Virus Research xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Virus Research

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

Subcellular localization of bacteriophage PRD1 proteins in *Escherichia coli*

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ARTICLE INFO

Article history:
Received 17 September 2013
Received in revised form
19 November 2013
Accepted 19 November 2013
Available online xxx

Keywords:
Membrane virus
Confocal microscopy
Protein interactions
Virus assembly
Bacteria

ABSTRACT

Bacteria possess an intricate internal organization resembling that of the eukaryotes. The complexity is especially prominent at the bacterial cell poles, which are also known to be the preferable sites for some bacteriophages to infect. Bacteriophage PRD1 is a well-known model serving as an ideal system to study structures and functions of icosahedral internal membrane-containing viruses. Our aim was to analyze the localization and interactions of individual PRD1 proteins in its native host *Escherichia coli*. This was accomplished by constructing a vector library for production of fluorescent fusion proteins. Analysis of solubility and multimericity of the fusion proteins, as well as their localization in living cells by confocal microscopy, indicated that multimeric PRD1 proteins were prone to localize in the cell poles. Furthermore, PRD1 spike complex proteins P5 and P31, as fusion proteins, were shown to be functional in the virion assembly. In addition, they were shown to co-localize in the specific polar area of the cells, which might have a role in the multimerization and formation of viral protein complexes.

1. Introduction

Bacterial cells have been mainly regarded as amorphous reaction vessels concealing a homogenous solution of proteins. Due to advances in bacterial cell biology, this traditional view has changed dramatically. Similarly to eukaryotes, bacteria deploy macromolecules such as proteins, lipids and nucleic acids into specific subcellular locations. This asymmetric architecture is spatially and temporally dynamic, enabling cells to respond to changing demands during their life cycle (Rudner and Losick, 2010). Accumulated data on bacterial proteins have revealed a variety of localization patterns (Amster-Choder, 2011). Whereas certain proteins oscillate from pole to pole (Gerdes et al., 2010; Leonardy et al., 2010; Loose et al., 2011), others form clusters on the bacterial cell surface or at specific sub-cellular locations (Amster-Choder, 2011). In addition, it is known that certain bacterial proteins, especially cytoskeletal, assemble into helical structures extending along the cell or construct ring-like structures at the mid-cell position (Vats et al., 2009). However, interpretation of the localization pattern data with fluorescent tagged proteins has been challenging and some artifacts have emerged (Swulius and Jensen, 2012). Q2

Recent studies have elucidated factors governing the asymmetric protein distribution in bacteria, which is presumably most commonly mediated by 'diffusion and capture', when proteins diffuse freely until interacting with other, so-called target proteins (Deich et al., 2004; Rudner and Losick, 2002). This raises the question about the primary factors directing the target proteins to their specific cellular sites, and emphasizes the need to reveal other mechanisms for protein targeting. For instance, self-assembly is a unique variation of the 'diffusion and capture' positioning, which does not require any pre-existing anchor structures. Cellular factors such as geometric cues and physical constrictions have a role in positioning a number of bacterial proteins into their specific intracellular sites (Rudner and Losick, 2010). Also localization signals can be found in certain bacterial proteins, similarly to their eukaryotic analogs (Russell and Keiler, 2007). Correspondingly, there is evidence of subcellular localization of certain mRNA transcripts correlating with the localization of their protein products (Nevo-Dinur et al., 2011). Yet another mechanism for protein positioning was brought up by the discovery of cytoskeletal proteins in bacteria

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0168-1702/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.virusres.2013.11.015

(Vats et al., 2009). These structures are also suggested to provide a track for other proteins to locate, resembling again the situation in eukaryotes (Nevo-Dinur et al., 2012). However, the underlying principles of targeting for most bacterial proteins remain elusive.

The studies indicate that the asymmetric protein distribution is particularly conspicuous at the chemically and physically unique cell poles (Lai et al., 2004). For instance, unequally distributed lipid composition and negative curvature of the membrane (Ramamurthi, 2010) are believed to contribute to encompassing proteins into these cellular areas (Nevo-Dinur et al., 2012). It has also been shown that a number of bacteriophages infect preferably at these extreme regions. These viruses bind to distinct cellular receptors on the surface of their Gram negative hosts such as *Escherichia coli*, *Yersinia pseudotuberculosis*, or *Vibrio cholera* (Edgar et al., 2008; Rothenberg et al., 2011) or Gram positive ones such as *Bacillus subtilis* (Jakutyte et al., 2011). This implies that the cell poles contain cellular components essential for DNA intake (Edgar et al., 2008). The hypothesis is supported by the fact that the poles are the preferred site of DNA intake in natural competent cells (Chen et al., 2005; Edgar et al., 2008). Moreover, in studies of *B. subtilis* infecting phage SPP1 (Jakutyte et al., 2011) and *E. coli* phage lambda (Rothenberg et al., 2011) it was observed that in addition to being injected, viral DNA is replicated at the poles. However, several proteins of replication machinery of bacteriophage ϕ29 infecting *B. subtilis* have been found localized in helix-like pattern near the membrane. It was also shown that the ϕ29 replication is dependent on cytoskeleton protein MreB, as also with phage PRD1 (Muñoz-Espín et al., 2009). Terminal protein of these phages has been shown to associate with bacterial nucleoid independently of other phage-coded proteins as well as localize in the nucleus of mammalian cells (Muñoz-Espín et al., 2010; Redrejo-Rodriguez et al., 2012). It seems that bacteriophages have evolved to exploit the internal asymmetry of their host cells in order to make the infection process more efficient.

One of the most extensively studied bacteriophages is Enterobacteria phage PRD1 (family: *Tectiviridae*, genus: *Tectivirus*), which infects various Gram negative bacteria, such as *E. coli* and *Salmonella typhimurium*, carrying P-, W- or N-type conjugative plasmid, whereas other tectiviruses can also infect Gram positive bacteria such as *Bacillus* (Grahn et al., 2006; Oksanen and Bamford, 2012). The PRD1 virion is formed by an icosahedral protein capsid surrounding a protein-rich membrane which, in turn, encloses the linear dsDNA genome (Abrescia et al., 2004; Cockburn et al., 2004; Olsen et al., 1974). Based on X-ray crystallographic analyses, PRD1 belongs to a certain structure based viral lineage with several other viruses such as: adenovirus, *Paramecium bursaria* chlorella virus 1 (PBCV-1) and *Sulfolobus* turreted icosahedral virus (STIV) (Abrescia et al., 2012; Benson et al., 1999; Khayat et al., 2005; Nandhagopal et al., 2002). All these viruses have a major capsid protein with a double β-barrel fold and similar virion architecture. In PRD1, the capsid is mainly composed of the major capsid protein P3, which forms pseudo-hexameric trimers (Abrescia et al., 2004; Benson et al., 1999). The receptor binding spike complex at the virion vertices contains the pentameric penton protein P31 forming the base structure from which the trimeric spike protein P5 and the monomeric receptor binding protein P2 protrude (Caldentey et al., 2000; Merckel et al., 2005; Rydman et al., 1999; Xu et al., 2003). The spike structure complex is stabilized by the integral membrane protein P16 linking the vertex to the underlying viral membrane (Jaatinen et al., 2004). In addition to several other PRD1 structural proteins, also a number of non-structural proteins have been identified, such as the tetrameric assembly protein P17 required for virion formation (Caldentey et al., 1999; Holopainen et al., 2000; Mindich et al., 1982). Despite the intensive structural and functional characterization, the interactions of a number of predicted PRD1 proteins are yet to be revealed.

Our aim was to analyze the localization and interactions of PRD1 proteins in its native host bacterium *E. coli*. The study included viral monomeric and multimeric structural proteins, an integral membrane protein and a soluble assembly protein. Special attention was paid to the receptor binding spike complex proteins P5 and P31 for which the structures at atomic resolution are known (Abrescia et al., 2004; Caldentey et al., 2000; Rydman et al., 1999). We localized the proteins in living cells by exploiting fluorescent fusion protein technology and confocal microscopy.

2. Results and discussion

2.1. Construction of bacterial expression vector library to produce fluorescent fusion proteins

We created a bacterial vector library for convenient production of fluorescent fusion proteins (Fig. 1; Table 1). The vectors were constructed by cloning genes encoding eGFP and its cyan and yellow variants eCFP and eYFP. We used two bacterial vectors pSU18 and pET24 bearing replicons p15A and ColE1, respectively, enabling simultaneous expression of two proteins. According to confocal microscopy the expression of the fluorescent protein genes in bacterial cells produced functional proteins (for eYFP see Section 2.3, for eGFP and eCFP data not shown). Using these vectors, it is possible to insert any gene of interest into either end of the fluorescent protein gene, thereby creating N-terminal fluorescent fusion protein (the fluorescent protein is linked to the N-terminus of the target protein) or C-terminal fluorescent fusion protein (the fluorescent protein is linked to the C-terminus of the protein). A linker sequence of six glycines was designed to these vectors to separate the fluorescent protein from the protein of interest reducing steric hindrance. In this study, we exploited the vector library to create cyan and/or yellow fluorescent fusion proteins of bacteriophage PRD1 proteins P2 (receptor binding protein), P3 (major capsid protein), P5 (spike protein), P16 (vertex stabilizing integral membrane protein), P17 (non-structural assembly protein), and P31 (penton protein) (Table 1). Genes were cloned into both vector types (Fig. 1) using either pSU18 or pET24 to produce both N-terminal and C-terminal fusion proteins, except gene *XVII*, for which only fusion P17-eYFP was available. Sequencing of the vectors revealed only minor changes in PRD1 genes (Supplementary Table S1).

2.2. Solubility and multimericity of viral fluorescent fusion proteins

In the fusion protein studies, the first concern is whether the fusion affects on the folding and functionality of the native protein. One way to evaluate this is to monitor changes in the protein solubility and find out whether the known multimeric proteins form multimers with fluorescent protein tags. The majority of the fusion proteins (P2, P3, P5, P17 and P31) were expressed as soluble (data not shown). These proteins were directed to sedimentation assay by a rate zonal centrifugation for the size determination.

Monomeric receptor binding protein P2 (Grahn et al., 1999; Xu et al., 2003) was expressed as a fusion protein in a monomeric form (Fig. 2A). Small fraction of smaller side-product was detected with both P2 fusions. More variation in the molecular mass distribution was detected with proteins, which can be released as multimers from the virion. The individual PRD1 spikes composed of the trimeric protein P5 form an elongated structure (Bamford and Bamford, 2000; Caldentey et al., 2000; Huiskonen et al., 2007; Merckel et al., 2005), and there is no obvious reason that the fluorescent tag at the C-terminus of the protein would interfere the folding. The N-terminal fusion protein eYFP-P5 (~61 kDa) sedimented as a monomer (Fig. 2B), but also a smaller multimeric side

Please cite this article in press as: Karttunen, J., et al., Subcellular localization of bacteriophage PRD1 proteins in *Escherichia coli*. Virus Res. (2013), http://dx.doi.org/10.1016/j.virusres.2013.11.015