methods are a good foundation for future studies on the crystal structure of TCCP-IRTKS. Meanwhile, recombinant TCCP was shown to directly bind to IRTKS in vitro, which provides additional evidence for the interaction between these two molecules.

Key words: Enterohemorrhagic Escherichia coli, Tir coupled cytoskeleton protein, insulin receptor tyrosine kinase substrate, protein complex.

INTRODUCTION

Attaching and effacing (A/E) lesions are a common change caused by many bacterial pathogens, such as EHEC (enterohemorrhagic Escherichia coli) (Phillips et al., 2000), EPEC (enteropathogenic Escherichia coli) (Moon et al., 1983), and Citrobacter rodentium (Schauer and Falkow, 1993), amongst others. A/E lesions are formed when bacteria attach to the host cell, causing the effacement or disruption of microvilli and the formation of...
actin pedestals below the attached bacteria (Frankel et al., 1998). Although the signal transduction pathway for A/E lesion formation differs amongst pathogens, it generally involves the polymerization of actin in the host cell, which can be visualized by fluorescent actin staining (FAS) assays (Shariff et al., 1993).

EHEC and EPEC are closely related and share a large number of similarities in signal transduction pathways for inducing A/E lesions. Both EHEC and EPEC translocate Tir (translocated intimin receptor) via their type III secretion system (T3SS), which interacts with intimin on the bacterial cell wall. The intimin-Tir interaction then activates N-WASP and induces actin polymerization (Frankel and Phillips, 2008). In EPEC, tyrosine residue 474 (Y474) is phosphorylated by mammalian kinases and activates N-WASP directly, by recruiting Nck (Campellone et al., 2002; Gruenheid et al., 2001). However, the pathway used by EHEC is quite different. In EHEC cells, Tir is not phosphorylated, but recruits IRTKS (insulin receptor tyrosine kinase substrate) from host cells (Vingadasalom et al., 2009). The IRTKS then interacts with TCCP (Tir coupled cytoskeleton protein), an effector also translocated by T3SS, followed by activation of N-WASP and enhancement of actin polymerization (Frankel and Phillips, 2008).

The interaction between IRTKS and TCCP is critical for EHEC to induce actin polymerization. The IRTKS is composed of two domains, namely IMD (IRSp53/MIM homology domain) and the SH3 domain. The IMD domain is responsible for Tir binding (de Groot et al., 2011), while the SH3 domain (SH3\textsubscript{IRTKS}) accounts for the interaction with the PRR (proline-rich repeat) region of TCCP [9]. TCCP from EHEC O157:H7 contains an N-terminus signal domain and seven almost identical PRRs. Previous studies have shown that the number of PRR regions in TCCP of EPEC varies from three to six and is related to the efficiency of actin polymerization (Garmendia et al., 2005). However, the underlying mechanism remains poorly understood (Sallee et al., 2008). Although the structure of the SH3 complex with one peptide from the PRR has been determined (Aitio et al., 2010), it is inadequate to fully explain the linkage between the number of PRRs and the level of actin polymerization. Therefore, we proposed that determination of the crystal structure of the SH3 domain, in complex with TCCP containing different PRRs, may help uncover the mechanism.

To determine the structure of a protein complex, efficient production of purified target complex is paramount. However, it is not economic to determine the complex structure of SH3 combined with all different types of TCCP. As a result we selected two TCCPs which containing three PRRs (TCCP3R) or five PRRs (TCCP 5R), because the two TCCP variants were proved to be significantly different in inducing actin polymerization (Sallee et al., 2008; Campellone et al., 2008; Wang et al., 2010). Here we report an efficient method for preparing SH3 from IRTKS in complex with TCCP3R (SH3\textsubscript{IRTKS}-TCCP3R), as well as a complex containing TCCP5R (SH3\textsubscript{IRTKS}-TCCP5R).

**MATERIALS AND METHODS**

**Plasmid construction**

The gene encoding the SH3 domain (Gln342-Glu401) of IRTKS was amplified using cDNA from IRTKS as template, and the primers P1(5′-CATATGCAGAAGATGACCA-3′) and P2(5′-CTCGAATTCATACATGACAAC-3′), Ndel and Xhol restriction endonuclease cleavage sites (underlined) were introduced into the ampiclon. The ampiclon was then digested with Ndel and Xhol and ligated to pET21a, to generate pET21a-SH3. To amplify DNA fragments encoding TCCP3R and TCCP5R, the annealing temperature was set to 56 and 50°C respectively, using primers P3 (5′-CCTAATGTGAGGAGCATATGATACA-3′) and P4 (5′-CCGCTCGAGCAGGCGCTTAGTGATT-3′). Genomic DNA from EHEC O157:H7 Sakai was used as the template for PCR. Amplons were then cloned into pET28a to generate pET28a-TCCP3R and pET28a-TCCP5R. All recombinant plasmids were verified by DNA sequencing.

**Expression and purification of SH3\textsubscript{IRTKS}, TCCP3R, and TCCP 5R**

Recombinant plasmids pET21a-SH3, pET28a-TCCP3R, and pET28a-TCCP5R were transformed into Escherichia coli BL21 (DE3), and positive transformants were selected on Luria-Bertani (LB) agar plates. Transformants were grown in LB medium at 37°C. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added when the optical density of the cultures reached OD\textsubscript{600}=0.6. After 5 h of induction, cells were harvested by centrifugation at 12, 000 × g for 15min at 4°C and resuspended in buffer A containing 50 mM imidazole, A Superdex 75 10/300 (GE Health) column was used for final purification. The purity of the protein was estimated using Quantity One software based on the band density of the proteins on SDS-PAGE gels. Then N-terminal sequencing was applied to confirm the composition of purified recombinant proteins.

**Generation of mouse anti-SH3\textsubscript{IRTKS} or anti-TCCP3R antisera**

Six (6)-week-old BALB/c mice were immunized with purified TCCP3R or SH3 formulated with complete Freund adjuvant or incomplete Freund adjuvant when needed. About 0.1 mg of each antigen was given subcutaneously in the inguinal groove on day 0, 14 and day 21 and serum was collected one week after last immunization and kept at −80°C until use.

**Production and identification of SH3\textsubscript{IRTKS}-TCCP3R and SH3\textsubscript{IRTKS}-TCCP5R**

To produce SH3\textsubscript{IRTKS}-TCCP3R, purified SH3\textsubscript{IRTKS} and TCCP3R were mixed at 4°C for 5 h. The final concentration of the mixture was determined to be 10 mg/ml, and the mole ratio of SH3\textsubscript{IRTKS} to TCCP3R was about 10:1. The mixture was loaded onto a Superdex 75 10/300 (GE Health) column at a flow rate of 0.6 ml/min. The fraction of each elution peak was collected and detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE. The method used to produce the SH3\textsubscript{IRTKS}-TCCP5R complex is similar.
TCCP5R complex was the same as that described for SH3\textsubscript{IRTKS}\textsubscript{SH3}\textsubscript{IRTKS}\textsubscript{TCCP3R}.  

**Western blot assay**

The putative SH3\textsubscript{IRTKS}\textsubscript{TCCP5R} and SH3\textsubscript{IRTKS}\textsubscript{TCCP3R} complex was further identified by Western blot using anti-TCCP3R or anti-SH3\textsubscript{IRTKS} antiserum as primary antibodies. In brief, purified TCCP5R, TCCP3R, SH3\textsubscript{IRTKS}, SH3\textsubscript{IRTKS}\textsubscript{TCCP5R} and SH3\textsubscript{IRTKS}\textsubscript{TCCP3R} were subjected to 12% native-PAGE and then electro-transferred onto a nitrocellulose membrane. Then the membranes were washed and incubated with anti-TCCP3R or anti-SH3\textsubscript{IRTKS} serum at a dilution of 1/10000 at 37°C for 1 h, after blocking with of 2% milk in TBST. Finally, color was developed with HRP-conjugated goat anti-mouse antibody.

**RESULTS**

**Production of TCCP fragments**

A DNA fragment encoding TCCP3R was successfully amplified from the EHEC O157:H7 genome (Figure 1A) and cloned into pPET28a. The recombinant PET28a-TCCP3R plasmid was digested with Ndel and Xhol and the resulting banding pattern was consistent with the predicted product sizes (Figure 1B). Results from DNA
sequencing analysis confirmed that TCCP3R was successfully cloned into the pET28a vector. PET28a-pET28a-TCCP3R was then transformed into E. coli BL21 and 1 mM of IPTG was added to induce expression of the recombinant protein. As shown in Figure 1C, recombinant TCCP3R was successfully expressed in a soluble form. Following purification by Ni²⁺-NTA chromatography and size-exclusion chromatography, the purity of TCCP3R was approximately 97.6% when evaluated by SDS-PAGE (Figure 1E). In addition, gel-filtration chromatography showed that the homogeneity of TCCP3R was acceptable, as the peak was almost symmetrical (Figure 1D), and its elution volume was approximately 12.4 ml.

The production of TCCP5R and SH₃_IRTKS was similar to that of TCCP3R. The PCR product of TCCP5R and SH₃_IRTKS was about 750 and 200 bp, respectively (data not shown). After successfully cloned into PET expression system and induced with IPTG, both TCCP5R and SH₃_IRTKS were expressed in a soluble form (data not shown). Following purification, the purity of recombinant TCCP5R and SH₃_IRTKS was approximately 96.3% and 94.8%, as determined by SDS-PAGE (Figure 2). In addition, size exclusion chromatography analysis showed that both TCCP5R and SH₃_IRTKS were correctly refolded, as no aggregation peak was observed. The elution peak of TCCP5R and SH₃_IRTKS was about 11.6 and 14.5 ml, respectively. The first ten amino acids of TCCP3R, TCCP5R and SH₃_IRTKS was determined by N-terminus sequencing and was totally identical to the expected (data not shown).

**Production and identification of SH₃_IRTKS-TCCP3R and SH₃_IRTKS-TCCP5R**

To prepare SH₃_IRTKS-TCCP3R and SH₃_IRTKS-TCCP5R complexes, purified recombinant TCCP3R and TCCP5R were individually co-incubated with SH3 at 4°C for 5 h. The mixtures were then purified by gel-filtration chromatography. As shown in Figure 3A, the elution volumes of TCCP3R and SH₃_IRTKS were 12.4 and 14.5 ml, respectively. A new peak appeared in the TCCP3R/SH₃_IRTKS mixture at approximately 11.5 ml (blank arrow). A similar result was also observed when purifying the TCCP5R/SH₃_IRTKS mixture (Figure 3A). The elution volume of TCCP5R was 11.6 ml, while the new peak was at 10.4 ml (solid arrow). These data indicated that the new peaks formed within the mixtures were likely to be SH₃_IRTKS-TCCP3R and SH₃_IRTKS-TCCP5R.

To further identify the compositions of the two new peaks, the peak fractions were analyzed by SDS-PAGE in advance. Results show that two bands were observed in each of the two peaks. In addition, the positions of the two bands from the putative SH₃_IRTKS-TCCP3R fraction were the same as those of TCCP3R and SH₃_IRTKS, respectively (Figure 4B). Similar results were observed from the putative SH₃_IRTKS-TCCP5R fraction. These results suggested that the two new peaks were composed of two proteins, namely TCCP3R and SH₃_IRTKS and TCCP5R and SH₃_IRTKS, respectively.

Later, native PAGE was used to analyze whether the complex was present (Figure 3C). A new band located between TCCP3R and SH₃_IRTKS was observed on native gels in the fraction from the mixture of the two proteins (arrow, Figure 3C left panel). Meanwhile, some smeared band was observed near TCCP5R on SDS-PAGE gel, which may be the degenerated TCCP5R. A similar result was observed for TCCP5R, SH₃_IRTKS, and their mixture (arrow, Figure 3C right panel). These data showed that the binding of TCCP3R or TCCP5R to SH₃_IRTKS was considerably strong, because both TCCP3R-SH₃_IRTKS and TCCP3R-SH₃_IRTKS were able to remained in a complex in native gel electrophoresis.

To further verify the composition of the two new bands on native page, its immune reactivity with anti-TCCP3R and anti-SH₃_IRTKS antibodies was detected by Western blot. As shown in Figure 4, on gel incubated with anti-TCCP3R antibodies, bands were visualized on lanes loaded with TCCP3R, TCCP5R, TCCP3R-SH₃_IRTKS and...
TCCP3R-SH3IRTKS, but not on that with SH3IRTKS (left panel). Nevertheless, on gel incubated with anti-SH3IRTKS antibodies, bands were only observed on samples containing SH3IRTKS fragments, namely TCCP3R-SH3IRTKS, TCCP3R-SH3IRTKS, and SH3IRTKS. Collectively, these data showed that both the two new bands was able to interacted with anti-SH3IRTKS and anti-TCCP3R antibodies simultaneously, which suggested that the new bands were composed of TCCP3R-SH3IRTKS and TCCP3R-SH3IRTKS.

DISCUSSION

There are two main strategies for determining the crystal structure of a protein complex. One way is to crystallize a large component and then soak the protein with the other components or molecules (Hassell et al., 2007). This strategy is usually applied to the structures of proteins in complex with small molecules. However, this cannot be used to solve complexes composed only of proteins of high molecular weight. The second method to obtain a structure is to purify the already formed protein complex and then crystallize it. In this study, the target proteins in the complex, TCCP combined with the SH3 domain of IRTKS, have molecular weights that are too large to use the soaking method. Therefore, we selected the second strategy for crystallization.

One method to prepare purified protein complexes is to co-express both components and purify them simultaneously (Kholod and Mustelin, 2001). The protein complex is formed in vivo, which closely mirrors the native form. Therefore, in advance of this study, we attempted this method using two plasmids. Unfortunately, the purity and quality of the protein complex did not meet the requirements needed for crystal formation. Therefore, to form the complex, we produced the two proteins separately and mixed them in vitro.

To provide as much structural information as possible, the full-length protein is preferred rather than truncated fragments. We attempted to clone full-length TCCP into a pET28a vector; however, it was expressed only as an inclusion body. In addition, we tried to refold full-length TCCP but failed. IRTKS is difficult to produce in E. coli because of its high molecular weight (56.8 kDa). As a result, we chose the truncated fragments of TCCP and IRTKS, namely TCCP3R, TCCP5R, and SH3IRTKS, to prepare protein complexes.

One interesting finding in our study is that the position of TCCP3R and TCCP5R on native PAGE gel is quite different from that on SDS-PAGE gel. One possible explanation is that the migration of a protein on native PAGE is affected by not only its molecule weight but also its electrical charge. The predicted isoelectric point (pI) of TCCP3R, TCCP5R and SH3IRTKS is 10.6, 10.1 and 7.3, respectively, therefore there is more negative charge of SH3IRTKS than that of TCCP3R, TCCP5R. This negative charge driven migration can also be used to explain why complexed TCCP3R-SH3IRTKS and TCCP5R-SH3IRTKS migrated longer than that of monomeric TCCP3R and TCCP5R, despite their larger molecule weight.

In this study, we established an efficient method to produce two TCCP-IRTKS complexes, namely TCCP3R-SH3 and TCCP5R-SH3. This work lays a solid foundation...
for determination of the crystal structure of TCCP-IRTKS. In addition, we showed that recombinant TCCP was able to bind to IRTKS in vitro directly, which provided additional evidence for the interaction between the two molecules.

ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (Grant No. 81271783).

REFERENCES


Figure 4. Western blot analysis of TCCP3R-IRTKS and TCCP5R-IRTKS complexes. Complexed TCCP3R-IRTKS, TCCP5R-IRTKS and monomeric TCCP3R, SH3IRTKS, TCCP5R after native-PAGE was detected with anti-TCCP3R and anti-SH3IRTKS antibodies, respectively. Both TCCP3R-SH3IRTKS and TCCP5R-SH3IRTKS were able to react with anti-TCCP3R and anti-SH3IRTKS antibodies simultaneously.