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SCIENCE DIRECT
Analytical Biochemistry 352 (2006) 61–67
ANALYTICAL BIOCHEMISTRY
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Cell-surface-localized ATP detection with immobilized firefly luciferase

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Received 6 October 2005
Available online 9 March 2006

Abstract

We demonstrate the application of an assay technique for the detection of ATP efflux from the cell surface. Until recently, the firefly luciferase assay has conventionally been used to detect ATP release because of its high sensitivity and wide detection ability. However, since this technique can be used only to infer the amount of diffused ATP in bulk solutions, it does not accurately reflect the dynamic ATP flux at the cell membrane. We therefore constructed luciferase fusion proteins that could be immobilized onto the cell surface. However, the low activities and limited application ranges of these proteins prompted us to use biotinylated luciferase given its attributes of strong and stable localization. Furthermore, luciferase can be immobilized strongly onto the biotinylated cell surface via streptavidin–biotin binding and can thus be used to determine the dynamic release of ATP near the cell surface.

Keywords: Extracellular ATP; Fusion protein; Immobilize; Luciferase

Extracellular ATP is known to modulate many cellular functions [1–4]. ATP efflux is an induced cellular response that occurs in response to a variety of unknown stimulatory mechanisms. Consequently, the determination of locally released ATP from cells is one of the steps required for resolving the primary modulation of cellular functions. There are three general mechanisms by which intracellular ATP can be released into extracellular spaces. The first involves direct and nonspecific cytolysis of healthy cells by physical damage, such as tapping, stretching, and hypotonic stress. The second mechanism is via vesicles under conditions of exocytosis, such as when ATP is copackaged in the secretory granules of many neuronal or endocrine cells and is rapidly released during synaptic neurotransmission and other types of regulated exocytosis [5]. The third potential route for ATP release is via the efflux of cytosolic ATP via plasma membrane transport proteins. Given that many cellular mechanisms are associated with ATP efflux, ATP is currently thought to be ubiquitous with respect to being the molecule responsible for modulating cellular functions. However, several factors complicate analyses associated with ATP release. For example, the ubiquitous presence of ATP in all cell types makes it difficult to determine whether extracellular ATP is derived from particular release mechanisms or whether it is derived from the nonselective cytolysis of cells under experimental conditions. In addition, most cells express ectonucleotidases that rapidly hydrolyze any extracellular ATP present at the cell surface [6]. Finally, nucleotides that are released are diluted rapidly by diffusion upon release. For these reasons, though the concentration of ATP in the proximity of the cell surface is expected to be dynamic, the measurement of ATP in extracellular fluids may result in a marked underestimate of the amount of ATP actually released. Consequently, the accurate measurement of localized ATP release is difficult in such diluted solution samples.

As a recent technique for ATP measurement, firefly luciferase is a widely used and extraordinarily sensitive bioluminescent ATP sensor protein [7,8]. This is due, in

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0003-2697/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2006.02.019

part, to the fact that this assay system can detect ATP concentrations at picomolar levels under optimal conditions. Unfortunately, however, such studies rely on the analysis of extracellular solution samples that, for the reasons described above, do not accurately reflect the dynamic ATP concentrations at the cell surface. Consequently, methods capable of estimating the ATP concentrations at levels such as those that occur near the cell surface and that are typical of cellular reactions are required. In a previous study [9,10] we described a technique using a fixed form of luciferase. The methods referred to in those studies were designed by fusing luciferase to a protein and then allowing these fusion proteins to bind to the cell surface. The luciferase localized in this way could then be used to determine the local concentration of ATP with high sensitivity. Recently, these localized fusion proteins have facilitated investigations of ATP release sites and ecto-ATPase activity on the cell surface of human astrocytes [11]. However, the activities of luciferase fusion proteins were much lower than those of native luciferase and their field of application was limited.

In this paper, we describe an improved extracellular ATP sensing system with a luciferase fusion protein whose activity is highly conserved. Methods for genetically engineering biotinylated proteins such as biotin acceptor peptide (BAP)[1] have recently been developed [12]. Proteins fused with BAP using such techniques were biotinylated in vivo in the posttranslational process with the modification in structure and bioluminescence activity confirmed by enzyme-linked immunosorbent assay [13]. This biotinylated luciferase was used for our local extracellular ATP sensing strategy. Streptavidin has a strong affinity for biotin and is widely used in ELISAs and other assays. Consequently, using biotinylated luciferase can improve the stability and the applicability of our previous systems [9,10]. Biotinylated luciferase was demonstrated to bind stably onto the biotinylated cell surface via interactions with streptavidin (Fig. 1) and could be used to distinguish the transient ATP signal from the background. This system will also prove useful for the online determination of the kinetics of ATP release from cells and for the analysis of many types of cellular functions. In this study, we examined the binding ability and bioluminescence activity of cell-surface-immobilized biotinylated luciferase for the detection of local ATP release.

Materials and methods

Materials

Restriction enzymes, ligase, and T4 DNA polymerase were obtained from Takara Shuzo (Shiga, Japan). *Escherichia coli* BL21 (DE3) and pET32c were from Novagen (Madison, WI). Picagene, which is the substrate solution for luciferase, was from Toyo Ink (Tokyo, Japan). All other chemicals were of analytical grade.

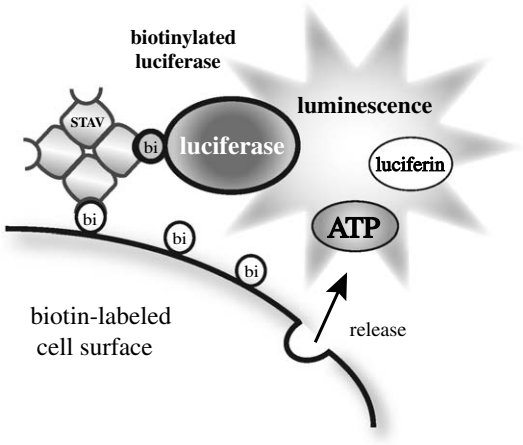

Fig. 1. Sensing diagram using biotinylated luciferase. The cell surface was biotinylated with sulfo-NHS-biotin, and streptavidin was bound to them. Biotinylated luciferase could be immobilized via streptavidin onto the cell surface. When the cells released ATP, the localized ATP sensor protein could detect the dynamics of ATP near the cell membrane.

Plasmid construction

The biotinylated luciferase gene fusion vector, pET-NHis-BAP-Luc (Fig. 2A), was constructed as follows. The synthetic DNA fragments encoding biotin acceptor peptide was inserted into the pBlueScript-SKIIgene carrying vector (Stratagene) in frame at *Bam*HI-*Pst*I. The luciferase gene was digested with *Pst*I and *Hin*dIII from pET32-NHis-STAV-Luc [10] and ligated into pBlueScript-BAP plasmid digested with *Pst*I and *Hin*dIII. To improve the stability of enzymatic activity in assay processes, we used the luciferase with a point mutation, from Glu to Lys at position 354 in its sequence (E354K). The E354K mutant was reported to have high thermostability compared with wild-type luciferase [14,15]. Then the BAP-Luc DNA fragment was fused into the *Bam*HI/*Hin*dIII site of pET32c expression vector, downstream of the T7 promoter. The T7 promoter is under control of lacUV5 and is induced by IPTG. For the purification, six repeats of the histidine sequence (His-tag) are coded to the N terminus of the fusion gene. The amino acid sequence of BAP is shown in Fig. 2B.

Expression and purification of biotinylated luciferase

The constructed pET-NHis-BAP-Luc plasmid was transformed into *E. coli* BL21 (DE3) competent cells by heat shock. Transformed *E. coli* cells (10 ml) were added to 1 L of LB medium in the presence of 50 μg/ml ampicillin and 50 μM biotin. Cells were grown to mid-log phase at 37 °C, at which time IPTG was added to 0.2 mM. After

[1] *Abbreviations used:* BAP, biotin acceptor peptide; IPTG, isopropyl β-D-thiogalactoside; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; HPAECs, human pulmonary artery endothelial cells; HBSS, Hanks' balanced salt solution; NHS, *N*-hydroxy succinimidyl.