Regulation of type II restriction-modification system Esp1396I

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Abstract. Fluorescence microscopy is a powerful tool which enables to observe a large number of processes at the level of single cells. A protein of interest could be selectively labeled using fluorescent proteins, thereby allowing direct observation of its accumulation in living cells. This work is devoted to study a restriction-modification system which is a protective system of bacteria against foreign DNA. The action of such a system is based on DNA degradation by an enzyme — restriction endonuclease — and is strongly regulated to prevent host cell DNA damaging. In current study, the restriction-modification system contains controller protein, which regulates amount of restriction-modification system enzymes. Changes in amount of restriction-modification system proteins may cause a change in protection level of bacteria against viruses. We use fluorescence microscopy for direct observation of restriction-modification protein levels and their changes depending on the amount of controller protein in bacterial cells.

1. Introduction

A huge amount of biological studies require direct observation of very small objects. Proteins have large diversity, they are crucial elements performing most major functions in the cell. To understand protein function it is often necessary to determine their amount, localization and synthesis dynamics in a cell. This process was long, difficult and the results were averaged over the cell population. Now, proteins amount could be directly observed in single cells using fluorescence microscopy and fluorescent proteins. In the current study, we utilized a restriction-modification system in which two enzymes were fused to different fluorescent proteins. In this case, the protein of interest amount is equal to fluorescent protein amount, which can be estimated using fluorescence intensity. Such modification was previously made for restriction-modification system II type Esp1396I [1]. This system protects bacterial cells from viral infection and contains methyltransferase (Mtase), restriction endonuclease (Enase) and controller protein (C-protein) [2]. Mtase protects bacterial DNA from degradation by Enase which in its turn cleaves unprotected foreign DNA (DNA of bacteriophages and plasmids). After entry of a restriction-modification system Esp1396I into the new host cell, Mtase starts to be synthesized first. Due to the weak promoter, C-protein and Enase accumulate slower. When C-protein concentration increases it suppresses Mtase producing by binding with the promoter region of Mtase gene. Also, the increasing concentration of C-protein activates production of Enase by binding to the first binding site in a promoter region of C-protein and Enase. As soon as C-protein concentration becomes sufficient to bind to a second binding site in the promoter region of C-protein and Enase their synthesis decreases.

The level of protection could depend on Mtase and Enase ratios inside the cell [3] that are obviously dependent on C-protein concentration. An optimal balance between Mtase and Enase concentrations during the life cycle of the cell could provide a high level of protection against bacteriophage. If the
balance is shifted to the increase of Enase, phage DNA could be degraded more effective but in some cases, this shift may lead to host DNA degradation. If the balance is shifted to the increase of Mtase, foreign DNA will be protected more often and it will lead to a resistant phage progeny appearance and further elimination of a whole bacterial population. Our previous study showed that there is a time delay between Mtase and Enase accumulation in a new host cell [1]. This information correlated with the concept of Esp1396I system regulation.

In the current study, Mtase and Enase genes were replaced by Venus and mCherry fluorescent protein genes respectively to avoid host cell genome degradation. This artificial system mimicks Mtase and Enase proteins amount through the accumulation of fluorescent proteins. In addition to this artificial system, C-protein gene was placed under the inducible promoter, to perform its controlled changes inside the cells using the different concentration of inducer.

The goal of this study was to estimate Mtase to Enase ratio specific for different concentrations of C-protein inside the cells.

2. Material and methods

In our experiments we used artificial restriction-modification system which consists of two plasmids, pUC-MV with genes of fluorescent proteins mimicking Mtase and Enase and pC, with an inducible gene of C-protein. The first plasmid carry fluorescent protein genes instead Mtase and Enase. Gene expression levels of this fluorescent proteins are equal to wild type restriction-modification gene expression. The second plasmid carry C-protein gene under inducible (arabinose) promoter control. Using such approach we were able to increase C-protein amount inside the cells by adding arabinose at the different concentrations and detect fluorescence of Venus and mCherry proteins, which is proportional to amount of Mtase and Enase.

E.coli cells were transformed with both plasmids and were grown for 16-20 hours in LB medium at 37 °C with addition of appropriate antibiotics and 2% glucose to inhibit arabinose promoter. Then cells were harvested and diluted 50-fold into a fresh medium six samples with the same cells and different induction parameters were made. Thereby 0.1 µM, 10 µM, 0.13 mM, 13 mM, 100 mM arabinose or 2% glucose were added to the samples. So in the sample with the addition of glucose there was no C-protein, in the sample with 0.1 µM of arabinose there was the smallest C-protein amount and in the sample with 100 mM arabinose — the largest amount of C-protein. Then all samples were cultivated in LB medium at 37 °C were collected every 1.5 hours. The fluorescence images were obtained using Nikon Eclipse Ti-E microscope equipped with Andor Zyla 4.2 sCMOS camera and Semrock filter sets for Venus and mCherry fluorescence detection. Exposures in fluorescence channels were 5ms and 20ms for Venus and mCherry proteins respectively. Images were made into three channels: transmitted light, in the fluorescence channel of Venus and in the fluorescence channel of mCherry.

All data analysis was performed using ImageJ (Fiji) software. The average intensity of more than 100 random cells at the selected area were measured. The total intensity of a cell population in the current time point was measured as the sum of average intensities for all cells in the population. The number of cells in the population was measured by optical density (OD600). As error there were calculated a standard deviation. For all experiments at least three replications were performed.

3. Results and discussion

As could be seen from graphs (Figure 1 and Figure 2) there is a correlation between our experimental results and existing model of how Esp1396I system is regulated [4]. Namely, if there is no C-protein inside the cells (case of 2% glucose) Mtase (Venus) have increased significantly by the end of the experiment, but Enase (mCherry) remains on the very low level. In cases when induction of C-protein is not too high (0.1 µM and 10 µM of arabinose), the final concentration of Mtase is lower than in the case of 2% glucose. Here we can suggest that C-protein starts to inhibit Mtase, but its amount is not enough for Enase gene expression activation. A larger amount of C-protein in the cells causes an increase in the level of Enase and a decrease in the level of Mtase. So 0.13mM and 13mM arabinose concentrations are those at which C-protein amount achieved such level which allowed to inhibit Mtase
gene expression and induce Enase gene expression. 0.13 mM arabinose concentration is enough for starting to inhibit Mtase, but Enase activation is still not very high. Moreover, 13 mM arabinose concentration inhibits Mtase more strongly than 0.13 mM, and also better activates Enase gene expression. 100 mM arabinose concentration quickly inhibits both Mtase and Enase genes expression.

**Figure 1.** Fluorescence intensity dynamics for Venus.

**Figure 2.** Fluorescence intensity dynamics for mCherry.
Figure 3. Fluorescent protein levels after 4.5 hours of inductions with different concentrations of arabinose. a – the last points for Venus from Figure 1. b – the last points for mCherry from Figure 2.

On the Figure 3 there are amounts of Mtase and Enase after 4.5 hours of induction. From left to right C-protein concentration increases. It could be seen that smaller amount of Mtase in the cells corresponds to higher amount of Enase. With an increase of C-protein in the cells, the number of Enase also increases, but this is correct only up to a some concentration at which C-protein starts to inhibit both Mtase and Enase gene expression. This observation confirms on a single cell level the previous results [1] and support the model of regulation of Esp1396I restriction-modification system.

4. Conclusion
In the current study, we experimentally confirmed that the concentration of C-protein significantly affects concentrations of Mtase and Enase in the cell. Also, we demonstrated the mechanism of regulation of type II Esp1396I restriction-modification system at the level of single cells. Briefly, Esp1396I restriction-modification system is regulated using different concentrations of C-protein. When the concentration of C-protein is low there is expression almost only from strong Mtase promoter. Then with the growth of C-protein concentration Enase and also C-protein expression becomes better. Further growth of C-protein concentration leads to the suppression of expression of Mtase and then Enase and C-protein.
This study allowed us to change the level of protection against viruses by accurate changing of the C-protein amount in the cells.

Further it is planned to estimate C-protein concentration in the cells with a certain concentrations of Mtase and Enase. Also, we want to estimate the concentration of C-protein in the cells during the appearance of a restriction-modification system in them.

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